STUDIES ON THE PREPARATION, PROCESSING AND PROPERTIES OF SOYMILKS

A Thesis
Presented in Partial Fulfilment
of the Requirements for the
Degree of Doctor of Philosophy
in Food Technology

at

Massey University

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1971

ABSTRACT

The effect that presoaking of soybeans in solutions of various chemicals has on the reduction of "beany" flavour in soymilk was investigated. Of the chemicals used, sodium carbonate and sodium hydroxide had a significant effect. Sodium carbonate soaking at 0.4 M concentration for 18 - 24 hours was significantly better than any other presoak treatment. Soymilk prepared from carbonate presoaked beans contained more protein and had a higher viscosity than milks prepared from water or sodium hydroxide presoaked beans. Beans presoaked in carbonate were easier to process than beans presoaked in water or hydroxide. There was no significant difference in the amino acid pattern of the proteins in the soymilks prepared by the three methods. The activity of lipoxidase as a major source of "beany" flavour in soymilk has been questioned.

The rate of inactivation of trypsin inhibitors in soymilks prepared from carbonate presoaked beans was faster than that in the water presoaked preparation when heated to 98°C. This effect was primarily associated with the rise in pH that occurred in the soymilks from carbonate presoaked beans. The effect of alkaline pHs at 98°C on the inactivation of trypsin inhibitor was examined and it was found that the rate of inactivation was changed from zero order at pH 6.8 to first order kinetics at pH 9.9. This effect of pretreatment was not noticeable when both milks were processed at 115°C in sealed cans because of the constancy of pH under these conditions.

The influence of heat processing conditions on the enzymatic digestibility of proteins in both soymilks was also studied. The digestibility with trypsin increased with the degree of heat treatment up to the point where the trypsin inhibitor had been destroyed, after

which further heating resulted in lower digestibilities. The optimum heat processed soymilks prepared from carbonate presoaked beans gave higher digestibilities than those of the water presoaked preparations under the conditions used.

Pepsin digestion (at 0.125% of 1 : 2,500 pepsin) showed no appreciable differences between soymilks prepared by either presoaking procedures. The degree of digestibility with pepsin plus trypsin of the proteins in the optimum heat processed carbonate presoaked preparation was similar to the digestibility of acid precipitated freeze-dried casein under identical conditions.

The changes in viscosity during processing of soymilks prepared from carbonate presoaked beans was different from that of the water presoaked soymilk. Of the factors which affect the viscosity of soymilk during processing, denaturation, aggregation and hydration-dehydration processes probably play the most important roles. Nevertheless, the proteins in both soymilks were very stable under the processing conditions used.

About 0.1 percent of sodium chloride was formed in the carbonate presoaked preparation during neutralisation with hydrochloric acid. When this amount of sodium chloride was added to the water presoaked preparation either in the raw state or during heat treatment, the proteins became unstable. This effect could be eliminated if the addition of sodium chloride was made after even slight denaturation of the proteins. Addition of up to 0.25 percent of sodium chloride had no effect on the stability of proteins in the carbonate presoaked preparation. The instability of proteins in soymilks in presence of sodium chloride was considered to be primarily due to aggregation.

Soymilk that has been heat treated to destroy the trypsin inhibitor is difficult to concentrate because its viscosity increases exponentially with increase of solids content. This imposes a concentration limit of around 17 - 18 percent total solids due to the formation of a gel-like structure beyond this. The possible factors which contribute to this gel formation during concentration were investigated. A sulphydryl-disulphide interchange reaction was mostly responsible for the increase in viscosity of the concentrate up to about 16 percent total solids but above this concentration other forces predominated. The possibility that gelation occurred through intermolecular cross-linkages by calcium ions was eliminated. Sodium sulphite and N-ethyl maleimide were effective in reducing this viscosity increase to some extent but when the concentrate was heat sterilised sodium sulphite ceased to have any effect on the viscosity and with N-ethyl maleimide the viscosity of the sterilised concentrate was about 50 percent of the unstabilised sample. Forewarming by heating to 115°C/5 min. caused an increase in viscosity during concentration but effectively stabilised the product against further increases in viscosity on sterilisation.

The effect of processing conditions on the nutritional quality of proteins in soymilks was studied using a number of in vitro methods. The results indicated that the nutritive values of the soymilks prepared from carbonate presoaked beans by three different processes (98°C for 40 minutes; 115°C for 18 minutes; preheated at 115°C for 5 minutes, concentrated to 15.5 percent solids content and then sterilised to a F_o value of 5.5) were about the same and were better than that prepared from the water presoaked preparation processed at 115°C for 18 minutes. Methods of process

control and quality assessment of soymilk are discussed. Flow and mass balance sheets for the process developed have been presented.

ACKNOWLEDGEMENTS

I desire to express my grateful appreciation to Dr G.M. Wallace, chief supervisor of this research project, whose experience, keen interest and untiring assistance proved invaluable. His help in the preparation of the manuscript for this thesis is gratefully acknowledged.

I am also indebted to the following:

- Dr W.R. Bannatyne, former Senior Lecturer of this department and Dr M.D. Earle, for their supervision of this work and also appreciation extended to Dr Earle for proof reading.
- Mr H.A.L. Morris, former Reader of this department, for his willingness to discuss some aspects of this work.
- Mr M.R. Patchell, Director of Poultry Research Centre, for providing facilities to use the amino acid analyser.
- Department of Chemistry and Biochemistry for their facilities and use of their equipment.
- Tasman Vaccine Laboratories for the donation of soybeans necessary for this study.
- The New Zealand Government, through the Colombo Plan arrangement, for providing the opportunity to undertake this project.

- Miss M.G. Campbell, Miss E.M. Green and other staff of the Massey University Library, for their help in seeking references for this thesis.
- Mr R.W.S. Leitch and Mr P. Herbert respectively for photography and printing the graphs.
- Miss Joan Quigan, for her excellent typing of this thesis.

I feel deeply grateful to my wife, Najma, for her constant help and endless encouragement during the course of this work.

A. KHALEQUE

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CHAPTER 1

GENERAL INTRODUCTION

It has long been recognised that proteins, essential constituents of living cells, are essential food components for man and other animals and that the protein requirements vary, sometimes considerably, during various stages of human life (14). Thus protein is required in different quantities for (i) maintenance and subsistence; (ii) for growth; (iii) for special purposes such as in pregnancy and lactation; and (iv) in various physiological and pathological conditions.

A large part of the human race does not consume sufficient protein foods of good biological value to sustain satisfactory health, growth and physical vigour (212). In the Far East, the Near East and in parts of Africa and Latin America consumption of protein of good biological value is particularly low. Figure 1 puts into sharp contrast the estimated per caput supply of total and animal proteins in developed and developing countries. While the total estimated amount in developed areas is high, its proportion from vegetable sources is low. On the other hand the bulk of the small amount of protein available in developing countries come from plant sources and is of low biological value.

Several reviews of protein deficiency diseases are now available (129, 222, 265), these summarise the hundreds of papers that have appeared over the last 25 years dealing with those diseases of both children and adults that are now thought to be due in whole

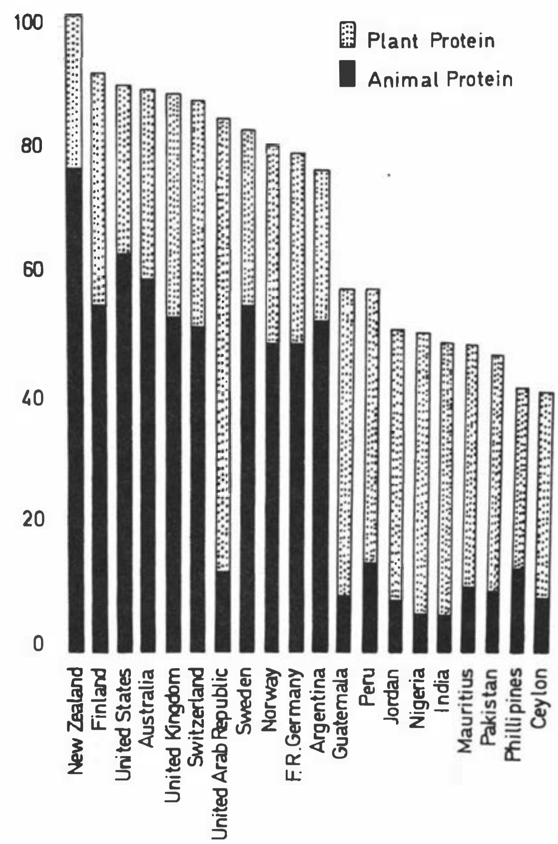

FIG. 1. ESTIMATED PROTEIN CONSUMPTION IN CERTAIN DEVELOPED AND DEVELOPING COUNTRIES (gm/caput/day)

Reproduced from : reference **212**

or in part to deficiencies in dietary protein with or without calorie deficiency. Special attention has been focused on kwashiorkor, which is a consequence of dietary imbalance in early childhood, that is, a diet which contains low protein but high carbohydrate calories and on nutritional marasmus which is a consequence of a diet very low in both protein and calories, so called balanced starvation (129). These nutritional defects are occurring in many parts of Africa, Asia and Central and South America (Figure 2). Surveys have shown that kwashiorkor and marasmus have their highest incidence of clinical symptoms in children between the ages of six months and three years, the period when the protein requirements are much higher than in older children and adults (224).

The weaning period is a critical and somewhat difficult phase in any part of the world and in the tropics and subtropics it is a time of extreme danger for the child and there is a high consequential mortality. It is during this phase that the infant crosses the precarious bridge between adequate growth while being fully breast-fed to later relative self-sufficiency on the adult diet. Some staple diets may be so poor in protein that a tiny child literally cannot eat enough of them to supply his protein needs (129). Protein calorie malnutrition also lowers the resistance to disease and facilitates infection. Under the circumstances, it is not surprising that in many populations as many as 40 percent of the children die before reaching school age (41). Furthermore, even where the sufferer from severe malnutrition survives, he or she is most likely to be both mentally and physically stunted, and so to be a liability rather than an asset to an advancing society. Today, there are over 300 million children suffering from protein and calorie malnutrition and it has been estimated that the world's population is going to be doubled by the

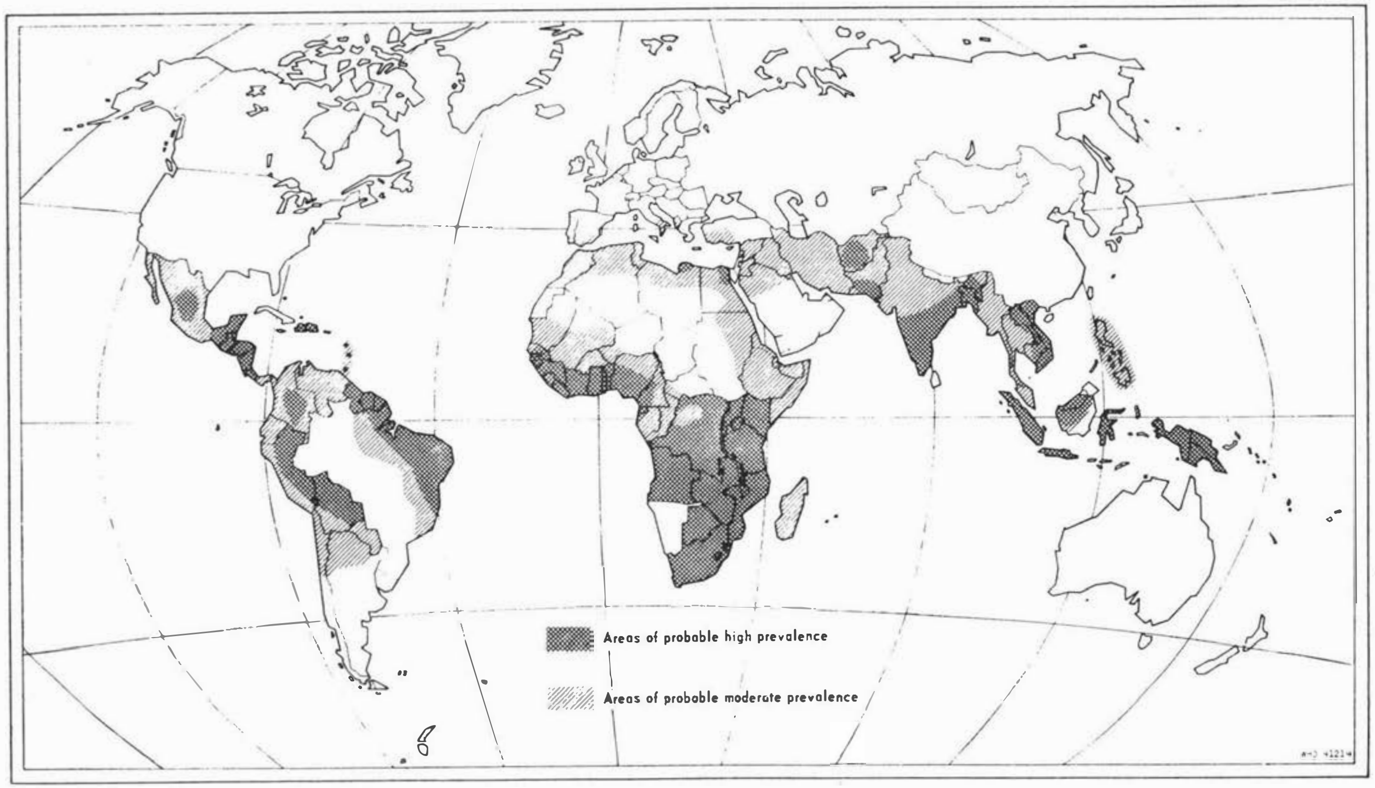

FIG. 2. GEOGRAPHICAL DISTRIBUTION OF PROTEIN–CALORIE MALNUTRITION IN YOUNG CHILDREN

Reproduced from : reference 129

year 2000! The U.N. publication, International Action to Avert the Impending Protein Crisis, states "it is essential that the United Nations family urgently takes action aimed at closing the present gap between world protein needs and protein supplies and at preventing even more widespread protein deficiency in future generations" (124). Dr Aaron M. Altschul, special assistant for nutrition improvement to the U.S. Secretary of Agriculture stated "we emphasize protein as the major quality component. There are other quality components - the vitamins and minerals - and these too are deficient in poor diets. We are confining this discussion to protein, not because the consequences of the other deficiences are less serious, but because the others are required in small quantities and can be made relatively cheaply, hence their deficiency is easier to remedy" (8).

Animal protein foods are scarce and expensive in the tropics and sub-tropics and from available evidence it would appear that this situation is not likely to improve significantly in the near future. The obvious solution to the problem of protein malnutrition must lie in the use of the more plentiful and cheaper plant protein foods, mainly pulses and oilseeds (41). Soybeans are a particularly attractive possibility for a number of reasons. (i) Soybeans grow relatively easily and widely; (ii) Soybeans have exceptionally high protein content and the protein is practically equivalent to animal protein in biological value; and (iii) the products made from soybeans have been used in human diets for centuries (see Appendix I). Lager (147) stated "the history of the soybean is a fascinating story of a little round bean that has literally been a golden nugget to the oriental world. Soybeans are perhaps the world's oldest food crop. For centuries, nutritionally they have meant meat, milk, cheese, bread and oil to the people of Asia. Soybeans can rightfully claim the honour of being one of the most concentrated and nutritive foods known to man". In recent years, the soybean has received a great deal of attention as a possible source of extra protein in the human diet in attempting to alleviate part of the world's continuing protein deficit (223).

The need of liquid nourishment following birth is evident from the fact that the young of all mammals, including the human, have no provision for taking solid food. As a starter food, a lacteal secretion is by nature supplied, and to meet the further needs of the growing child various animal milks have been used. However, the supply of animal milk is meagre in Asia, Africa and Latin America. Even in countries where cow's milk is available, 7-15 percent of infants, children and adults show a sensitivity to it (185). The problem of providing a substitute for animal milks in the diet of infants and children has been engaging the attention of research workers in different countries (64, 110). Soymilk is of particular interest since it can be prepared to yield a readily digestible food for infants and young children in those countries where protein malnutrition is a serious problem. It can also be used for babies who are allergic to cow's milk, even in highly developed dairying countries. Dr Harry W. Miller (185), Director of the International Nutrition Research Foundation, stated "soymilk, with physical characteristics and formula similar to human milk, has proved to be the most dependable milk food in all cases of allergies in humans. Soybeans are grown in large quantities in many lands where we do not find a dairy industry. Soymilk can also be marketed within the economic range of most nationals in non-dairy countries".

Although soybeans are becoming an increasingly important source of high quality proteins, they possess a characteristic "beany" flavour which affects their use as food for human consumption (238). Even in the orient, where other soybean foods are traditionally accepted, soymilk has never achieved much popularity because of the absence of a method to reduce its beany flavour (241). The very numerous attempts to improve the flavour of soymilk date from ancient Chinese history up to the present time and have met with but little success (see Chapter 2).

With an aim of developing a wholesome soymilk of acceptable quality, this study was undertaken to examine the preparation and processing of soymilk and to establish whether important changes take place in the chemical, physical and nutritive characteristics of the milk during preparation and processing.

CHAPTER 2

DEVELOPMENT OF A METHOD FOR THE MANUFACTURE OF BLAND-FLAVOURED SOYMILKS

2.1 Introduction

Soymilk is the name given to aqueous extracts from soybeans or to fine emulsions of soybean flour because of their milky appearance. The pH of this soymilk is very similar to cow's milk, about 6.7, and when the milk is allowed to sour in a normal way, the pH will drop to about 4.9 and cause precipitation of most of the protein. Soymilk, as ordinarily produced, does not have the bland flavour or smooth texture of cows milk. Casein, the principal protein of cow's milk has the characteristics of contributing consistency to the milk but soy proteins lack this property and therefore the soymilk possesses an unpleasant watery taste and poor mouthfeel.

Soymilk has been known for a long time, probably over many centuries in a few Far Eastern countries. It is mostly produced in the homes and sometimes on a cottage industry scale. The milk prepared by the oriental method, possesses an unpleasant "beany" flavour and that is why soymilk is one of the minor uses compared with other soybean products. This flavour is less obvious when prepared as bean curd. Bean curd is prepared by coagulating soymilk with calcium salts and the product is popular with oriental people.

2.2 Review of literature

The original method used in the Far Eastern countries for the preparation of soymilk is known as the oriental method. The method is simple and straightforward. The soybeans are first thoroughly washed with water and then soaked in water for about 10 hours in summer and 24 hours in winter, after which they are ground using a stone mill and the crushed mass is extracted with water at a ratio of about 8 parts of water to 1 part of dry beans. Undissolved solids are removed with a coarse cloth filter and the milk is boiled for 20-30 minutes (242).

Several methods have been developed aimed at improving the composition for better nutritional quality or modifying the flavour either by incorporating certain flavour components or treating the milk with enzymes or bacterial cultures to impart a more acceptable flavour, or expelling the beany flavour or removing the bitter principles.

Early workers attempted to volatilise the "beany" flavour by boiling or steaming, and to improve the nutritional balance of the milk by addition of sugars and vegetable fats. For example, Miller (184) boiled the finely ground slurry after soaking and then centrifuged it to remove the undissolved solids. Also, to reduce the beany flavour, he used the variety of soybeans used in the Orient as a vegetable and not the "field" soybeans grown for oil production. Sugar, vegetable fat, and salt are added in order to make a balanced milk. The mixture is then actively boiled with agitation for 30-60 minutes under conditions that prevent coagulation of the protein on the surface of the milk. This boiling process is credited with improving the flavour of the milk which is finally homogenised and bottled or spray-dried. Horowitz et al (118) have worked out the following procedure. The ground soybeans are soaked for eight hours and treated with steam at 100°C for deodorisation. This is followed by the extraction with water and the separation of the residue. The emulsion is cooked for five minutes and then 0.25% sodium chloride, 0.5% glucose, 2% soyoil, and 0.02% vanilla or 0.2% almond essence are added.

Tso (259) also modified the soymilk formula prepared by the oriental method. The protein constitutes 20-22% of the calories, he supplemented it with cane sugar, corn or rice starch, cod liver oil, calcium lactate, sodium chloride and cabbage water - the later as a source of vitamin C. Monahan and Pope (192, 193) improved the flavour of soymilk by using malt, chocolate or cocoa. Thevenot (256) suggested that the mashed beans should be digested with proteolytic enzymes or the beans be treated with alcohol to remove some of the undesirable principles. Melhuish (177) improved the flavour of soy milk by incorporating milk ripening bacteria or by using butyric acid as flavouring material.

Wilkens et al (269) modified the oriental method by using the temperature of water during grinding above 80°C to inactivate lipoxidase present in soybeans which is thought to be responsible for the oxidative off-flavour in soymilk.

De and Subrahmanyan (63) and Desikachar et al (66) effected a number of improvements in the oriental method of preparing the soymilk. The soybeans are soaked overnight in water. They are then germinated and the skin is peeled by gentle rubbing. The beans are then extracted with 0.04% sodium bicarbonate at 70°C for half an hour to remove the bitter principle and colouring matter. The beans are then made into a fine paste, stirred up with about 6 volumes of water and boiled for 20-30 minutes. The mixture is allowed to settle and then filtered through cloth. Bieringer (22) devised a procedure in which the soaked beans were ground to a slurry and shaken with an aqueous chlorophyllin solution in an attempt to absorb bitter material and to deodorise sulphur containing compounds. Sakata et al (235) attempted to reduce the off-flavour of soymilk by treating it with a weak solution of sodium sulphite at 80-90°C. It has also been reported that soaking the soybeans in a 1% solution of sodium bicarbonate gives a slight improvement in flavour of the beans as a pre-treatment for producing soyflour (199). After the completion of the present work, patent literature has come to our notice in which Okumar and Wilkinson (205) developed a method to remove the beany flavour by treating the sprouted soybeans with sodium hydroxide solution. Badenthop and Hackler (15) also indicated that sodium hydroxide markedly reduces the off-flavour of soymilk.

Tan (255) investigated various processing steps used in the oriental method and reported that extraction of soaked flour in a 10 percent ratio, gave milk containing 7.6-7.9 percent dry matter, about half of which was crude protein. The yield of dry matter was about 59 percent and about 68 percent of the total protein was recovered in the milk. Extraction of soaked flour yielded more milk than extraction of beans. With the flour as well as with the beans better grinding yielded more milk and increased the dry matter and crude protein content of the milk. The influence of the extraction ratio is important for if the ratio of beans to water increased, the dry matter and crude protein content of the milk also increased. The yields of both, however, decreased. The extraction pH also had an important influence, for, if the pH was increased the dry matter content of the milk did not increase at the same rate as the crude protein content. At pH 9.5 the yield of crude protein was 80 percent. The extraction temperature had little effect on the yield in range 20° to 50°C. After a certain minimum (about 2½ minutes) the extraction time had little influence on the yield.

Using the original oriental method, the yield of soybean solids in the form of soymilk is limited to about 65 percent. To improve the yield of soymilk solids, a number of methods have been developed recently. Hand et al (111) reported a method that involved dehulling and fine grinding of steam-dried soybeans and slurrying the powder with water to produce a soymilk. This utilised 90 percent of the soybean solids. Mustakas and Mayberry (200), in cooperation with the Wenger machinery company, developed another process for manufacturing soymilk. By this procedure dehulled soybean flakes, properly conditioned with moisture, are fed into an extruder and forced through an orifice under conditions of short-time, high temperature, high-pressure treatment from which the bean mixture emerges cooked, puffed and dried. The puffed material is then finely ground and can be slurried with water to form soymilk.

Miles (182) described a method in which the hulls are removed at the outset from the unmoistened soybeans by cracking and winnowing. The dehulled cracked beans are passed through flaking rolls between which they are rolled under heavy pressure into very fine flakes of a thickness in the range of 0.003 to 0.008 inch. Water is added to the flakes at a ratio of 1 pound of beans to 10 pounds of water. A stabiliser of phosphate type or a sequestering agent such as EDTA is added to the slurry. The slurry is then cooked at a temperature in the range of 220° to 250°F for a period of time in the range from a mere flash to a maximum of 10 minutes. The cooked slurry is subjected to homogenisation at a pressure in the range of 5000 to 8000 psi. The slurry is then clarified by centrifugation. Oil, carbohydrate (including sugar), vitamins, and minerals are added in accordance with the requirements for a whole type milk or a skim type milk.

To improve the palatibility and nutritional quality, Johnson (134) developed a method comprising three principal ingredients: soybean flour, sesame flour and coconut meal. The three components are ground to extremely fine powder with the soybean being provided preferably in four parts by weight to three parts by weight of the sesame seed and two parts by weight of the coconut meal. A small amount of lecithin, usually in the range of 0.5 to 2 percent by weight of the three principal ingredients, is desirably incorporated as a water dispersing agent, and a better product is obtained by incorporating a small amount of an edible emulsifying agent.

Hand (110) noted that the yield of soymilk can be increased to 90 percent by suspending all of the solids of dehulled soybeans. The fluid milk thus produced is not as smooth or as stable in suspension as the milk made by water extraction.

Miller (183) stated that some researchers have made milk from the whole dehulled soybeans used entirely, giving 3 percent fibre on a dry basis. Older children and adults can do well on this when formulated. Miller objected to the presence of fibre on two grounds, (i) that animal milks are fibre free, and (ii) on coagulation a rough, tough curd is obtained as compared with a smooth, jelly like curd when made from a fibre-free liquid. He believed that human nutrition was so important that he could afford to sacrifice to animal feed the fibre and small amount of ingredients that cling to it in order to obtain the important ingredients in soluble form. The non-fibrous product was still economical when compared with other similar products on the market.

Modern trends in manufacturing soymilk start with isolated water soluble soyprotein and make an emulsion by adding emulsifiers, oil, minerals, vitamins and sugars. The method permits much better control of the composition and concentration of milk, also the milk has a less beany flavour than when the whole bean extract is used. In making isolated soyprotein, the beany flavour is mostly removed, part of it goes into the whey solution and part is destroyed or steam-distilled during drying. The milk prepared by this method will be more expensive because isolated protein may cost 3-5 times an equal amount of protein in the form of soyflour (243).

2.3 Objective and Experimental Plan

From the literature, it appears that many attempts have been made to improve the quality of soymilk but as yet no simple procedure has been found which will adequately reduce the beany taste to give a bland-flavoured product. A number of workers have employed some form of chemical pretreatment of soybeans or of the gruel to improve the flavour. No systematic study of the effect of presoaking soybeans in dilute chemical solutions appears to have been made. The purpose of the present work was to investigate this effect on the beany flavour of the resulting soymilk.

A wide range of chemicals, whose effect on the product will not result in it becoming inedible, was selected for the pretreatment of the soybeans. The intensity of the beany flavour of milks prepared from these beans presoaked in chemical solutions were compared with that of a standard milk prepared from water presoaked soybeans using a taste panel. The effect of temperature during the grinding operation on the lipoxidase activity and the beany flavour of the milk was also investigated.

2.4 Experimental

2.4.1 Materials

Soybeans (Harasoy variety) grown in New Zealand were used throughout the experiment. Their proximate composition on a dry basis was fat 17.3%, protein 45.6%, ash 5.5% and crude fibre 7.6%. Moisture content averaged 11.3%.

2.4.2 Methods

Preparation of soymilk

100g of soybeans were washed with water and then soaked in various solutions of differing concentrations for specific time periods at room temperature (17-21°C). The soaked beans were then dehulled by gently rubbing them on a rough surface (6-mesh sieve) and the skins were removed by flotation in water. The dehulled soybeans were washed thoroughly with water, drained, and then ground into a slurry, after adding 250 ml water, in a Waring Blendor (3-4 min). This slurry was then made up to 700 ml with water. After adjusting the pH to 7.0, when necessary, the extract was boiled for 30 min and then filtered through a double layer of 80 lb cheese cloth. The pH of the resulting milk was adjusted to 6.8-7.0, if necessary, by adding 0.1 M-HCl, and the volume was made up to 800 ml with water. In the hot grinding procedure the drained beans were added to 250 ml of boiling water to bring them up to grinding temperature.

Determination of sodium chloride

Sodium chloride content in soymilks was determined according to the standard method (86).

Flavour evaluation

The beany flavour of the various soymilk preparations was evaluated by a trained taste panel of eight judges who had demonstrated an ability to distinguish correctly the intensity of beany flavour in different dilutions of the same soymilk sample. The sodium chloride content of all soymilks within each series was adjusted to an equal level before presenting them for flavour evaluation. The judges were asked to score samples within a range from 6 points for very strong beany flavour to 1 for no beany flavour.

Lipoxidase activity

Lipoxidase activity was determined by the cup-plate technique of Blain & Todd (25) in which the extent of enzyme activity is indicated by the diameter of the zone of carotene bleaching.

2.4.3 Results

Effect of nature of the soaking agent on the beany flavour of soymilk

A series of soymilks was prepared from beans soaked in solutions of Na_2CO_3, $NaHCO_3$, NaOH, Na_2SO_4, Na_2SO_3, Na_2HPO_4, Na_3PO_4, NaCl, or $Na_2S_2O_5$ plus NH_4OH. These milks were then presented to the taste panel for evaluation of intensity of the beany flavour. The control milk sample used in the experiments was made from soybeans soaked in water. The results are given in Table 1. Of the different soak solutions examined, at the concentrations used, sodium carbonate and sodium hydroxide were found to produce highly significant reductions in beany flavour. The results also indicate that the effect of sodium carbonate is significantly greater than that of sodium hydroxide. During the experiments, it was found impracticable to use sodium hydroxide concentrations higher than 0.2 M and still maintain constant conditions of processing because beans soaked in stronger caustic solutions absorbed all the available water during grinding and the gel could not then effectively be re-dispersed, or if originally dispersed in a greater quantity of water it would still not filter. Centrifugal separation was possible but the product was still not as good as that from beans presoaked in 0.4 M sodium carbonate.

TABLE 1. Mean sensory scores for beany flavour of soymilks prepared from beans soaked for 24 hours in the pretreatment media.

Soaking medium	Mean scores[a] (n = 8)	Significance of difference from ‡
Water	5.75‡	
0.5 M-Na_2CO_3	2.65†	***
0.2 M-NaOH	4.12††	**
0.5 M-Na_2SO_4	4.90	n.s.
0.5 M-Na_2SO_3	5.00	n.s.
0.5 M-Na_2HPO_4	5.25	n.s.
0.5 M-$NaHCO_3$	4.90	n.s.
0.5 M-Na_3PO_4	4.75	*
0.8 M-NaCl	5.25	n.s.
0.5 M-$Na_2S_2O_5$ + NH_4OH	5.12	n.s.

[a]Score of 6 = very strong beany flavour; 1 = no beany flavour

*, **, *** significant at $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively

Difference between † and †† is significant at $P < 0.05$

Effect of concentration of soaking agent and time of soaking on reduction of beany flavour of soymilk

The beany flavour of soymilks prepared from beans soaked in varying concentrations of either sodium carbonate or sodium hydroxide was also assessed and the results are shown in Table 2. In the case of preparations soaked in sodium carbonate, the best scores in terms of reduced beany flavour were obtained using concentrations of 0.5 M and 0.4 M in the soaking medium. Since both milks concerned received identical mean scores there was no advantage in using sodium carbonate concentrations higher than 0.4 M under the conditions used. The other milks tested received significantly higher scores in terms of beaniness.

TABLE 2. Effect of variation of concentration of Na_2CO_3 and NaOH in soak solution on reduction of beany flavour in soymilks prepared after 24 hours of soaking.

Soaking medium	Mean scores[a] (n = 8)	Significance of difference from ‡
Water	5.75‡	
0.5 M-Na_2CO_3	2.62	***
0.4 M-Na_2CO_3	2.62	***
0.3 M-Na_2CO_3	3.75	**
0.2 M-NaOH	4.12	**
0.1 M-NaOH	4.75	*

[a]Score of 6 = very strong beany flavour; 1 = no beany flavour

*, **, *** significant at $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively

TABLE 3. Effect of variation in time of soaking in 0.4 M-Na_2CO_3 and 0.2 M-NaOH solution on reduction of beany flavour in soymilks.

Soaking medium	Time of soaking, h	Mean scores[a] (n = 8)	Significance of difference from ‡	Significance of difference from †
Water	24	5.75‡		
0.4 M-Na_2CO_3	24	2.62†	***	
	18	3.00	***	n.s.
	12	4.00	***	**
0.2 M-NaOH	24	4.12	**	-
	18	4.25	**	-
	12	4.25	**	-
	8	4.50	*	-

[a]Score of 6 = very strong beany flavour; 1 = no beany flavour

*, **, *** significant at $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively

Table 3 shows the results of the taste panel evaluation of the intensity of beany flavour in various soymilk preparations from beans soaked in 0.4 M sodium carbonate or 0.2 M sodium hydroxide for various times. These demonstrate that there is no significant difference in the beany flavour of soymilk whether the beans were soaked for 18 or 24-h (sodium carbonate) or 12, 18 or 24-h (sodium hydroxide). Once again it was found that sodium hydroxide, at the maximum possible concentration used for the optimum time period, was considerably less effective than sodium carbonate in reducing the beany flavour.

Effect of extraction temperature on the lipoxidase activity and beany flavour of soymilks

Soybeans were soaked in solutions of water for 24-h, 0.4 M sodium carbonate for 24-h and 0.2 M sodium hydroxide for 12-h. Two series of soymilks were then prepared from these beans, using in one series a grinding temperature above 70°C to destroy the lipoxidase activity whilst the other grinding was done at room temperature. Both milks were presented to the taste panel for evaluation of the intensity of beany flavour and were also tested for lipoxidase activity. The results, reported in Table 4, show that there is no significant difference in intensity of beany flavour in soymilks prepared using either cold or hot grinding temperatures, although lipoxidase activity was not measurable after grinding at the higher temperature.

TABLE 4. Effect of water temperature during grinding on the lipoxidase activity and the beany flavour of soymilks

Soaking medium	Temperature during grinding, °C	pH during grinding	Diameter of the zone, cm	Mean scores[a] (n = 8)
Water	19 - 30	6.8	2.05	5.11x
	80 - 82		0	5.11x
0.4 M-Na_2CO_3	19 - 30	9.7	1.68	2.88y
	70 - 75		0	2.55y
0.2 M-NaOH	19 - 30	10	1.60	4.33z
	70 - 75		0	3.78z

[a]Score of 6 = very strong beany flavour; 1 = no beany flavour

Mean scores associated with the same letter are not significantly different.

2.4.4 Discussion

The results demonstrate that soaking soybeans in dilute solutions of sodium carbonate and sodium hydroxide as a pretreatment for the production of soymilk markedly reduces the beany flavour when compared with milk prepared by the standard water soak procedure.

The effect of sodium hydroxide soaking was not as marked as that of sodium carbonate.

The optimum conditions for reducing the beany flavour in soymilk were: presoaking the beans in 0.4 M-Na_2CO_3 for 18-24-h. Under these conditions a very significant reduction in beany flavour was achieved over that in soymilks prepared from beans presoaked in water or from beans presoaked in 0.2 M-NaOH for 12-24-h.

In discussing oxidative off-flavours of soymilk Wilkens et al (269) state that an acceptable, bland milk can be produced by grinding unsoaked, dehulled soybeans with water at temperatures between 80 and 100°C and by maintaining this temperature for 10 min to inactivate the lipoxidase enzyme completely. Mattick and Hand (173) state that the oxidative activity of lipoxidase contributes to the overall total raw bean flavour of the soymilk; they identified ethyl vinyl ketone as one of the raw, bean-like flavour components and they postulate that this is derived from linolenic acid through lipoxidase enzyme action.

The results from this study suggest that the contribution to the beany flavour by lipoxidase activity is relatively small, as indicated by the small difference in flavour scores of the extracts after cold and hot grinding of the alkaline presoaked beans. With the beans presoaked in water, lipoxidase activity did not appear to affect the beany flavour at all unless its action had occurred during the presoak period or during the fraction of time before the soaked beans reached the grinding temperature.

Lipoxidase activity in the samples ground at the lower temperature was similar for the two alkaline soaks and the pH of the slurry was within the range for optimum activity of soybean lipoxidase, yet there was a significant difference between the beany flavour scores of these two milks. Despite the milder conditions of the carbonate soaking system which should have favoured lipoxidase activity more than the conditions in the hydroxide soak, the beany flavour is much less in the former sample. It seems very likely that there are more significant factors than lipoxidase activity responsible for this beany flavour.

Recently Sessa et al (238) reported that the beany, bitter, green and other flavour characteristics of raw full-fat, and defatted flakes may preexist in the whole soybeans. The apparent low level of oxidation that does occur contributes little to the overall soybean flavour.

CHAPTER 3

EFFECT OF PREPROCESSING CONDITIONS ON THE COMPOSITION AND THE QUALITY OF THE RESULTING SOYMILKS

3.1 Introduction

The composition of soymilk will have a relatively wide variation for several reasons. There are some marked variations in the composition of different varieties of soybeans (197), but more important deviations will result from those factors which influence the extraction procedures such as the fineness of grinding of the mass, ratio of the water to beans (255), and the temperature of the extraction (162). The addition of oil or fat, sugar, salt and flavouring materials may be the cause, however, of the greatest variation in the milk composition. The composition of soymilk, compiled from various sources by Burnett (46), is compared with cow milk in Appendix 2.

Wilkens and Hackler (270) studied a range of possible preprocessing soaking conditions to determine the influence of such parameters on the composition and yield of soymilk using water as the soaking medium. They showed that the time and temperature of soaking as well as the temperature during grinding affects the yield and composition of soymilk. It has long been known that the dispersibility of soyprotein is higher in alkaline solution than it is either in potable waters or in neutral salt solutions and usually the extraction is carried out at pH 9.0 (244).

Cystine is unstable in the presence of alkalies (62, 100).

Deaminisation, elimination of sulphur from the molecule, racemisation and reduction to cysteine are known to occur. Jones and Gersdorff (136) showed that the method commonly used for the preparation and purification of casein by dissolving in dilute sodium hydroxide and precipitating the protein from the alkaline solution by addition of acid causes an appreciable destruction of cystine. The amino acids of casein other than cystine are not greatly affected by this alkaline extraction process.

3.2 Objective and Experimental Plan

The method developed for the production of bland-flavoured soymilk involves presoaking the beans in alkaline solutions. This pretreatment may cause variation in crude composition of soymilk as well as the amino acid pattern of proteins. The work reported in this chapter was, therefore, initiated to investigate the effect of pretreatment on the product quality and processing characteristics.

3.3 Experimental

3.3.1 Methods

Preparation of soymilk

Soymilks used in the present study were prepared from beans presoaked either in 0.4 M-Na_2CO_3 solution for 24 hours or 0.2 M-NaOH for 12 hours or water for 24 hours according to the method described in Chapter 2.

Proximate analysis of soymilk

Moisture, ash and crude fibre contents were determined by the methods of analysis of the A.O.A.C. (13). Nitrogen was determined by the Kjeldahl method using a macro-digestion procedure followed by a semi-micro distillation of the ammonia produced into two percent boric acid solution containing mixed indicator (203) and protein content was calculated using a conversion factor of % N x 6.25. The fat content was determined by the Gerber method (42) and carbohydrate level was estimated by difference.

Viscosity measurement

The viscosity of the soymilk samples was measured at 25°C with an L.V. Model Brookfield Synchro-lectric viscometer at a speed of 30 rev/min using U.L. adapter. The absolute viscosity ($\eta_{abs.}$) was calculated using appropriate factor as follows:

$$\eta_{abs.} = (\text{Dial reading} - 0.1) \times 0.2 \text{ cps.}$$

The specific viscosity (η_{sp}) was then calculated by using the formula (132).

$$\eta_{sp} = \frac{\eta_{coll} - \eta_{sol.}}{\eta_{sol.}}$$

where η_{coll} = absolute viscosity of the colloidal solution and

η_{sol} = absolute viscosity of the solvent.

The reduced viscosity is then obtained by dividing the specific viscosity by the concentration of the colloid, i.e. $\frac{\eta_{sp}}{C}$. The concentration C is usually expressed in grams per 100 cm^3 of solution.

Amino Acid Analysis of Proteins in Soymilks

(a) Preparation of sample for amino acid analysis other than for cystine and tryptophan. Sample, containing 36 mg protein, was introduced into a pyrex-glass tube of 40 ml capacity followed by 36 ml of 6N-HCl. The tube had been constricted at the neck by the use of heat before connecting with a vacuum pump. The mixture was frozen by placing the tube in liquid air and the tube was evacuated to a residual pressure of about 50 microns of Hg, the contents were then allowed to melt so that any entrapped air bubbles could escape; after refreezing the contents, the tube was sealed. Hydrolysis of the sample was carried out at 110 $\pm$ 2°C in a hot air oven for 24 hours. After removal from the oven and cooling to room temperature, the tubes were broken open. The acid from the hydrolysate was removed under vacuum to near dryness with a Buchi rotary evaporator using a bath temperature of 50°C, followed by evaporation three times with small amounts of added water. The hydrolysate was a very pale yellow and any insoluble humin was removed by filtration through Whatman No.52 filter paper. The concentrate was made up to 10 ml with sodium citrate buffer, pH 2.2, and the sample was kept frozen until analysed. The samples were analysed within a week of preparation.

(b) Preparation of sample for the analysis of cystine. Cystine content in soymilk was determined in the form of the more stable derivative, cysteic acid, by using the method of Schram et al (237).

Sample, containing 36 mg protein, was weighed into a 250 ml round-bottom flask, 25 ml of performic acid reagent, previously cooled to 0°C, were added. (The performic acid reagent was prepared by the addition of 1 vol. of 30 % ($^{W}/_{W}$) H_2O_2 to 9 vol. of 88 % ($^{W}/_{W}$) formic acid. The solution was allowed to stand for 1 hour at room temperature to permit the performic acid concentration to reach maximum value). The oxidation was allowed to proceed at 0°C for 4 hours. At the end of the reaction time most of the reagent was removed under reduced pressure at a bath temperature of 40°C on a rotary evaporator. To hydrolyse the proteins, the residue from the oxidation was immediately dissolved in 36 ml of 6N-HCl and boiled under reflux for 24 hours at atmospheric pressure in an open system. The rest of the procedure was the same as described in (a).

(c) Preparation of sample for the analysis of tryptophan. The tryptophan content was measured in an alkaline hydrolysate. To a sample representing 36 mg protein in a pyrex-glass tube (0.9 x 25 cm), 2.0 g of barium hydroxide octahydrate, previously ground to a fine powder was added, followed by 1 ml of water. The contents were frozen, the tube was evacuated to a residual pressure of 50 microns of Hg, sealed and hydrolysis was then carried out at $110^{o} \pm 2^{o}C$ in a hot air oven for 24 hours as in (a). After hydrolysis, 6N-HCl was added slowly to the mixture until the pH had dropped to 2 and then 1.0 g. of anhydrous sodium sulphate was added to remove Ba^{2+} by precipitation as $BaSO_4$ (186). The precipitate was removed by filtration through Whatman No.52 filter paper. The rest of the procedure was the same as in (a).

(d) Amino Acid Analysis. Analyses for the amino acid content were made by ion exchange chromatography according to the procedures of Spackman et al (249) and Moore et al (194) with a Beckman-Spinco model 120C amino acid analyser. Type PA-35 resin was used for the separation of basic amino acids and type PA-28 resin for all acidic and neutral amino acids. Basic amino acids were separated on a 5.5 cm column using pH 5.28 buffer. The acidic and neutral amino acids were separated on a 56 cm column using pH 3.25 buffer followed by pH 4.26 buffer (for composition of buffers, see Appendix 3). The standard 4 hour procedure was used and the operation conditions are summarised in Appendix 4.

(e) Calculations. The amount of each component amino acid in the sample analysed was determined by measuring the area enclosed by its corresponding peak on the chromatograms. The height-width (HW) method was used for the integration of peaks. The height of the peak was easily determined from the chart. The width of a peak was measured in terms of time by counting the number of dots printed above the half-height of the peak. The micromoles of each amino acid were calculated by using the following formula, which relates the peak area (H x W), the concentration (in μ moles), and the HW constant C_{HW}, i.e. $\mu \text{ moles} = \frac{H \times W}{C_{HW}}$.

3.3.2 Results

Effect of soak solution on the composition and viscosity of the soymilk and on the protein recovery

The composition of soymilks prepared from beans soaked in water for 24-h, 0.4 M sodium carbonate for 24-h and 0.2 M sodium hydroxide for 12-h are compared in Table 5. The gross composition of soymilk prepared from beans soaked in sodium carbonate does not show measurable differences from that of the control milk prepared from beans soaked in water. The milk prepared from beans soaked in sodium hydroxide contains lower percentages of protein, fat, ash and carbohydrate (by difference) than either of the others.

Table 6 shows that the viscosity of the soymilk prepared from beans soaked in sodium carbonate is much higher than that of the control milk or of the milk prepared from beans soaked in sodium hydroxide. The recovery of protein in the form of soymilk is slightly higher for sodium carbonate than for the control water soak but in the case of sodium hydroxide soak it was significantly reduced.

TABLE 5. Proximate analysis of various prepared soymilks.

	Soymilks from beans presoaked in		
	Water	0.4 M-Na_2CO_3	0.2 M-NaOH
Moisture, %	93.36	93.62	95.01
Crude protein, %	3.62	3.76	2.87
Fat, %	1.50	1.50	1.20
Ash, %	0.50	0.43	0.36
Crude fibre, %	0.05	0.06	0.09
Carbohydrate, % (by difference)	0.97	0.63	0.47

TABLE 6. Effect of soaking as a pre-treatment on the protein recovery and viscosity of the prepared soymilks.

Soaking medium	Time of soaking, h	Protein recovery as % of protein in cotyledon fraction	Reduced viscosity of milk($\frac{\eta_{sp}}{C}$)
Water	24	74.0	0.32
0.4 M-Na_2CO_3	24	76.8	0.83
0.2 M-NaOH	24	45.1	-
0.2 M-NaOH	12	52.3	0.42

Loss of crude proteins in the soaking solution

The loss of soluble nitrogen, calculated as crude protein, in the soaking solution is shown in Table 7. The data indicate that the loss of crude protein into the sodium hydroxide soak solution is 7 - 8 times more than the loss in water and is 4 times more than the loss in the sodium carbonate soak solution. The loss in the sodium

TABLE 7. Loss of soluble nitrogen, expressed as crude protein, into soaking solution.

Soaking medium	Time of soaking at room temperature, h	Protein loss, g/100 g soybean
Water	24	0.43
0.4 M-Na_2CO_3	24	0.77
0.2 M-NaOH	24	3.36
0.2 M-NaOH	12	3.01

carbonate soak solution was less than twice that with the water soak and amounted to less than 2 percent of the original nitrogen in the beans.

Effect of soaking on the amino acid composition of protein in soymilks

The amino acid analysis of soyprotein in the form of various soymilk preparations are set out in Table 8. The results show that histidine content in soymilk prepared from beans presoaked in sodium hydroxide is less than that either in water or carbonate presoaked preparations. The recovery of cystine is slightly lower in milk from carbonate presoaked beans and even lower in hydroxide preparation than it is in milk prepared from water-presoaked soybeans. However, statistical analysis of the results showed that there was no significant difference (at the $P = 0.05$ level) between the amino acid contents in milks prepared by the three different procedures. Therefore, the pretreatment given to the soybeans by soaking in 0.4 M sodium carbonate solution for 24 hours and 0.2 M sodium hydroxide solution for 12 hours before manufacturing soymilk had no significant effect on the amino acid composition when compared with the milk prepared from water presoaked soybeans.

TABLE 8. Amino acid composition of proteins in various soymilk preparations (g amino acid/100 g protein)

	Soymilk prepared from beans soaked in		
	Water	0.4MNa_2CO_3	0.2MNaOH
Lysine	5.42 ± 0.16	5.72 ± 0.20	5.48 ± 0.17
Histidine	1.71 ± 0.02	1.70 ± 0.05	1.42 ± 0.02
Arginine	5.89 ± 0.03	6.18 ± 0.08	5.67 ± 0.29
Aspartic acid	12.51 ± 0.03	12.18 ± 0.05	12.43 ± 0.06
Threonine	3.69 ± 0.33	3.24 ± 0.01	3.21 ± 0.00
Serine	4.31 ± 0.31	4.51 ± 0.17	4.13 ± 0.02
Glutamic acid	23.57 ± 0.35	23.49 ± 0.04	24.30 ± 0.43
Proline	7.98 ± 0.29	8.07 ± 0.14	8.33 ± 0.12
Glycine	3.44 ± 0.07	3.63 ± 0.02	3.47 ± 0.10
Alanine	3.41 ± 0.03	3.50 ± 0.03	3.51 ± 0.07
½-Cystine	1.39 ± 0.11	1.34 ± 0.04	1.22 ± 0.00
Valine	3.90 ± 0.06	4.03 ± 0.06	4.05 ± 0.04
Methionine	1.00 ± 0.02	1.03 ± 0.04	0.95 ± 0.06
Isoleucine	4.60 ± 0.04	4.61 ± 0.12	4.78 ± 0.02
Leucine	8.25 ± 0.06	8.26 ± 0.00	8.21 ± 0.05
Tyrosine	3.17 ± 0.04	3.04 ± 0.09	3.21 ± 0.08
Phenylalanine	4.50 ± 0.22	4.26 ± 0.20	4.45 ± 0.07
Tryptophan	1.28 ± 0.03	1.20 ± 0.03	1.20 ± 0.08

3.3.3 Discussion

Lo et al (162) have reported that there is a loss of soluble solids into the water presoak liquor and that the extent of this loss increases with time. The present results confirm that a loss of soluble nitrogen occurs into the soak solution; that the loss increases in the presence of alkali; and the loss in 0.2 M-NaOH after only a 12-h soak is four times greater than that in 0.4 M-Na_2CO_3 after a 24-h soak.

Soaking in sodium hydroxide solution reduced the yield of soluble protein, fat and ash when compared with both the beans soaked in water and in sodium carbonate. The yield of both fat and protein was lower by about 20 percent. The smaller amount of protein extracted into solution and the lower viscosity of the milk prepared from the beans soaked in sodium hydroxide suggest that the protein had swelled or interacted to a far greater extent during the soaking than it did in the sodium carbonate soak. This modified protein was then unable to diffuse from the cotyledon residue during the subsequent grinding and extraction operations. The loss of nitrogen into the presoak medium was also much greater in the hydroxide presoak system. The effects are a disadvantage if soymilks having the maximum soluble protein are to be produced economically. In the hydroxide-soaked system, there was also a lesser extraction of fat into the milk, suggesting that there was a direct interference with the diffusion of small molecules as well as of the larger protein molecules. Wilkens and Hackler (270) also report a drastic reduction in the total solids of milk prepared after soaking in water at 80°C or higher. The mechanism here is probably different.

The soymilks prepared from beans after alkali presoaks both possessed higher reduced viscosities than that made from beans presoaked in water, and the milk after carbonate soaking had a viscosity which was appreciably higher than that of milk prepared after hydroxide soaking. This again suggests that there had been selective diffusion of only the smaller protein molecules into the milk after sodium hydroxide soaking.

The increased viscosity of the milks prepared after alkali presoaking was noted by the taste panel who expressed preference for the carbonate presoaking process as giving a milk with a better mouthfeel and body.

It was also found much easier to dehull beans soaked in carbonate solution than those soaked in water, there being less breakage of the cotyledons and less loss of cotyledon material. On the other hand, beans soaked in 0.2 M-NaOH became very soft and there was a greater loss of cotyledon material during dehulling.

The treatment given to the beans in the present study does not significantly affect the amino acid composition of the protein in soymilk.

3.3.4 Conclusion

Presoaking in 0.4 M sodium carbonate thus has advantages in three important aspects - better flavour and body, better yield of protein and fat, and easier processing - and this has been achieved without significant effect on the amino acid composition.

CHAPTER 4

EFFECT OF PROCESSING CONDITIONS ON THE DESTRUCTION OF TRYPSIN INHIBITOR ACTIVITY IN VARIOUS SOYMILK PREPARATIONS

4.1 Introduction

It is ironic that nature having generously provided man with a liberal supply of plant protein foods, has as the same time seen fit to contaminate these foods with a variety of "toxic" or "antinutritional" substances. The soybean contains a number of antinutritional substances which interfere with the utilisation of its protein. The tremendous amount of work which has been performed on these antinutritional substances stems from the important role which the soybean has assumed in the fields of animal and human nutrition.

4.2 Review of literature on the "antinutritional" substances

Osborne and Mendel (208) observed that young rats fed on a diet in which the soyflour component had been heated in an autoclave, grew much better than those receiving the same diet but with the soyflour in the raw state. This observation has been confirmed by numerous investigators who established that the favourable effect of heat treatment upon the nutritive value of soyproteins appears not only in rats but also in other monogastric species including humans (154). Three constituents are often associated with the poor nutritive value of unheated soybeans: a) trypsin inhibitors; b) haemagglutinin; and c) saponins.

a) Trypsin Inhibitors. In 1944, the presence of a trypsin inhibitor in soybeans was discovered independently by Ham and Sandstedt (109) and by Bowman (36). A year later, Kunitz (143) reported crystallisation of a trypsin inhibitor. The general molecular properties of the Kunitz inhibitor are well established. The molecular weight is close to 21,500 (275). The molecule consists of a single polypeptide chain, cross-linked by two disulphide bridges (252). The solubility properties are those of a globulin, with a distinct minimum in solubility at the isoelectric point, pH 4.5 (144). The best known property of the Kunitz inhibitor is its ability to form, at neutral pH, a one-to-one stoichiometric complex with trypsin (148). The complex which can be crystallised is devoid of proteolytic activity. While appreciable interaction also occurs with chymotrypsin, the magnitude of the association does not approach that for trypsin (148).

Bowman (37-39) believes that soybeans contain three different substances capable of inhibiting trypsin. These substances are distinguished on the basis of their solubility but have not been isolated in a very highly purified form. Recently, four (227) to seven (251) inhibitor fractions have been separated from soybean whey proteins by diethyl aminoethyl (DEAE) cellulose chromatography. One fraction had properties similar to the crystalline inhibitor (227).

Several biological activities are attributed to the trypsin inhibitor. The literature on this subject is voluminous and often controversial (see review in Appendix 5).

b) Haemagglutinin. Liener (157) observed that a concentrate of the crude extract of soybean trypsin inhibitor caused the death of rats when administered by intraperitoneal injection, while the crystalline material was innocuous under the same conditions. Continuing his studies Liener (158) was able to isolate and purify another protein from the crude inhibitor concentrate which, when injected into rats was found to have toxic properties.

In contrast to the crystalline trypsin inhibitor, the haemagglutinin is an albumin, being soluble in water at its isoelectric point. Its isoelectric point is 6.1 and molecular weight is between 89,000 - 105,000, which is higher than that of the trypsin inhibitor. Another major difference is in its multichain structure (213).

Liener (159) reported that haemagglutinin comprising 1 percent of a diet containing heated soybean meal caused a significant depression in the growth of rats. It was estimated that the soybean haemagglutinin accounted for about one half of the growth inhibition that is obtained when raw soybean replaces the heated soybean in a diet. Growth impairment by haemagglutinin was originally attributed to a decrease in the quantity of food consumed.

Experiments by Jaffé and co-workers (127, 128) provide a possible explanation for the deleterious effects of the orally ingested haemagglutinin. Jaffé believes that the action of the haemagglutinin is to combine with the cells lining the intestinal wall (in much the same fashion as it combines with red blood cells), thus causing a non-specific interference with the intestinal absorption of all nutrients. Liener is also in agreement with Jaffé's theory (160).

c) Saponins. Saponins are known to cause haemolysis of red blood cells in vitro (23). Potter and Kummerow (221) reported that incorporation of saponin into feeds for chicken caused retardation of growth. Birk et al (23) found saponins inhibit to some extent the proteolytic activity of chymotrypsin and of *Tribolium castaneum* larval midgut enzyme solution but in a later report, Ishaaya and Birk (125) showed that considerable amounts of saponins can inhibit these enzymes to only a certain extent and this inhibition is not a specific one but results from a protein-saponin interaction and this enzyme inhibitory effect can be overcome by adding casein and soybean proteins. Recently Gestetner et al (98) showed no growth impairment of chicks, rats or mice when soybean saponins are added to their diets. Birk et al (23) presented evidence for the heat stability of soybean saponins in contrast to the earlier suggestion that cooking of meal caused hydrolysis of saponin (222).

Recently, it has been concluded (23, 271) that soybean saponin may be considered as not interfering with digestion or metabolism.

Elimination of Anti-nutritional Effect

The literature on the anti-nutritional factors of the soybean suggests that the retardation of growth using raw soybean products is the result of the inhibitors first accentuating the digestive losses of nutritive material and secondly of increasing the need for certain nutrients.

There is no doubt that by correcting the nutritional deficiencies of a raw soybean product, the retardation effect on growth can be cancelled or at least mitigated (31, 32, 84, 114, 115) but this symptomatic treatment is neither specific nor profitable. Since they do not act upon the inhibitors, the addition to the diet of sulphur amino acids or of a mixture of essential amino acids, although preventing inhibition of growth, do not prevent either the hypertrophy of the pancreas with the consequent loss of endogenous nitrogen, or the increased consumption of cystine. Moreover, the supplementation with these amino acids is very expensive. Therefore, it was concluded that this solution is not practicable (99).

The possibility of inactivating anti-nutritional substances contained in raw soybeans was indicated by the fact that trypsin inhibitors as well as haemagglutinins are thermolabile and numerous experiments have shown that heat treatment improves the nutritional value of soybeans (154).

Klose et al (142) showed that the effect of heat treatment on the nutritional quality of soybean products depends on the intensity (temperature and duration of exposure) of heat and upon the state of the substrate exposed (presence or absence of water) to the heat. Osborne and Mendel (208) reported that dry heat in an oven at 110°C for up to 4 hours produced no improvement in growth promoting activity of soybeans. However, if the beans were cooked with water as a thick paste at 80 - 90°C for 3 hours and incorporated in the ration, they permitted rats to grow at a normal rate. Maximum improvement in the protein efficiency ratio (PER) occurred when the raw soybean meal was steamed at atmospheric pressure for 30 minutes. When autoclaved at 15 psi (121°C) maximum improvement in the meal occurred after 15 minutes of heating. Longer heating produced a linear reduction in PER with the meal autoclaved for 180 minutes having the same value as the unheated meal.

In contrast to this, Smith et al (246) reported that regardless of whether the soybean flakes containing 19 percent moisture, were steamed at 100°C for 15 or 120 minutes, the same improvement in biological value occurred. When soyflakes containing only 5 percent moisture were steamed at 100°C for 15 minutes they produced as good growth as those that had 19 percent moisture prior to heating. However, heating the soyflakes that had 5 percent moisture for periods longer than 15 minutes reduced both body weight gains in rats and the biological value of the protein.

Jacquat (126) showed that using the same temperature and time of treatment, the improvement in nutritive value obtained by dry heat amounted to 25 percent whereas in the presence of at least 10 percent of water the improvement reached 360 percent and when water was in excess the improvement rose to 500 percent.

Fritz et al (93) reported that the best biological value was obtained when ground soybeans were autoclaved at 15 lbs pressure (121°C) for 20 - 30 minutes whereas Rackis (228) showed that at 100°C only 15 minutes of steaming is required to obtain maximum protein efficiency values and to inactivate the trypsin inhibitors of either full-fat or defatted soybean flakes of about 0.01 inch thickness. These differences may merely represent the effects of heat transfer consequent on the different thicknesses of the raw materials used.

Diser (66) reported that maximum nutritional value is obtained when raw soybeans are treated with steam at 15 lbs pressure (121°C) for 10 - 15 minutes or at atmospheric pressure for 30 minutes or when soybean flour is heated at 5 lbs pressure (108°C) for 15 - 30 minutes. Gontzea and Sutzeseu (99) stated that it has been observed that for soy products the maximum advantage can be obtained by autoclaving at 115°C for 20 minutes or at 107 - 108°C for 40 minutes, but with some varieties of beans it may be necessary to keep them for at least 30 minutes at 121°C.

Hackler et al (104) reported on the effects of both temperature and time on the destruction of the trypsin inhibitor in soymilk prepared from water presoaked beans. Their data gives such a wide range in the percentage destruction of the trypsin inhibitor both at 93°C and 121°C in different experiments that no consistent pattern is apparent. They attribute these variations to inadequate cooling in some of their treatments.

All of the heat treatments discussed above were carried out at neutral pH values. Kunitz (145) studied the effect of heating at acid pH on the inactivation of trypsin inhibitor and reported that when a solution of the soybean trypsin inhibitor in dilute acid at pH 2.5 to 3.5 is heated to 90°C, the inhibitor is immediately and completely denatured. The denaturation was reversible since the protein on cooling gradually reverted to the native, soluble state. The reversal is complete if the cooling is initiated within a minute or so after the protein solution has been brought to 90°C. The longer the protein solution is kept at 90°C, the less will be the reversal on cooling.

Since haemagglutinating activity in raw soybean is more readily destroyed by moist heat than is the trypsin inhibitor (161), then there should not be any problem with it in soymilk that has received an adequate heat treatment to inactivate the trypsin inhibitor. Van Buren et al (261) stated "the level of trypsin inhibitor remaining in the soy product can serve as an index of the adequacy of heat treatment".

Westfall and Hauge (267) stated "the determination of the inhibitor potency of soybean products may be used as a practical index of the effectiveness of the heating necessary to increase the protein quality, providing that the heating is not continued beyond the time required for the total destruction of inhibitors".

4.3 Objective and Experimental Plan

The literature indicates that very little information is available on the inactivation of trypsin inhibitor in soymilk. Most of the reports on the effect of heat-treatment on nutritional quality were centered on soybean meal. There is, however, no unanimity as to the quantitative effect of different heating conditions. The method developed in this study for the manufacture of soymilk is different from the conventional method, and therefore, it was necessary to establish the optimum processing conditions for completely destroying the anti-nutritional factors and for avoiding over-processing which could cause damage to the proteins (see Chapter 5). The purpose of this aspect of the study was to determine the rate and extent of the destruction of the trypsin inhibitor under various heat processing conditions applied to milk prepared from carbonate presoaked beans as well as from beans presoaked in water.

The use of the alkaline soaking method for the production of bland-flavoured soymilk made it necessary to establish more clearly the action of heat on the destruction of trypsin inhibitor activity under alkaline conditions. From the literature, it appears that the destruction of the trypsin inhibitor activity of soybeans has been studied at near neutral pH and at acid pH but the effect of alkaline pH at elevated temperatures on the trypsin inhibitor activity has not been reported. A study was therefore made of the effect of a high temperature (98°C) at pH's between 6.8 and 10 on the rate and extent of destruction of the trypsin inhibitor activity of soybean milk.

4.4 Experimental

4.4.1 Materials

Haemoglobin - Laboratory Reagent, Hopkin & Williams Ltd., England.

Folin & Ciocalteu's Reagent - Laboratory Reagent, The British Drug House Ltd, England.

Trypsin - 10.54 Anson Unit per gramme, The British Drug House Ltd, England.

Tyrosine - Mann Research Laboratories, Inc. New York.

4.4.2 Methods

Preparation and processing of soymilks

The soybeans were soaked in 0.4 M sodium carbonate solution at pH 11.1 or in water for 24 hours as a pretreatment. The soymilks were prepared from the presoaked beans according to the method described previously (Chapter 2). The prepared soymilks, either from carbonate or from water, presoaked beans were adjusted to pH 6.8 where necessary and were then divided into two portions. One portion was processed at 98°C and a sample was taken after every 10 minutes up to 60 minutes. The other portion was divided among seven 211 x 309 cans with a net headspace of 3/16" and processed for 5, 10, 15, 20, 25, 30 and 40 minutes at 115°C. Each milk sample after processing at the specified time and temperature was cooled immediately to 15°C. The pH of all the samples was measured and adjusted to 6.8. Each sample was then freeze-dried.

For the study on the effect of pH and heat on the destruction of trypsin inhibitor activity, the pH of the milk prepared from water presoaked soybeans was adjusted to different levels by adding 1 M-NaOH solution and then processing at 98°C for varying lengths of time. Thereafter the processed milk samples were treated in the same way as above.

Nitrogen determination

The nitrogen contents of the freeze-dried soymilks were determined according to the method described earlier in Chapter 3.

Preparation of trypsin inhibitor extract

Freeze-dried soymilk samples each containing 85 mg N were suspended in 10 ml of 0.05 N HCl and were shaken vigorously for 15 minutes using a Griffin flask shaker. The mixture was kept overnight at 34°F (1°C) and the insoluble materials were removed by centrifugation at 10,000 x g for 10 minutes (35). The supernatant was used as the trypsin inhibitor extract.

Determination of trypsin inhibitor activity

The method of Anson (11) as modified by Borchers et al (35) was used throughout these studies. As carried out in this experiment, 1 ml of the trypsin inhibitor extract was added to 1 ml of 0.1 percent trypsin solution and then made up to 5 ml with distilled water. This was then incubated with 5 ml of 2.2 percent haemoglobin solution, pH 7.6 (See Appendix 6) at 37°C for 7 minutes. The reaction was stopped by adding 10 ml of 0.3 M trichloroacetic acid. The precipitated proteins were removed by filtration through Whatman No.3 filter paper. To 5 ml of the digestion filtrate were added 10 ml of 0.5 M sodium hydroxide solution and 3 ml of the dilute Folin and Ciocalteu's reagent (1 part reagent plus 2 parts water). These were mixed thoroughly and allowed to stand for 5 minutes to develop colour. The

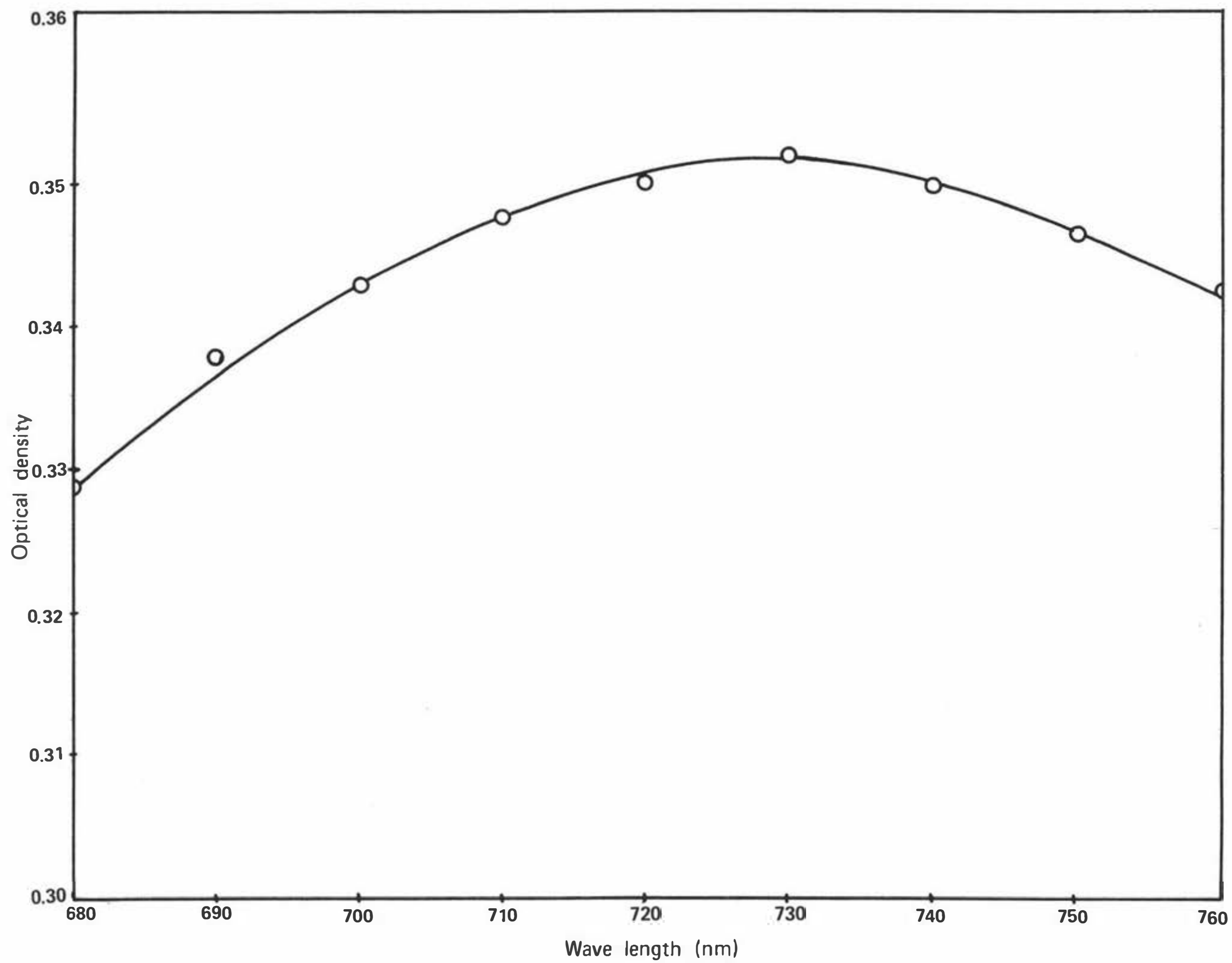

FIG. 3. ABSORPTION SPECTRUM OF THE COLOUR DEVELOPED BY THE CHROMOGENIC SUBSTANCES WITH FOLIN AND CIOCALTEAU'S REAGENT.

intensity of the developed colour was read on a Hitachi spectrophotometer model 101 at 730 nm after experimental establishment that this was the optimum wavelength for adsorption. For experiments without incorporation of trypsin inhibitor, the inhibitor extract was replaced by distilled water. The blank determination was carried out in the same way as above except that trichloroacetic acid was added immediately without prior incubation. The level of chromogenic substances, expressed as tyrosine, liberated due to the action of the trypsin were obtained by reference to a standard curve, By using the trypsin inhibitor activity present in raw soymilks prepared from beans presoaked in water for 24 hours as the maximum activity present (i.e. 100 percent trypsin inhibitor activity) the percentage inhibition resulting from the heat treatment has been calculated.

Determination of optimum wave length for maximum absorption

The wave length of maximum absorption might have changed from that reported in the literature (750 nm (167)) because the system used in this study contains compounds other than the specific chromogenic substances. The absorption spectrum of a typical system was measured from 680 - 760 nm (Fig. 3). The optimum wave length was found to be 730 nm and this was used throughout this study.

Preparation of standard curve

Into a series of test tubes, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ml of standard tyrosine solution containing 1 mole tyrosine per litre were introduced. To make the contents 1 ml in each tube, distilled water was added. In each tube 4 ml of filtrate from the haemoglobin precipitation, 10 ml of 0.5 M sodium hydroxide and 3 ml of dilute Folin and Cioculteau's reagent were added. The blank was the same as above except that the tyrosine solution was

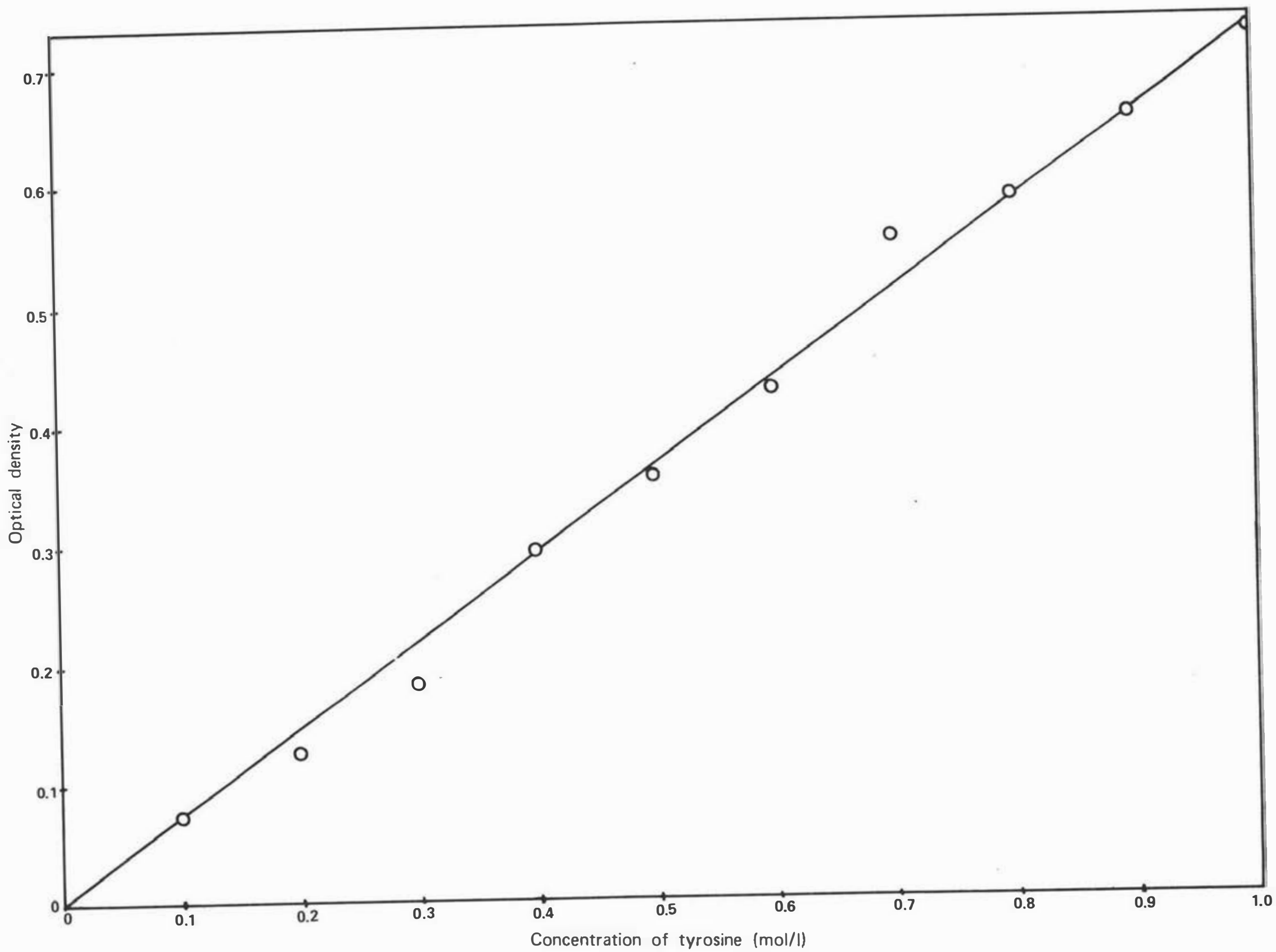

FIG. 4. STANDARD CURVE FOR THE CHROMOGENIC SUBSTANCES EXPRESSED AS TYROSINE

replaced by distilled water. The intensity of the developed colour was measured. Optical density is plotted against the concentration of tyrosine in Fig. 4.

4.4.3 Results

Effect of heat treatment on the inactivation of trypsin inhibitor in soymilks

The results are presented in Fig. 5 and Fig. 6. Fig. 5 shows that there exists a linear relationship between the time of heating at 98°C and the percentage of trypsin inhibitor inactivation in soymilk prepared from water presoaked beans but the relationship is curvilinear in the case of milk prepared from carbonate presoaked soybeans. The rate of inactivation of trypsin inhibitor is much faster with the latter pretreatment. The time taken for complete destruction of the inhibitor activity in soymilk prepared from water presoaked beans was 76 minutes whereas 40 minutes were a sufficient treatment for the milk prepared from carbonate presoaked soybeans. Since the inactivation of trypsin inhibitor in soymilks is slow at 98°C, an experiment was designed to use a higher temperature (115°C) and the results are given in Fig. 6. The rate of inactivation in both soymilks was about the same irrespective of the pretreatment and about 17 minutes were taken for 100 percent inactivation of the inhibitor. The change of pH in soymilks during processing has also been shown in Fig. 5 and Fig. 6. The change was significant only with the milk prepared from carbonate presoaked soybeans when processed at 98°C.

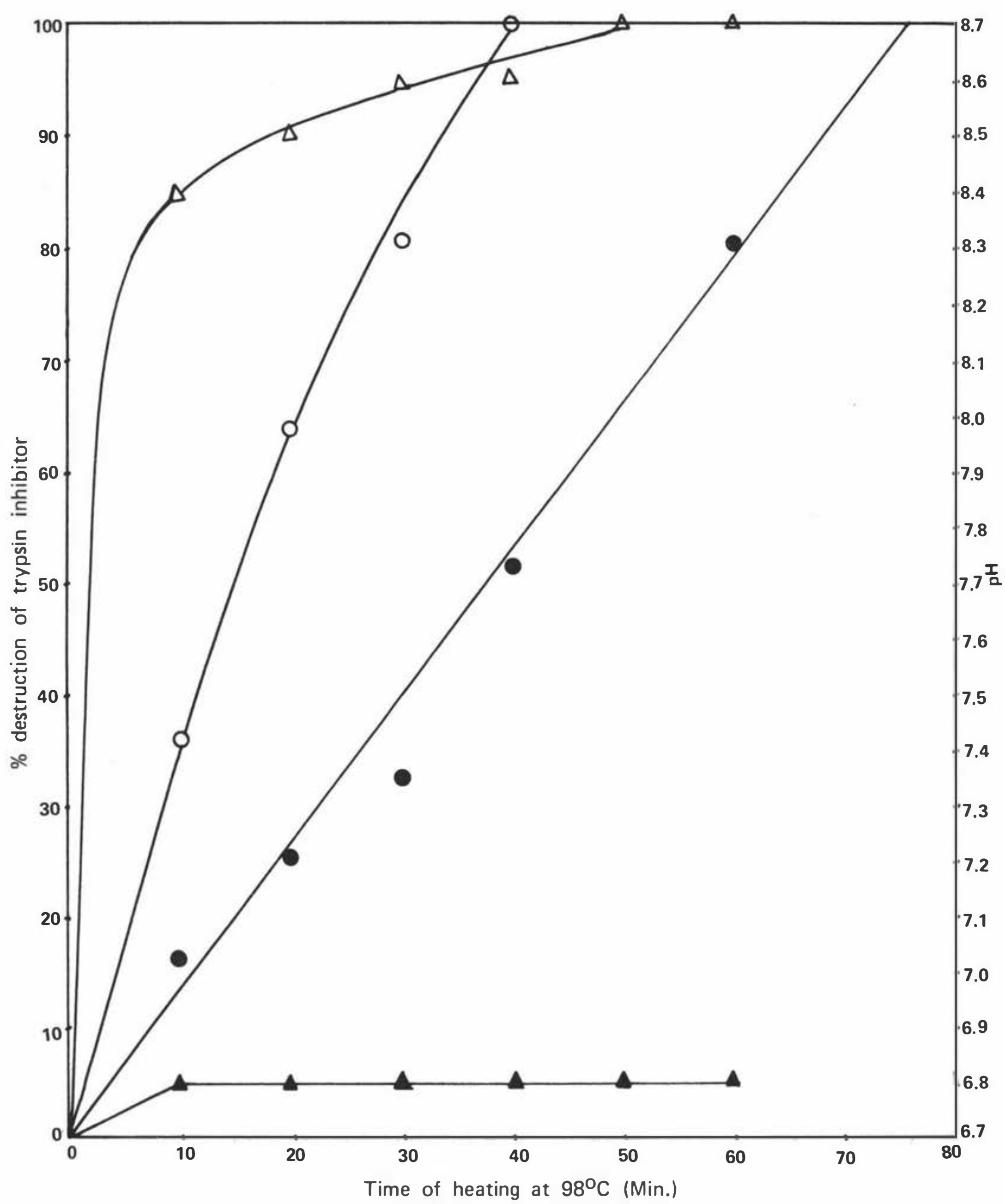

FIG. 5. DESTRUCTION OF TRYPSIN INHIBITOR ACTIVITY AND CHANGE OF pH OF SOYMILKS WITH TIME OF HEATING AT 98°C.

● — ● water presoaked preparation, destruction of trypsin inhibitor;
○ — ○ carbonate presoaked preparation, destruction of trypsin inhibitor;
▲ — ▲ water presoaked preparation, pH change;
△ — △ carbonate presoaked preparation, pH change.

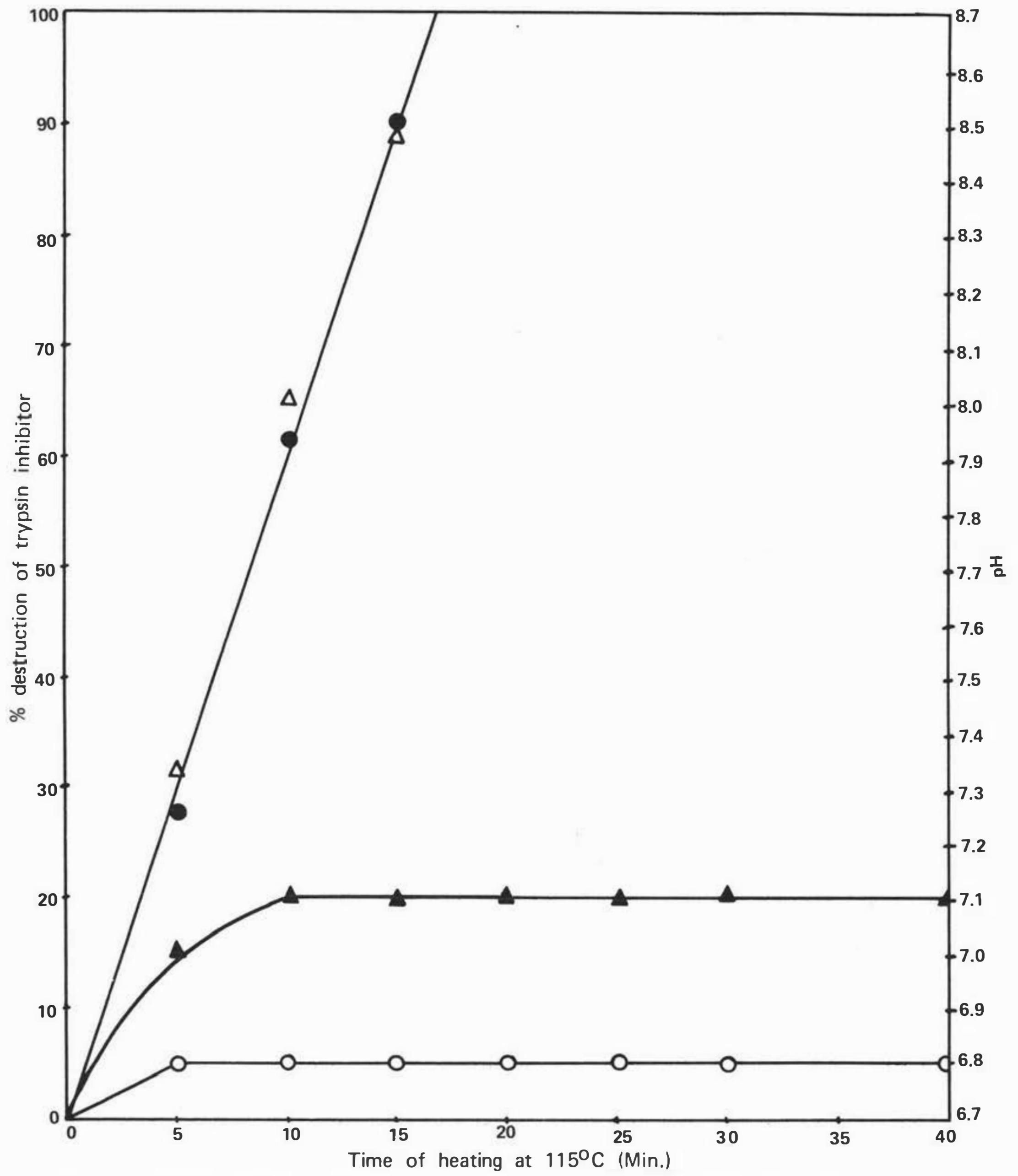

FIG. 6. DESTRUCTION OF TRYPSIN INHIBITOR ACTIVITY AND CHANGE OF pH OF SOYMILKS WITH TIME OF HEATING AT 115°C.

●—● water presoaked preparation, destruction of trypsin inhibitor;
△—△ carbonate presoaked preparation, destruction of trypsin inhibitor;
○—○ water presoaked preparation, pH change;
▲—▲ carbonate presoaked preparation, pH change.

Effect of alkaline pH at elevated temperature (98°C) on the destruction of trypsin inhibitor activity of soymilks

The results are given in Figs. 7 - 9. Fig. 7 shows that when the samples of soymilk were heated at 98°C there was an increased rate of inactivation of the trypsin inhibitor with increase in pH. The time taken for complete destruction of the inhibitor activity in soymilk was reduced from 76 minutes to 11 minutes by increasing pH from 6.8 to 9.9 under the same conditions of heat-treatment. Fig. 8 illustrates that as the pH of the soymilk samples was raised from 6.8 to 9.9, the rate of inactivation of trypsin inhibitor at 98°C progressively approximates more closely to that of first order kinetics. In Fig. 9 the relationship between the time at 98°C and pH is plotted for different levels of inactivation. The range of pH for which these plots apply is limited to the alkaline side of neutrality, because Kunitz (144) has shown that for a specified time and temperature, inactivation also increases with increased hydrogen ion concentration. At 98°C and zero time, the extent of inactivation is indicated by extrapolation. This inactivation at zero time may be reversible, Kunitz (145) working with a temperature of 90°C up to pH 11.0 and time of less than 2 minutes, found that the inactivation could be reversed on cooling, but the extent of this reversal became less as the time of processing extended beyond 2 minutes. Our minimum time of heat treatment was 5 minutes, but at zero time and at the pHs used no trypsin inhibitor destruction was observed prior to heating.

The regression equation between pH and time for 100 percent inactivation at 98°C is

$$Y = 10.63 - 0.05\,X$$

where Y = pH and X = time

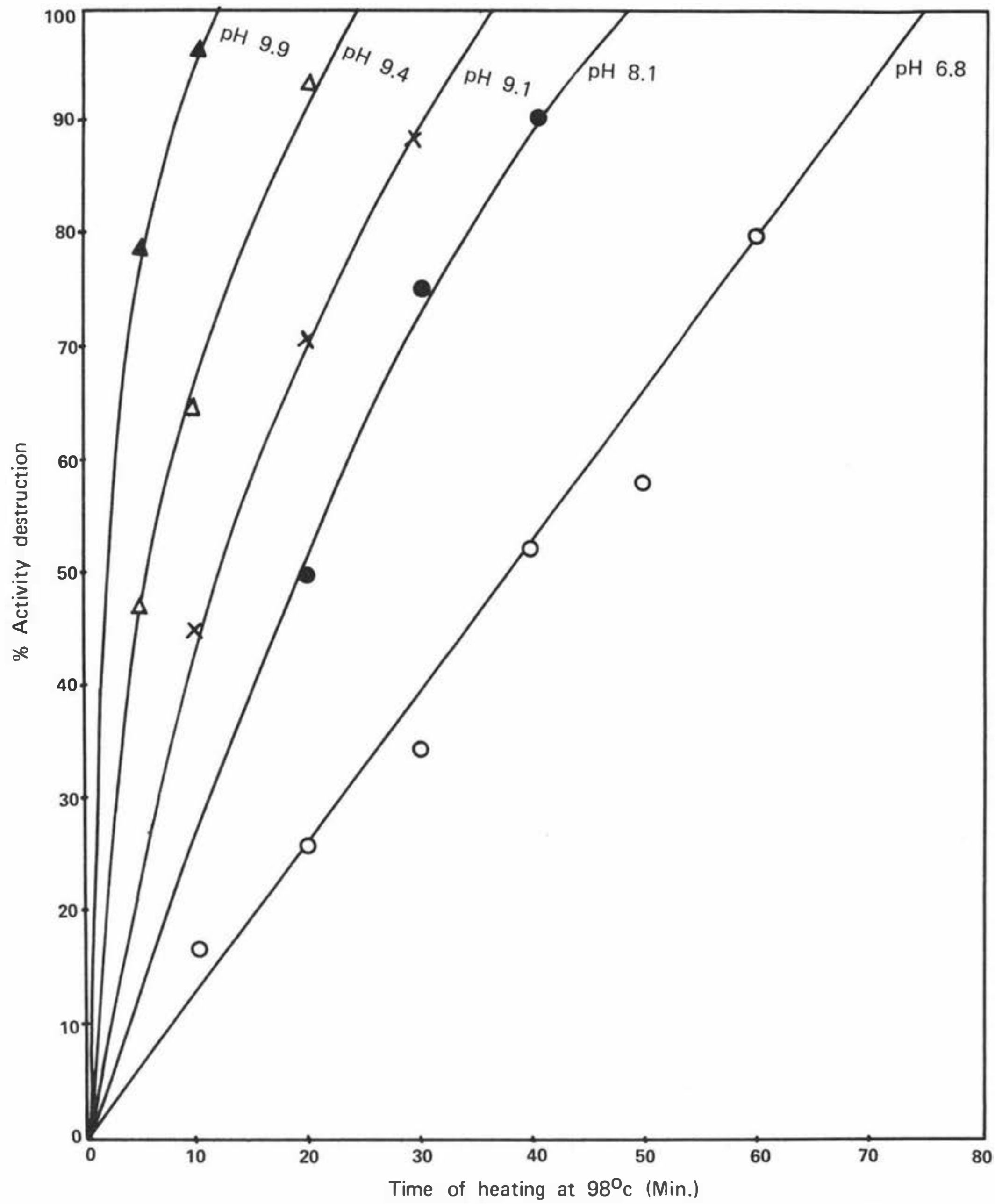

FIG. 7. INACTIVATION OF TRYPSIN INHIBITOR WITH TIME OF HEATING AT 98°C RELATIVE TO pH OF SOYMILK.

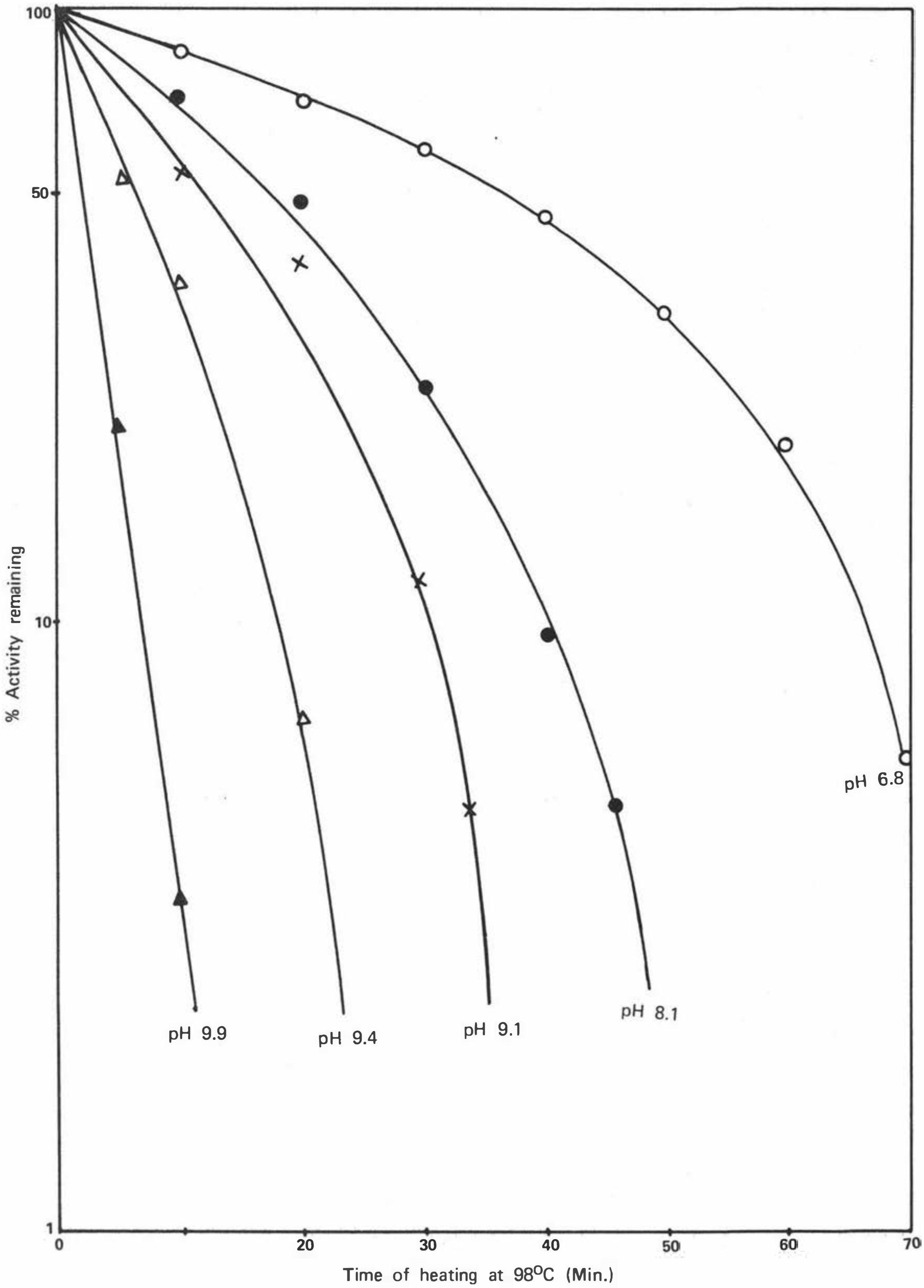

FIG. 8. LOSS OF TRYPSIN INHIBITOR ACTIVITY WITH TIME OF HEATING AT 98°C RELATIVE TO pH OF SOYMILK.

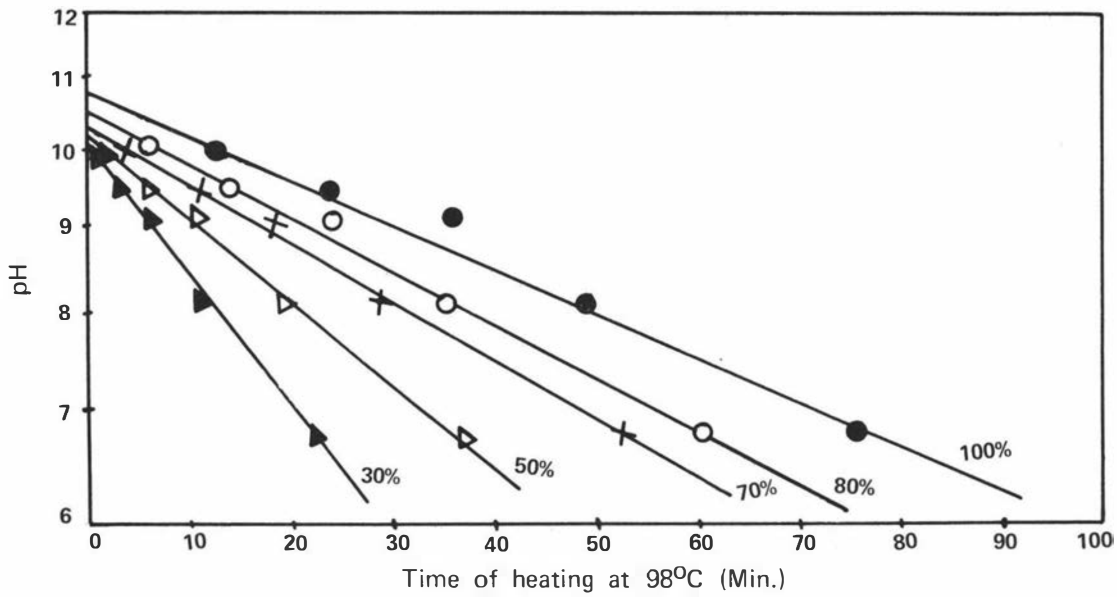

FIG. 9. RELATION BETWEEN TIME OF HEATING AT 98°C AND pH OF SOYMILK ON THE EXTENT OF INACTIVATION OF TRYPSIN INHIBITOR.

4.4.4 Discussion

The inactivation of the trypsin inhibitor in the milk prepared from water presoaked soybeans (pH 6.7) was very slow at 98°C and 76 minutes were necessary to complete destruction of the inhibitor activity. Results of other studies on the inactivation of trypsin inhibitor in solvent extracted soybean meal (35), soybean oil cakes (276) and soymilk (104) support our findings. The rate of inactivation of the inhibitor in soymilk prepared from carbonate presoaked beans was faster than that in the water presoaked preparation when both were processed at 98°C (Fig. 5). This is due to the fact that during heating, the pH of the carbonate presoaked preparation increased from 6.7 to 8.7 due to loss of dissolved CO_2. It is evident from Fig. 8 that using the same temperature (98°C) for processing, the rate of inactivation of the trypsin inhibitor increased as the pH of the milk was increased from neutrality and approached first order kinetics at pH 9.9. When both the milks were processed at 115°C in 211 x 309 cans with standard net head space, there was no opportunity for CO_2 to be lost from the system, the pH remained about the same for milks from both pretreatments and the rate of inactivation of the inhibitor was found to be about the same.

The alkaline conditions during presoaking in 0.4 M sodium carbonate solution (pH 11.1, room temperature 15 - 18°C) cause no irreversible loss of activity of the inhibitor (Fig. 5 and Fig. 6). This could be predicted as Kunitz (144) showed that the soybean trypsin inhibitor is stable over a wide range of pH at temperatures below 30° but it is gradually denatured if heated to higher temperatures. This denaturation is reversible if the degree of heating has been brief. However, prolonged heating causes irreversible denaturation of the inhibitor. Steiner and Edelhoch (253) have also shown that at pHs up to 12 and at 25°C there is no indication of any irreversible or time-dependent structural change occurring in the soybean trypsin inhibitor.

CHAPTER 5

EFFECT OF PROCESSING CONDITIONS ON THE DIGESTIBILITY IN VITRO OF PROTEIN IN VARIOUS SOYMILK PREPARATIONS

5.1 Introduction

From the literature reviewed in the previous chapter (Chapter 4), it appears that for soybeans to provide a maximal contribution to nutrition, deleterious substances must be removed from the raw beans. Fortunately, through the application of heat, it is possible to inactivate such harmful materials, but at the same time, there is a danger of over-processing which could cause damage to the proteins as a nutrient source, particularly through the destruction or modification of certain amino acids and an increased tendency of the protein to be resistant to enzymatic digestion (230). Since the value of soybeans as a food is due in large part to their high content of protein, it is important to be able to measure the extent to which the nutritional quality of the protein may have been harmed during processing.

Evans (75) studied the influence of autoclaving soybean oil meal on the liberation of amino groups by different enzymes or enzyme combinations and found that when trypsin or trypsin and erepsin were used, a marked increase in digestibility resulted from autoclaving the soybean oil meal at 100°C for 30 minutes. Autoclaving at 130°C for 60 minutes very markedly decreased the liberation of amino groups by any of the enzyme combinations.

In later studies, Evans et al (76) reported that the soybean oil meals which had been autoclaved at temperatures between 100°C and 120°C for 30 minutes were more completely digested by the chick, or by trypsin and erepsin in vitro, than the raw meal or the meals which had been autoclaved at 130°C for 30 or 60 minutes using undigested total protein, or sulphur, cystine or methionine in the undigested protein as criteria. Significant correlations were observed between in vitro trypsin and erepsin digestion and chick digestion of these soybean oil meals when digested protein, organic sulphur or cystine were the measured criteria.

Clandinin et al (59) indicated that a meal of as high nutritive value could be provided by processing solvent extracted soybean flakes in an autoclave at 4 pounds pressure (about 105°C) for 45 minutes as by processing similar raw flakes at 15 pounds pressure (about 121°C) for 4 minutes. Working with soymilk prepared from water presoaked beans, Hackler et al (105) reported that the highest protein efficiency ratio (PER) was obtained when the milk was processed at 121°C for 5 - 10 minutes.

In a study of the release of essential amino acids by enzymatic hydrolysis from properly heated and overheated soybean oil meals, Riesen et al (231) reported that the decreased nutritive value of the overheated meals was associated with a decreased liberation of essential amino acids and that in vitro enzymatic hydrolysis values may constitute an index of the relative nutritive value of different meals.

Melnick and Oser (179) stated "for optimal utilisation of protein all essential amino acids must not only be available for absorption but also be liberated during digestion in vivo at rates permitting mutual supplementation. Heat-processing influences the relative rate of liberation of the amino acids."

From growth studies as well as studies of in vitro release of amino acids and alpha-amino-nitrogen, Clandinin and collaborators (60, 61) showed that once the peak of enzymatic release is reached, meals of progressively decreasing quality are produced. Venkatesan and Rege (262) also noted that the extent of breakdown in vitro of proteinaceous materials by enzymes often gives valuable information on the availability of the amino acids to the host in vivo and on the presence of any enzyme inhibitors as well as on the effects resulting from processing conditions.

5.2 Objective and Experimental Plan

The purpose of the work reported in this chapter was to compare the digestibility in vitro of proteins in various heat-treated soymilks prepared either from carbonate or water presoaked soybeans, to determine whether any change of protein quality takes place due to pretreatment in alkaline solution and also to evaluate the optimum heat processing conditions. The rate of enzymatic hydrolysis of various optimum heat-processed soymilks was compared. A comparison was also made on the susceptibility to enzyme attack of optimum heat-processed soymilks and of casein.

5.3 Experimental

5.3.1 Materials

Freeze-dried soymilk samples used in this study were the same as used in the previous chapter (Chapter 4).

Pepsin - 1 : 2500, The British Drug House Ltd., England.

Trypsin - same as in Chapter 4.

Casein - Freshly prepared in the laboratory by isoelectric precipitation using the method of Wallace and Aiyar (263)

5.3.2 Methods

In vitro digestion studies using pepsin and trypsin

The enzymatic hydrolysis was carried out according to the method of Venkatesan and Rege (262) with minor modifications. Freeze-dried soymilk or casein samples each containing 0.19 g protein were suspended in 20 ml of 0.05 M HCl containing 25 mg pepsin at pH 1.6 or in 20 ml of 0.2 M-Na_2HPO_4-HCl buffer containing 25 mg of trypsin at pH 8.2. The mixture was incubated at 37^{o}C for 24 hours with occasional swirling. 0.5 ml of toluene was added to the mixture before incubation as preservative. The undigested proteins and larger peptides were precipitated by adding 6 ml of 50 percent trichloroacetic acid and the precipitates were removed by filtration.

Pepsin followed by trypsin digestion was carried out by incubating samples containing the same amount of protein with the same amount of pepsin as above in 18 ml of 0.05 M-HCl at 37^{o}C for 24 hours at pH 1.6. After pepsin digestion, the pH of the digests was brought to about 8 with 0.5 M-NaOH and 1.0 g of $NaHCO_3$ was added to bring the pH 8.2. The final volume was made up to 20 ml, then 25 mg of trypsin were added and the digestion mixtures were incubated for an additional 24 hours at 37°C. Enzyme blanks were prepared by incubation under the condition described above with the soymilk or casein omitted.

In the experiment for the determination of rate of hydrolysis by pepsin or trypsin, only optimum heat processed soymilks were used and samples were taken after specified times of digestion to determine the degree of hydrolysis.

Acid hydrolysis of the samples

Samples either of freeze-dried soymilks or of casein containing 0.095 g of protein were introduced into pyrex glass tubes followed by 26 ml of 6 M-HCl. After the contents were frozen each tube was evacuated and then sealed. Hydrolysis was carried out under the conditions described in Chapter 3. The insoluble humin was removed by filtration.

Examination of the digests

The amino nitrogen in all the digests was determined by using the method of Pope and Stevens (219). The degree of hydrolysis of the proteins by enzyme(s) was calculated by considering acid hydrolysis to be 100 percent. The results reported have been corrected for the enzyme(s) blanks.

5.3.3 Results

Effect of heat-treatment on the enzymatic hydrolysis of proteins in soymilks

The influence of heat-treatment on the digestibility of proteins in soymilks prepared from water or carbonate presoaked beans by trypsin is shown in Fig. 10. The results demonstrate that the

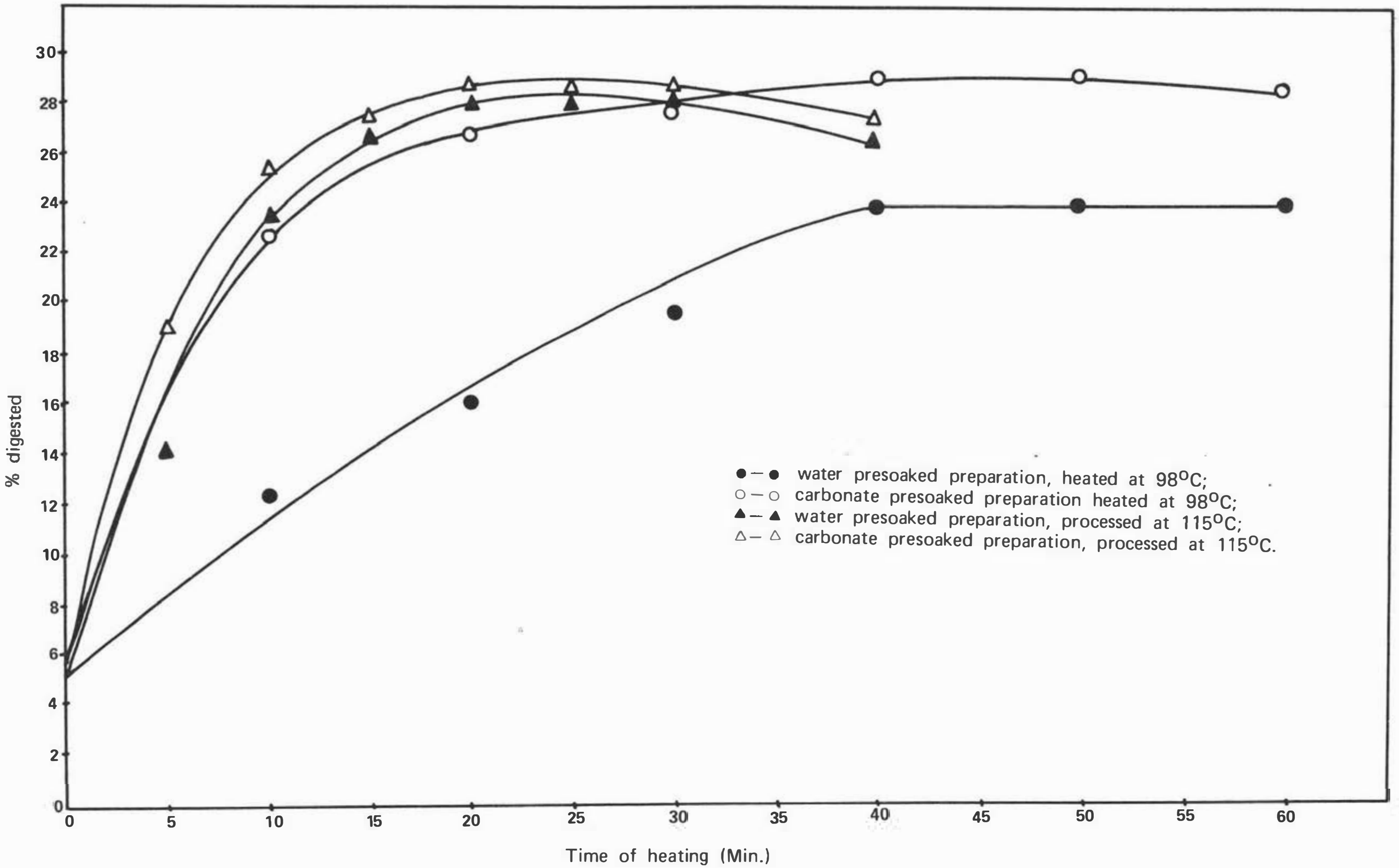

FIG.10. EFFECT OF HEAT TREATMENT ON THE DIGESTIBILITY OF PROTEINS IN SOYMILKS BY TRYPSIN.

digestibility of proteins in soymilks increases with increase of heat-treatment and coincides with the decrease of trypsin inhibitor activity. Once the trypsin inhibitor has been destroyed further heating results in a lower digestibility. The extent of hydrolysis of proteins under the specified conditions is higher in soymilk prepared from carbonate presoaked beans than in milk prepared from water presoaked soybeans. This difference is 19.1 percent of the total enzymatic hydrolysis when both the milks have been processed at 98°C but the difference is only 5.6 percent when both milks were processed at 115°C. With the soymilks prepared from water presoaked beans the increase of hydrolysis is 14.1 percent when the milk had been processed at 115°C instead of 98°C, but with the carbonate presoaked beans the maximum digestibility was the same, irrespective of the temperature used in processing.

In the study of pepsin digestion of the various heat-processed soymilks, it was found that digestibility of the proteins in the unprocessed soymilk was the highest, and after treatment at both 98°C and 115°C this gradually decreased as the time of heating increased (Table 9).

TABLE 9. Effect of processing on the peptic digestibility of proteins in soymilks.

Time of processing min.	Processing Temperature 98°C		Processing Temperature 115°C	
	Water presoaked preparation percent hydrolysis	Alkali presoaked preparation percent hydrolysis	Water presoaked preparation percent hydrolysis	Alkali presoaked preparation percent hydrolysis
0	26.3	27.2	26.3	27.2
10	25.9	26.7	25.9	26.3
20	25.9	26.3	25.4	25.9
30	25.9	26.3	24.6	25.0
40	25.4	25.9	24.6	24.6
50	25.0	24.7	-	-
60	24.7	24.7	-	-

This decrease amounted to about 6.3 percent using milk prepared from water presoaked soybeans whether processed at 98°C for 60 minutes or 115°C for 40 minutes and there was a 9.4 percent decrease when milk from carbonate presoaked soybeans was processed under the same conditions.

Rate of enzymatic digestion of proteins in various optimum heat-processed soymilks

Fig. 11 shows that the rate of hydrolysis, by pepsin, of the proteins in various soymilks was about the same irrespective of pretreatment or method of heat processing but Fig. 12 shows that the tryptic digestion of the protein in milk prepared from carbonate presoaked beans was faster and more extensive than that of milk prepared from water presoaked soybeans.

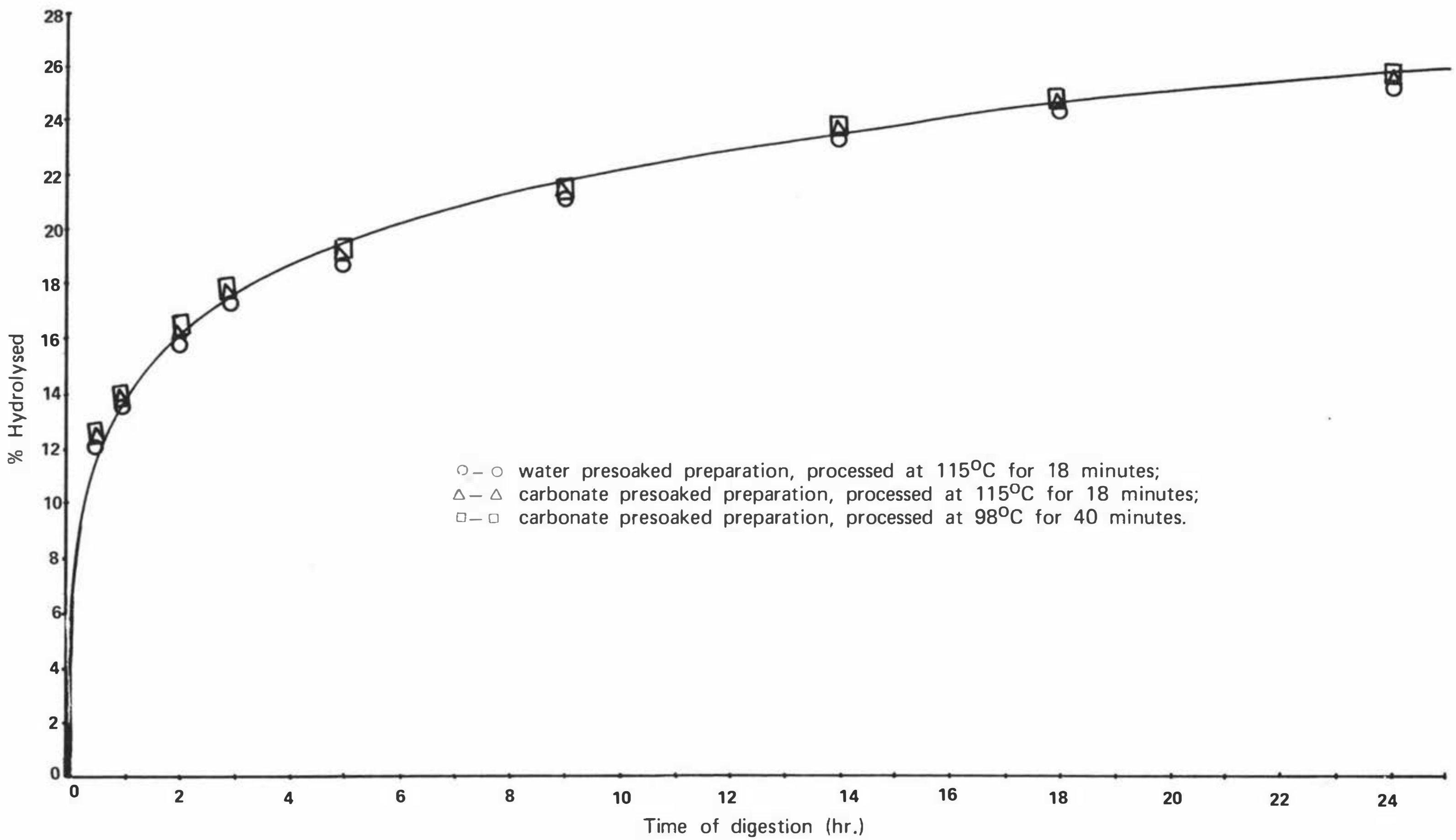

FIG 11 HYDROLISIS OF PROTEINS BY PEPSIN AGAINST DIGESTION TIME IN VARIOUS OPTIMUM HEAT PROCESSED SOYMILKS.

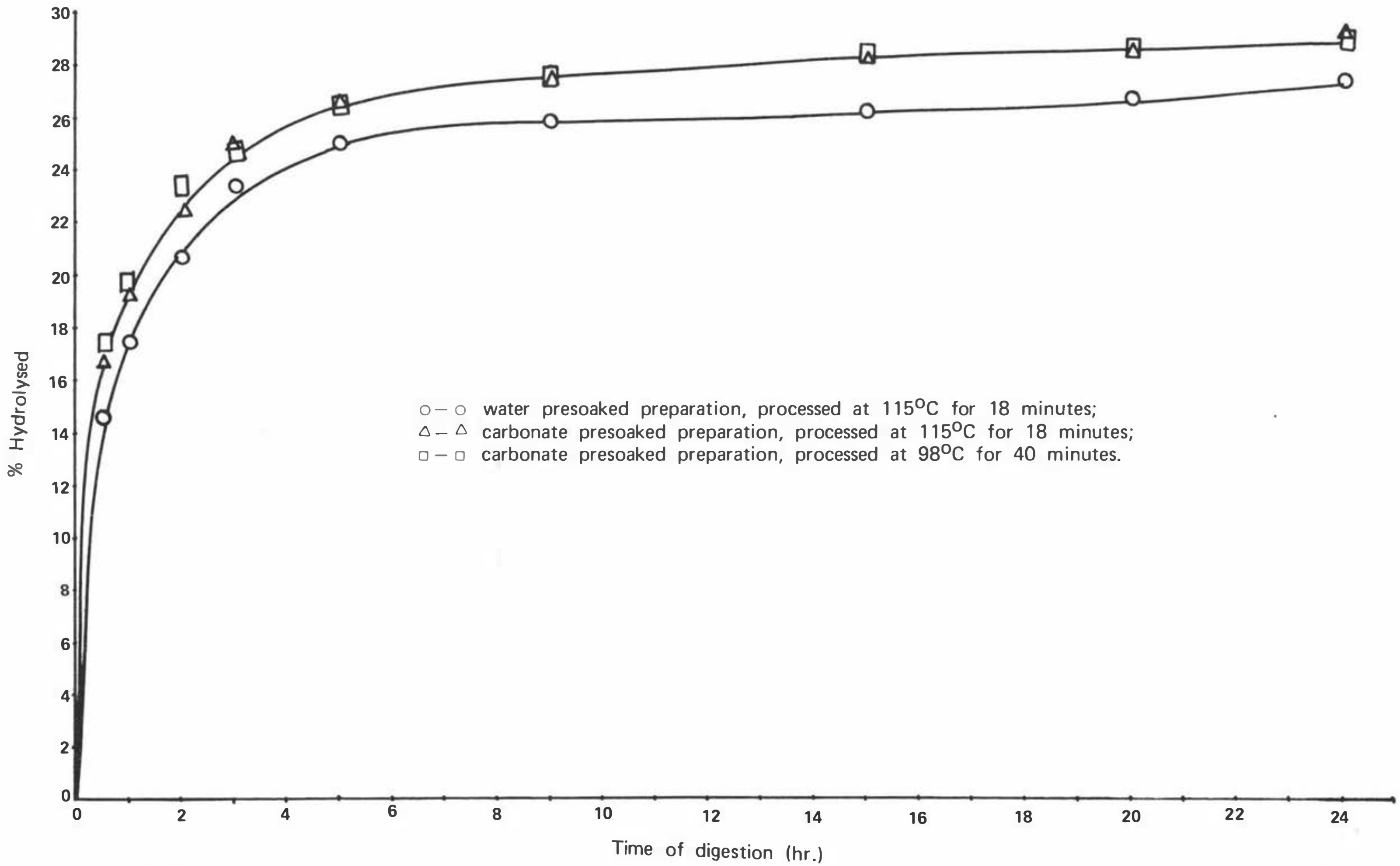

FIG 12 HYDROLISIS OF PROTEINS BY TRYPSIN AGAINST DIGESTION TIME IN VARIOUS OPTIMUM HEAT PROCESSED SOYMILKS.

Comparison of enzymatic hydrolysis of heat-processed soymilks with that of casein

The results of pepsin, trypsin and combined sequential digestions of various properly heat-processed soymilks and of casein are set out in Table 10.

TABLE 10. Digestibility in vitro of heat-processed soymilks and casein.

Substrate	Heat processing		Percentage hydrolysis with		
	Temp. °C	Time min.	Pepsin	Trypsin	Pepsin + Trypsin
Milk prepared from water presoaked soybeans	98	60	25.4	23.7	34.0
	115	18	25.3	27.4	37.9
Milk prepared from carbonate presoaked soybeans	98	40	25.9	28.9	39.5
	115	18	25.9	29.1	40.3
Casein	-	-	16.8	30.2	37.9

The data indicate that casein was less susceptible to pepsin attack and slightly more susceptible to trypsin attack than the proteins in soymilks. The proteins of soymilks were susceptible to pepsin attack to about the same extent irrespective of method of presoaking or heat-treatment. The trypsin digestibility of proteins in soymilk prepared from water presoaked beans was increased when the higher processing temperature (115°C) was used and approached that of soymilk prepared from carbonate presoaked beans. The extent of hydrolysis with pepsin plus trypsin of proteins in soymilk prepared from water presoaked beans and processed at 98°C was appreciably lower than that of the other processed soymilks and of casein.

5.3.4 Discussion

Using soymilk prepared by the water presoaking method this study has shown that a processing temperature of 115°C instead of 98°C had the advantages not only of destroying the trypsin inhibitor more quickly (see Chapter 4), but also of increasing the digestibility in vitro of the protein by trypsin. Although complete inactivation of trypsin inhibitor occurred when the milk was processed at 98°C for 76 minutes (see Fig. 5, Chapter 4), there was no increase in trypsin digestibility after 40 minutes processing and the maximum trypsin digestibility was still only 80 percent of that found with the other soymilks. Melnick and Oser (179) showed that heating soybean meal in boiling water caused no improvement in the rate and degree of digestibility of the protein component whereas autoclaving progressively increased digestibility concomitant with an increase in biological value up to a limiting time-temperature combination after which both values decreased. Riesen et al (231) stated that, in order to obtain a meal of maximum nutritive value, sufficient heat must be applied not only to destroy the trypsin inhibitor but also to alter the protein in such a way that it may be more readily attacked by proteolytic enzymes. Fukushima (95) reported that most soybean proteins are globular, the molecules being compactly folded, including in the interior a hydrophobic region, and the chains have little susceptibility to proteinases before disruption of the internal structure. The same author (96) also showed that when soybean flour, to which an equal volume of water had been added, was autoclaved at 100°C a small amount of native protein still remained after three hours heating whereas the native protein completely disappeared after a few minutes heating at either 110°C or 120°C. Hayashi and Ariyama (113) showed that the in vitro pancreatic digestibility of soybean protein is higher when autoclaved with water at 110°C, than when boiled with water. Hackler et al (105) reported that the effect of processing soymilk at 121°C was very striking, the peak of nutritional quality was reached in 5 - 10 minutes of heating at 121°C, whereas essentially no change in nutritive value was noted for soymilk protein cooked at 93°C even after 90 percent of the trypsin inhibitor was inactivated.

The trypsin digestibility of the protein from the soymilk prepared from carbonate presoaked beans was much higher than that of the water presoaked beans when both samples had been processed at 98°C to adequately destroy the trypsin inhibitor activity. This is due to the fact that the combined effects of heat and alkalinity resulted in more complete unfolding of the globular proteins. This was also observed but to a lesser extent when milks prepared by the two methods were autoclaved at 115°C. This suggests that the action of soaking and grinding the beans under alkaline conditions results in a change in protein sturcture further favouring trypsin attack and this is apparent even after the protein has been exposed to an appreciable heat treatment.

On the other hand, heat treatment was not found effective in improving the digestibility by pepsin of proteins in soymilks under the conditions used, since the proteins in unheated soymilks showed slightly higher digestibility possibly because at lower pH (i.e. pH 1.6) the native proteins are unfolded anyway (236). The peptic digestibility of the alkali presoaked sample was 3.4 percent higher than that of the water presoaked sample prior to heat processing but during processing the digestibility of the alkali presoaked sample dropped more quickly than that of the water presoaked sample until all samples reached the same level of digestibility (Table 9). The effect of the alkali presoaking on the peptic digestibility was not directly related to the higher pH the milk reached during heat processing because a greater loss of digestibility was observed for both the milk processed at 98°C where the pH reached 8.7 and for the milk that was autoclaved where the pH was the same at the beginning and the end of the process. The alkaline presoaking must thus result in a structure more exposed for pepsin attack prior to heat treatment. Table 10 demonstrates clearly that the digestibility of soymilks prepared by the water presoak method and autoclaved at 115°C and of soymilks prepared by the alkaline presoak method and then processed at 98°C were similar to the digestibilities obtained with acid precipitated freeze-dried casein under identical conditions. This suggests that the soymilk protein in these milks was as available to enzyme attack as was the reference casein sample.

This study has shown that the alkaline presoak treatment followed by processing at 98°C gives digestibilities which can be obtained only when milks prepared by the water presoak method are autoclaved at 115°C.

5.3.5 Conclusion

In addition to the advantages mentioned in the conclusion of Chapter 3, the alkaline presoaking treatment for the manufacture of soymilk has the further advantage that there is a more rapid destruction of trypsin inhibitor and this can be achieved using a simple system of heat processing and is without any deleterious effect on the digestibility as measured by the systems described.

CHAPTER 6

EFFECT OF PROCESSING CONDITIONS ON THE STABILITY OF PROTEINS IN VARIOUS SOYMILK PREPARATIONS

6.1 Introduction

It has been known for a long time that treatment of solution of proteins with heat, acid or alkali causes denaturation of the protein and this generally involves uncoiling of the relatively tightly folded native protein molecule to a more open and extended form (112). Due to the exposure of functional groups previously buried in the interior of the molecule, the potential energy of interaction increases when the protein undergoes denaturation. As a consequence of this increase in potential interaction energy, the state of dispersion of the protein molecules in solution may change, for example, aggregation or even coagulation may occur during the denaturation (135).

The nature of the bonds between the molecules in the aggregate or in the coagulum varies between proteins, but frequently the molecules seem to be linked together through hydrogen bonds formed between the carboxyl and amino groups of neighbouring peptide chains which were liberated during the configurational perturbation of the intramolecular secondary structure (130). In some proteins aggregation occurs through the sulphydryl-disulphide interchange reactions between the molecules (277). Divalent metals such as calcium and magnesium are able to form inter-chain or inter-polymer links between proteins resulting in a progressive increase in size of the protein particles. The process of polymerisation, if permitted to occur, must necessarily increase the tendency of the protein to coagulate (24).

Moist heat treatment is necessary to inactivate the anti-nutritional factors present in raw soybeans (see Chapter 4). One of the important changes that takes place during heat treatment of soybean meal in an aqueous solvent is the insolubilisation of the major proteins (17). Mann and Briggs (171) reported that heating aqueous extracts of soybean meal resulted in the precipitation of protein in varying amounts, the quantity being precipitated increasing with increase in temperature and with increase in length of heating period. This precipitation was probably the result of a heat accelerated interaction of the protein components and involved primarily those protein fractions other than globulins. However, although the isolated globulins alone were unaffected in their dispersibility by heat treatment up to 90°C for 2 hours, they became increasingly more non-dispersible when heated in the presence of the other protein components of the soybean extract.

Since the formation of a Soybean Protein Nomenclature Committee under the sponsorship of the Oilseeds Division, American Association of Cereal Chemists, the names used in the past for soybean proteins are discontinued except for Glycinin, the name given by Osborne and Campbell to the major reserve protein of soybeans. It appears that various proposals are under study as possible solutions to the nomenclature problem. Most of them are based on the ultra-centrifugal behavior of the proteins (272). Ultracentrifugal studies showed that the soybean proteins consist of four components having $s_{20}w$ of 2, 7, 11 and 15S respectively, of which the 7S and 11S components constitute the major part of the proteins (for detail see the recent review by Wolf (273)). The 11S protein corresponds to glycinin.

Catsimpoolas et al (55) showed that on heat treating a 0.3 percent solution of glycinin at temperatures above 70°C it dissociates at first into subunits and the solution then becomes increasingly turbid due to the aggregation of the dissociated subunits and the protein partially precipitates when heated to 90°C. Glycinin appears to be stable to heating up to 50°C and very little change is observed between 50° and 70°C.

Wolf and Tamura (274) reported that heat treatment disrupts the quaternary structure of the 11S protein and separates the subunits into two fractions. Denaturation of 11S protein at 100°C proceeds through at least three steps designated (a) to (c) in the following reation scheme:

$$11S \xrightarrow{(a)} \text{A-subunits} + [\text{B-subunits}]$$

$$[\text{B-subunits}] \xrightarrow{(b)} \text{soluble aggregates} \xrightarrow{(c)} \text{insoluble aggregates}$$

6.2 Objective and Experimental Plan

It appears from the above discussion that soy proteins undergo reaction or interaction during heat treatment and the extent of this depends on the degree of heat treatment. In Chapter 4, we demonstrated that heat processing at 115°C for about 17 minutes must be applied to destroy the antinutritional factors in soymilks prepared either from water or carbonate presoaked beans. The work reported in the present chapter was carried out to investigate the effect of this optimum heat treatment on the viscosity and the insolubility of the solids which will indicate any changes of protein structure in soymilks. Any significant role calcium ions had in the aggregation process was also investigated.

6.3 Experimental

6.3.1 Materials and Methods

Preparation and processing of soymilks

Soymilk was prepared either from water or carbonate presoaked beans according to the method previously described (Chapter 2). Distilled water was used throughout the study starting from the washing of the beans through to diluting the slurry. The prepared milk was further filtered through a sintered glass funnel no.1 (pore dia. 100 - 120 μ), followed by no.2 (pore dia. 40 - 50 μ). The processing of the resulting soymilk was carried out at 115^{o}C for 17 minutes in a 211 x 309 can with the standard net head space.

Viscosity measurement

The viscosities of the various soymilks were measured according to the method described earlier (Chapter 3).

Determination of insolubility of solids in soymilks

The quantity of insoluble solids in soymilks either raw or after processing at 115^{o}C for 17 minutes was determined by two methods. The first method was the New Zealand standard method for the determination of the solubility index of dry milk powder (250). Results of this method would indicate suitability of the processed soymilks as a commercial product. The second method involved centrifugation at 38,000 x g for 20 minutes, using the high speed attachment to a model UV International centrifuge. The results from this method gave a measure of the extent of aggregation of the protein occuring during processing. In both methods the dry weight of sediment was recorded as a percentage of total solids and, where indicated in the table, the volume of sediment per 10 ml of milk is recorded.

Determination of volume of the sediment

Transparent nitrocellulose centrifuge tubes of 10 ml capacity were carefully calibrated into 0.1 ml division with water. The volume of the sediment was recorded as millilitres in the tube to the nearest graduated scale division.

Determination of calcium content in soymilks

The calcium content in the soymilk was determined according to the method of the A.O.A.C. (13).

Treatment of the milk with metal chelating agents

(a) Sodium hexametaphosphate-$(NaPO_3)_6$. 0.05, 0.10, 0.15, 0.20, 0.25, and 0.50 g of $(NaPO_3)_6$ was added respectively to 100 ml samples of raw soymilk prepared by the water presoaking procedure and to samples of the same milk which had previously been heat treated to 115°C for 17 minutes. The raw soymilk to which $(NaPO_3)_6$ had been added was then processed at 115°C for 17 minutes.

(b) Ethylene diamine tetra-acetic acid-(EDTA). 0.05, 0.10, 0.15, 0.20, and 0.25 g of EDTA (disodium salt) was dissolved respectively in 10 ml aliquots of distilled water and the pH of the solution was adjusted to 7.0 by adding a few drops of 1M-NaOH solution. To this were added 90 ml of soymilk prepared from water presoaked beans either raw or previously heated at 115°C/17 min. The total solids content in 90 ml of milk was the same as in the 100 ml of milk used in (a). The raw soymilk which had been treated with EDTA was then processed at 115°C for 17 minutes as in (a).

(c) Trisodium polyphosphate-($Na_3P_3O_{10}$) and sodium dihydrogen orthophosphate-($NaH_2PO_4.2H_2O$). $Na_3P_3O_{10}$ and $NaH_2PO_4.2H_2O$ (1 : 1) were used to treat the soymilk prepared from water presoaked beans. The pH of this combination was about 6.8. Using these two chemicals combined, the treatment given to the soymilk (water presoaked preparation) was the same as in (a).

6.3.2 Results

Effect of processing on the viscosity of soymilks

The results are given in Table 11.

TABLE 11. Effect of processing conditions on the viscosity of soymilks.

Samples	Processing conditions		Reduced viscosity
	Temp. °C	Time min	(η red)
Soymilk prepared from water presoaked beans	Nil	Nil	0.23
Soymilk prepared from water presoaked beans	115	17	0.36
Soymilk prepared from carbonate presoaked beans	Nil	Nil	0.41
Soymilk prepared from carbonate presoaked beans	115	17	0.41

Table 11 shows that the viscosity of soymilk prepared from carbonate presoaked beans prior to heat-treatment is very much higher (about double) that of the soymilk prepared by water presoaking procedure. On heating the viscosity of water presoaked preparation is increased whereas that of the soymilk prepared by the carbonate presoaking procedure remains the same. After identical heat processing conditions (115°C/17 min) the viscosity of the carbonate presoaked preparation is still higher than that of the soymilk prepared from water presoaked beans.

Effect of processing and time of ageing after processing on the viscosity of soymilks

The similar viscosities of raw and heat processed (115°C/17 min) soymilks prepared by carbonate presoaking procedure as shown in Table 11 made it necessary to further investigate the effect of time of heat-treatment at 115°C and the time of ageing on the viscosity of soymilk and the results are set out in Table 12.

TABLE 12. Effect of processing and time of ageing after processing on the viscosity of soymilks.

Samples	Processing conditions		Apparent viscosities of soymilks after different time intervals after processing, cps			
	Temp. °C	Time min	½-h	2-h	4-h	8-h
Soymilk prepared from water presoaked beans	Nil	Nil	2.24	-	-	-
Soymilk prepared from water presoaked beans	115	17	2.60	2.60	2.66	2.63
Soymilk prepared from carbonate presoaked beans	Nil	Nil	3.28	-	-	-
Soymilk prepared from carbonate presoaked beans	115	5	2.88	2.88	2.90	3.10
Soymilk prepared from carbonate presoaked beans	115	10	2.88	2.96	3.06	3.22

Table 12 continued over page..........

Table 12 continued......

Samples	Processing conditions		Apparent viscosities of soymilks after different time intervals after processing. cps			
	Temp. °C	Time min	½-h	2-h	4-h	8-h
Soymilk prepared from carbonate presoaked beans	115	17	3.06	3.20	3.28	3.26
Soymilk prepared from carbonate presoaked beans	115	30	3.20	3.22	3.28	-

The results demonstrate that the viscosity of raw soymilk, prepared by the carbonate presoaking procedure decreased from 3.28 to 2.88 after 5 minutes of heating at 115°C and remained the same at 10 minutes of heating and then gradually increased as the time of heat-treatment increased. The viscosity of the soymilk heated at 115°C/17 minutes was still lower than that of the same milk in the raw state. This viscosity measurement was done within half an hour after processing. As the time of ageing was increased the viscosities of the heat treated soymilks also increased. It is also interesting to note that the viscosity on ageing reached that of the raw sample more quickly in the soymilks which had been processed for the longer times.

Effect of processing on the insolubility of the total solids in soymilks

Soymilks, raw and heated (115°C/17 min), prepared either from water or carbonate presoaked beans were centrifuged using a relatively low speed centrifuge according to the solubility index method and also using a high speed centrifuge (38,000 x g). The dry weight of the residues obtained by both test methods and the wet volumes of the residue in the high speed centrifuge method are presented in Table 13.

TABLE 13. Effect of processing on the insoluble solids content of soymilks.

Samples	Processing conditions		Insolubility of solids content		
			Using solubility index method	Using high speed centrifuge at 38,000xg	
	Temp. °C	Time min	Dry wt. %	% of total solids present in the sediment	wet vol. of sediment per 10 ml of milk. ml
Milk prepared from water presoaked beans	Nil	Nil	Nil	3.52	0.70
"	115	17	<0.01	6.00	0.40
Milk prepared from carbonate presoaked beans	Nil	Nil	Nil	5.50	0.60
"	115	17	<0.01	7.16	0.45

The centrifugal separation of solids from raw soymilks prepared either from water or carbonate presoaked beans is nil and less than 0.01 percent of the total solids is sedimented from the heated soymilks in both cases when measured by the solubility index method.

Using the high speed centrifuging method, both the raw and the heated soymilk samples prepared by carbonate presoaking procedure gave higher quantities of sediment when compared with the corresponding samples prepared from the water presoaked beans. The results also indicate that the insoluble solids content increased due to heat-treatment (115°C/17 min) in the same order of magnitude in the soymilks prepared from both the water and the carbonate presoaked beans.

The wet volumes of the sediments are higher in the raw soymilks than those of heated soymilks with both presoaking procedures. The difference between the raw and heated samples is higher with the water presoaked preparation than with that of soymilk prepared from carbonate presoaked beans.

Calcium content in soymilks

The results are shown in Table 14.

TABLE 14. Calcium content in soymilks

	mg/100 ml milk
Soymilk prepared from water presoaked beans	28
Soymilk prepared from carbonate presoaked beans	25
Cow's milk	120

The Table 14 shows that the soymilks prepared either from water or from carbonate presoaked beans contained about the same level of calcium which is much less than that in cow's milk.

Effect of treatment with calcium chelating agents on the viscosity of soymilks

The soymilk prepared from water presoaked beans was treated with various chelating agents such as sodium hexametaphosphate $(NaPO_3)_6$, ethylene diamine tetra-acetic acid (EDTA), and trisodium polyphosphate $(Na_3P_3O_{10})$ plus sodium dihydrogen orthophosphate (NaH_2PO_4), to establish what effect complexing of calcium would have on the aggregation process. The viscosities of the variously treated soymilks are reported in Tables 15 - 17.

TABLE 15. Effect of treatment with sodium hexametaphosphate-$(NaPO_3)_6$ on the viscosity of soymilk (water presoaked preparation).

Addition of $(NaPO_3)_6$ %	Reduced viscosity ($\eta_{red.}$)		
	After addition to raw soymilk	After addition to milk previously heated at 115°C/17 min	After addition to raw soymilk and then heated at 115°C/17 min
Nil	0.23	0.36	0.36
0.05	0.24	0.36	0.51
0.10	0.25	0.36	0.57
0.15	0.24	0.37	0.58
0.20	0.24	0.37	0.56
0.25	0.24	0.38	0.58
0.50	0.23	0.39	Gel

The results in Table 15 show that addition of $(NaPO_3)_6$ to raw or heated (115°C/17 min) soymilks results in no change in viscosity from the control samples which have not been treated with $(NaPO_3)_6$. But on heat-treatment, the viscosity of raw soymilk which has previously been treated with $(NaPO_3)_6$, increased with the increasing quantities of $(NaPO_3)_6$ and at 0.5 percent concentration the milk gels.

TABLE 16. Effect of combined treatment with trisodium polyphosphate ($Na_3P_3O_{10}$) plus sodium dihydrogen orthophosphate (NaH_2PO_4) on the viscosity of soymilks (water presoaked preparation).

Addition of polyphosphate and phosphate (1:1) %	Reduced viscosity ($\eta_{red.}$)		
	After addition to raw soymilk	After addition to soymilks previously heated at 115°C/17 min	After addition to raw soymilk and then heated at 115°C/17 min
Nil	0.23	0.36	0.36
0.05	0.25	0.36	0.39
0.10	0.25	-	0.40
0.15	0.24	-	0.40
0.20	0.24	-	0.40
0.25	0.24	0.36	0.39
0.50	0.24	0.36	0.39

Table 16 shows that the viscosities of phosphate treated soymilk samples either raw or preheated at 115°C/17 min are about the same as those of the control samples. A slight increase in viscosity is observed when the raw soymilks pretreated with phosphates are heated at 115°C for 17 minutes.

TABLE 17. Effect of treatment with ethylene diamine tetra-acetic acid-(EDTA) on the viscosity of soymilks (water presoaked preparation).

Addition of EDTA %	Reduced viscosity ($\eta_{red.}$)		
	After addition to raw soymilks	After addition to soymilks previously heated at 115°C/17 min	After addition to raw soymilks and then heated at 115°C/17 min
Nil	0.23	0.36	0.36
0.05	0.23	0.37	0.62
0.10	0.24	0.37	0.98
0.15	0.31	0.38	Gel
0.20	0.36	0.39	
0.25	0.54	0.40	
0.50	0.70	0.44	

The results in Table 17 show that treatment of raw soymilk samples with EDTA caused an increase in viscosity. Subsequent heat-treatment of these samples increased the viscosities more rapidly and gelation occurred at 0.15 percent concentration. When the previously heated samples are treated with EDTA at the concentrations used, the viscosity is affected to a much smaller extent than in the other two cases.

6.3.3 Discussion

Soymilks prepared from water presoaked beans behave differently in their changes in viscosity when processed at 115°C from the milks prepared by the carbonate presoaking procedure.

At high alkaline pHs (soaking at pH 11.1 and grinding at pH 9.7), protein particles hydrate more extensively and swell thus contributing to the increase in viscosity (1). If the particles are compact, hydration is restricted to surface layers. If the particles have a loose structure, hydration does not depend only on the surface area of the particles because the water molecules can now penetrate the frame work of the molecules (133). It is known that the soy proteins are globular, compactly folded and include an interior hydrophobic region (95). In the present study at the high alkaline pH during the carbonate presoaking, the -COOH groups of proteins would be expected to be dissociated into ions and the protein particles swell or unfold due to the mutual repulsion of the negatively charged groups. Glutamic and aspartic acids together represent around 32 percent of the total amino acid content and could thus be significant sources of these ionisable carboxyl groups. Since the protein particle is electrically charged and its structure is consequently loosened, it is rendered capable of becoming more hydrated. Nakajma and Scheraga (201) showed that the degree of hydration of ribonuclease at pH 9.53 is more than double that at pH 5.87 at 60°C. McAnelly (176) found that increasing the pH of spent soybean flakes before desolventising and toasting, and heating the treated flakes for a short period of time greatly improved the water absorption properties. This is supported by the data presented.

In summary the following changes appear to be occuring during the preparation and processing of the two types of soymilk

	Apparent viscosities (cps)	
	Water presoaking	Carbonate presoaking
Original milk	2.24(1)	3.28(2)
Heated 115°C/10 min	-	2.88(3)
Heated 115°C/17 min	2.60(5)	3.06(4)
Rehydration	2.66(7)	3.28(6)

These effects may be explained as follows:

(1) and (2)- this difference is probably the consequence of the swelling of the soy proteins under the effect of the alkaline conditions and the persistence of this enlarged structure through increased hydration - a structural change as a consequence of the carbonate presoaking has already been suggested in Chapter 5.

(2) and (3)- this difference represents reduction in the size of the protein particles due to dehydration during heating and suggests that at this point there is no effect of heat denaturation on the protein structure because of the prior effect of the alkaline treatment.

(3) and (4)- this difference represents further unfolding of the protein and possibly some aggregation due to heating to give a larger apparent volume despite the volume reduction effect of dehydration.

(1) and (5)- this difference consequenton unfolding due to heat denaturation (130) suggests that the unfolding of the protein and possibly some aggregation have made a greater contribution in this case to the increase in "swept" volume than the relative loss in particle volume consequent on dehydration.

(4) and (5)- the effect of carbonate presoaking on the swelling of the protein is permanent and unfolding due to heat denaturation does not contribute as much to the increase in "swept" volume as does the effect of alkali.

(4) and (6)- rehydration occurs on cooling and ageing for on denaturation protein does not lose its water binding capacity to any great extent (112, 135). The return to the same apparent viscosity is probably coincidental in view of the obvious heat denaturation that has occurred with its contribution to viscosity changes.

(5) and (7)- further rehydration in this system contributes very little to viscosity changes.

(6) and (7)- heat denaturation and rehydration has less effect than alkaline soaking on the viscosity.

There is thus evidence that carbonate presoaking causes a readily discernible change in the structure of the protein.

The viscosity of soymilk prepared from carbonate presoaked beans reported in Chapter 3 is higher than that in the present study because of the differences in both the filtration and the processing of the resulting soymilks.

The proteins in the soymilks prepared either by the water or by the carbonate presoaking procedures are highly stable under the processing conditions used in the present study as indicated by the negligible precipitates obtained even when using high speed centrifugation (38,000 x g). The wet volumes of the sediment in the case of raw soymilks are higher (highest in water presoaked preparation) than those of heat processed soymilks in both the presoaking procedures. This higher volume of the sediment from the raw soymilks is principally due to the higher quantity of water both bound and free which accompanied the protein particles. It was observed during the experiment that the sediment from the raw water presoaked preparation was more loosely packed at the bottom of the centrifuge tube than that from the raw carbonate presoaked preparation. The sediment from the heated soymilks were firmly packed in the sediments from both the presoaking procedures probably because lower quantities of water accompanied the sediment. It appears that measuring the volume of the precipitate after high speed centrifugation does not give a true measure of the extent of insolubility caused by processing.

The possibility of involvement of calcium ions in the aggregation process and the increase in viscosity during heat-processing is ruled out. This is probably due to the low levels of calcium present in the soymilk (Table 14).

It can be seen in Tables 15 - 17 that the viscosities are increased during treatment with calcium chelating agents. This is probably due to electrostatic effects. Similar effects will be studied in detail in Chapter 7.

CHAPTER 7

EFFECT OF ELECTROLYTES ON THE STABILITY OF PROTEINS IN SOYMILKS

7.1 Introduction

The viscosities of heated protein solutions, in general, are very much greater than the viscosities of native protein solutions, particularly when salts are present (277). The heat alters the shape of the protein molecule by denaturation with concomitant viscosity increases and also cross-reactions between the protein molecules affected by the heat are increased by the presence of salts and are greatest near the isoelectric point. Due to these cross reactions an abrupt increase of viscosity takes place. The extent of cross reactions which lead to this viscosity increase is limited by the presence of like electric charges on the molecule since these lead to electrostatic repulsion between molecules (130), but this effect is overcome by an increase in ionic strength.

Zittle and Della Monica (277) showed that maximum viscosity is achieved when a solution of 1.8 percent β-lactoglobulin is heated at 90°C for 30 minutes with 0.25 M sodium chloride at pH 6.7. Below pH 6.7 the solution gels. Zittle (278) reported that an aqueous solution of K-casein at pH 7.0 is not affected by heating at 100°C for 5 minutes. If, however, 0.05 M sodium chloride is present the K-casein is liable to heat.

Frensdorff et al (92) studied the effects of various electrolytes on the gelling time of a 3 percent solution of ovalbumin denatured in 10 M urea at pH 7.6 - 8.0. They reported that two important effects may be noted: (a) increasing the electrolyte concentration accelerates gelling; and (b) electrolytes with more highly charged anions are more effective gelling agents than those with monovalent anions. This effect is so strong that gelling occurs at a protein concentration as low as 2 percent in the presence of 0.2 M sodium sulphate. The cationic charge seems to be unimportant, since the gelling time is the same in 0.1 M-NaCl as in 0.05 M-$CaCl_2$.

Zubay and Doty (279) showed that heating a solution of 0.5 percent bovine serum albumin at pH 5.5 caused partial heat coagulation as evidenced by the formation of a milky opacity in the solution (Note: isoelectric point of BSA is 4.5) but at pH 6.5, no heat coagulation occurs. They suggest that as the pH becomes higher, the negative charge on the albumin rises and apparently the repulsion between albumin molecules becomes great enough above pH 6.0 to prevent effective contact. The repulsion caused by this charge effect should be overcome in a medium of high ionic strength, and accordingly when the albumin at pH 6.5 is heated in 0.04 M-NaCl coagulation occurs.

Smith et al (245) found that sodium chloride disperses less of the nitrogenous materials from soybean meal than is dispersed by water and that soybeans have a minimum point of dispersion at about 0.1 M-NaCl. On increasing the concentration of the sodium chloride extractants over 0.1 M, the extractability of the nitrogenous materials from soybean meal also increases and reaches a maximum of 0.5 M-NaCl but is still much less than that extracted by water. They attributed the lower dispersibility of the nitrogenous materials to the flocculation of the protein during extraction.

7.2 Objective and Experimental Plan

The literature, reviewed above, clearly indicates that the stability of proteins is affected by the presence of electrolytes. The method developed by us for the production of bland flavoured soymilk involves presoaking and grinding the beans in an alkaline medium (sodium carbonate, see Chapter 2). Neutralisation of the extract from pH 9.7 present during grinding to pH 6.7 with hydrochloric acid before heat-treatment results in the formation of about 0.1 percent of sodium chloride. Therefore, a study was undertaken to investigate the effect of electrolytes on the viscosity of soymilks with particular reference to sodium chloride.

7.3 Experimental

7.3.1 Materials

Electrolytes

1) Sodium chloride (NaCl) - Analar grade
2) Sodium nitrate ($NaNO_3$) - Analar grade
3) Calcium nitrate ($Ca(NO_3)_2$) - Reagent grade

7.3.2 Methods

Preparation of soymilk samples

Soymilk was prepared from carbonate and water presoaked beans according to the method described in Chapter 2. Distilled water was used for the washing, soaking the beans and diluting the slurry after grinding. The milk was finally filtered through a sintered glass funnel no.1 porosity followed by filtering through a no.2 porosity as in Chapter 6.

Treatments of soymilk with various electrolytes

0.05, 0.10, 0.15, 0.20, 0.25, and 0.50 g either of NaCl, $NaNO_3$ or $Ca(NO_3)_2$ was added at room temperature (17° - $20^\circ C$) respectively to 100 ml of raw soymilks or preheated ($115^\circ C$/17 min) soymilks prepared from water presoaked beans. The raw soymilks treated with electrolytes were then processed at $115^\circ C$ for 17 minutes.

Conductivity measurement

The measurement of electrical conductivity in the various soymilk samples was carried out according to the British Standard Method (43) at a temperature of $20^\circ C$ and a frequency of 1000 c/s. The apparatus used was a Philip resistance meter type-GM 4249/10 which measures the resistance between the electrodes of the cell (Philips, PR 9510). From this information the conductivity was calculated (see Appendix 7).

Viscosity measurement

The viscosity of the various soymilks was determined according to the method described previously (Chapter 3).

Determination of degree of aggregation of protein in soymilks treated with sodium chloride

The extent of aggregation of the proteins in the raw soymilk after treating with different concentrations of sodium chloride at room temperature (17° - $20^\circ C$) was determined by centrifugation at 38,000 x g for 10 minutes and the results were compared with data from control sample.

7.3.3 Results

Effect of electrolytes on the stability of proteins in soymilk (water presoaked preparation)

Three electrolytes, sodium chloride, sodium nitrate and calcium nitrate, were used. Their effects on the stability of proteins were measured in terms of changes in viscosity and in the formation of a coagulum. The results are reported in Tables 18 - 20. Table 18 shows that with the increase in concentration of NaCl in raw soymilk the viscosity also increases and reaches a maximum at a concentration of 0.2 percent. On further increase in concentration of NaCl over 0.2 percent, the viscosity of the sample decreased and a coagulation occurred. When these sodium chloride treated raw soymilk samples were heated at 115°C for 17 minutes, the viscosities of the samples were greatly increased even at the lower concentrations of NaCl and maximum viscosity was reached in the sample which contains 0.1 percent NaCl. The proteins were coagulated in samples which contained NaCl concentration over 0.1 percent. On the other hand if sodium chloride was added to samples of the same soymilks which had been preheated at 115°C for 17 minutes, no change in viscosity was observed even at a concentration of 0.5 percent NaCl.

The results in Table 19 demonstrate the effect of sodium nitrate on the viscosity and the protein coagulation of soymilk samples (water presoaked preparation) which had been treated in the same way as in the case of sodium chloride. The behaviour of sodium nitrate in increasing the viscosity of soymilks, and in coagulating the proteins was similar to that of NaCl except that the effect was milder in the case of $NaNO_3$. The maximum reduced viscosity of raw soymilk treated with $NaNO_3$ was 0.60 at 0.25 percent concentration

TABLE 18. Effect of sodium chloride on the stability of proteins in soymilk (water presoaked preparation).

NaCl added %	Viscosity of solvent* cps	Addition to raw soymilk		Addition to soymilk Preheated at 115°C/17 min		Addition to raw soymilk and then heated at 115°C/17 min	
		Conductivity μmhos/cm	Reduced viscosity (η_{red})	Conductivity μmhos/cm	Reduced Viscosity (η_{red})	Conductivity μmhos/cm	Reduced viscosity (η_{red})
Nil	0.893	2.25×10^3	0.23	2.25×10^3	0.37	2.25×10^3	0.37
0.05	0.894	2.88×10^3	0.27	3.00×10^3	0.38	3.00×10^3	0.67
0.10	0.895	3.60×10^3	0.60	3.60×10^3	0.38	3.60×10^3	1.15
0.15	0.897	4.50×10^3	0.76	4.60×10^3	0.38	4.50×10^3	coagulation
0.20	0.898	5.14×10^3	0.87	5.10×10^3	0.38		
0.25	0.899	5.33×10^3	0.79**	5.50×10^3	0.38		
0.50	0.905	9.00×10^3	0.75**	9.00×10^3	0.38		

* Taken from: Handbook of Chemistry, N.A. Lange (Chief Editor), 10th edition, McGraw-Hill Book Company (1966) p.1677

** Slight separation of the phases

TABLE 19. Effect of sodium nitrate on the stability of proteins in soymilks (water presoaked preparation).

$NaNO_3$ Added %	Viscosity of solvent* cps	Addition to raw soymilk		Addition to soymilk preheated at 115°C/17 min		Addition to raw soymilk and then heated to 115°C/17 min	
		Conductivity µmhos/cm	Reduced viscosity (η_{red})	Conductivity µmhos/cm	Reduced viscosity (η_{red})	Conductivity µmhos/cm	Reduced viscosity (η_{red})
Nil	0.893	2.00×10^3	0.23	2.10×10^3	0.36	2.10×10^3	0.36
0.05	0.893	2.60×10^3	0.24	2.70×10^3	0.35	2.60×10^3	0.49
0.10	0.894	3.10×10^3	0.31	3.10×10^3	0.36	3.10×10^3	0.74
0.15	0.895	3.60×10^3	0.43	3.40×10^3	0.35	3.60×10^3	0.85
0.20	0.896	4.00×10^3	0.57	4.00×10^3	0.37	4.00×10^3	coagulation
0.25	0.897	4.40×10^3	0.60	4.50×10^3	0.37		
0.50	0.898	6.50×10^3	0.58**	6.50×10^3	0.36		

* Taken from: Handbook of Chemistry, N.A. Lange (Chief Editor), 10th edition, McGraw-Hill Book Company (1966) p.1677

** Slight separation of the phases

compared with 0.87 at 0.20 percent concentration of NaCl. When the raw samples pretreated with $NaNO_3$ were heated at 115°C for 17 minutes, the maximum reduced viscosity was 0.85 at 0.2 percent concentration whereas under identical conditions of processing the maximum reduced viscosity is 1.15 at 0.15 percent concentration of NaCl. Once the maximum viscosity was reached, proteins in samples which contained high concentration of $NaNO_3$ were coagulated.

Table 20 shows that the effect of calcium nitrate on the viscosity of soymilk and on protein coagulation was more pronounced than that of sodium chloride. Unlike sodium chloride and sodium nitrate, calcium nitrate affected the viscosity and protein coagulability of preheated soymilk. To coagulate the proteins in preheated soymilk less calcium nitrate was required than was necessary for the raw sample.

The effect of these three electrolytes on the viscosity of raw soymilk (water presoaked preparation) is shown in Fig. 13 in which the conductivities of the samples are plotted against their corresponding reduced viscosities. The figure indicates that the degree of change in viscosity is not dependent on the conductivity of the sample, for example, up to a conductivity of 3.25×10^3 µmhos/cm, the viscosities in samples treated with either NaCl or $NaNO_3$ are about the same but as the conductivity is increased over this range the viscosities are more rapidly increased in the samples treated with NaCl than those with $NaNO_3$. At a conductivity of 3.0×10^3 µmhos/cm, the viscosity of calcium nitrate-treated sample is about 4.5 times higher than that either of NaCl-or $NaNO_3$-treated sample.

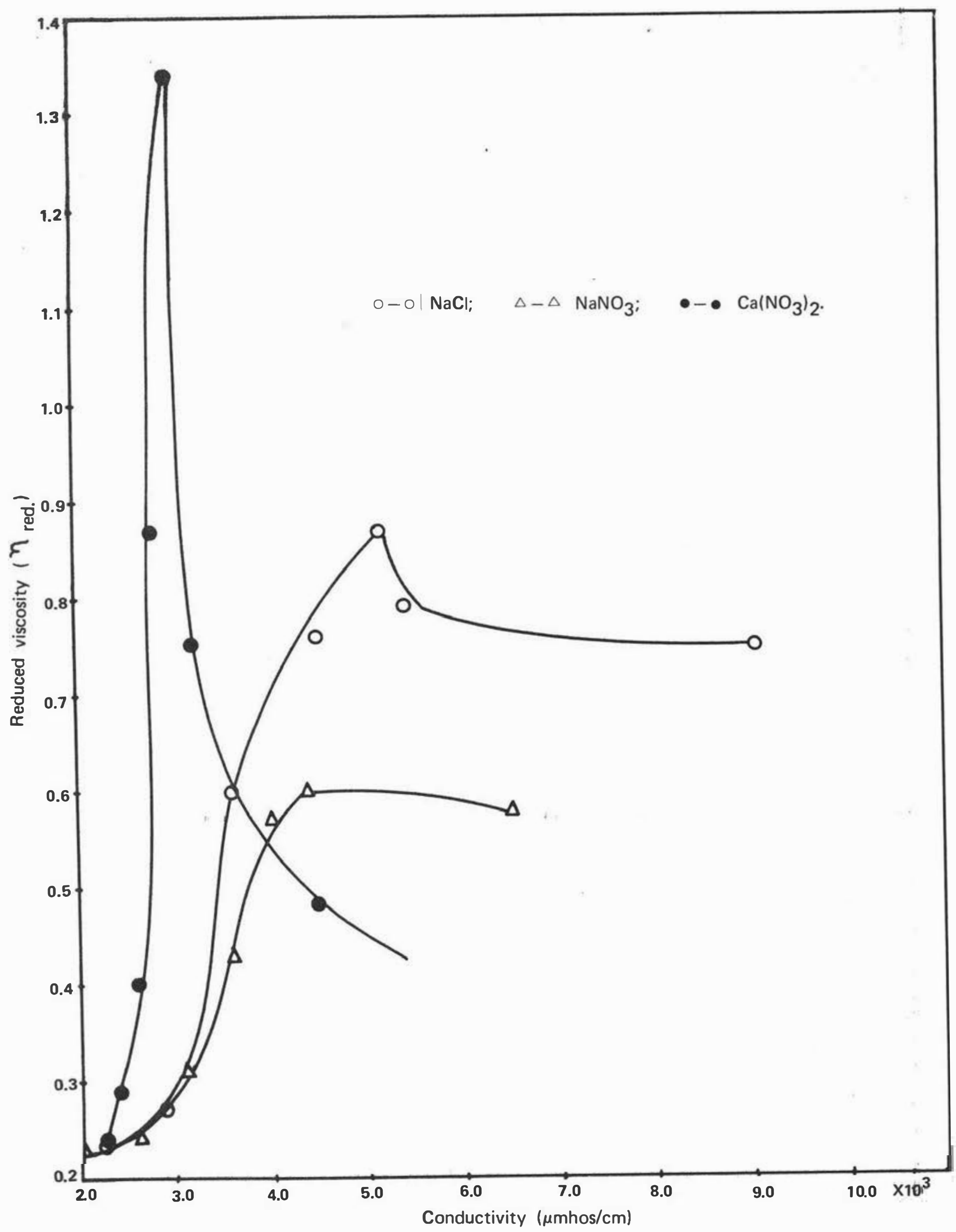

FIG.13. EFFECT OF ELECTROLYTES ON THE VISCOSITY OF RAW SOYMILK (WATER PRESOAKED PREPARATION).

TABLE 20. Effect of calcium nitrate on the stability of proteins in soymilks (water presoaked preparation).

$Ca(NO_3)_2$ added %	Viscosity of solvent* cps	Addition to raw soymilk		Addition to soymilk preheated at 115°C/17 min		Addition to raw soymilk and then heated to 115°C/17 min	
		Conductivity µmhos/cm	Reduced viscosity (η_{red})	Conductivity µmhos/cm	Reduced viscosity (η_{red})	Conductivity µmhos/cm	Reduced viscosity (η_{red})
Nil	0.893	2.25×10^3	0.24	2.30×10^3	0.36	2.30×10^3	0.36
0.05	0.893	2.40×10^3	0.29	2.50×10^3	0.38	2.50×10^3	0.83
0.10	0.894	2.60×10^3	0.40	2.70×10^3	0.46	2.60×10^3	coagulation
0.15	0.895	2.80×10^3	0.87	2.90×10^3	0.60		
0.20	0.895	3.00×10^3	1.34	3.00×10^3	coagulation		
0.25	0.896	3.20×10^3	0.75**				
0.50	0.897	4.50×10^3	0.48**				

* Taken from: Handbook of Chemistry, N.A. Lange (Chief Editor), 10th edition, McGraw-Hill Book Company (1966) p.1677

** Slight separation of the phases

Effect of sodium chloride on the stability of protein in soymilk (carbonate presoaked preparation)

Three soymilk samples differing in sodium chloride level were prepared from the carbonate presoaked beans. The different concentrations of sodium chloride were made by differing the degree of washing of the cotyledons with water before grinding. The sample whose NaCl level was 0.12 percent was divided into five portions and four of them were further treated with additional quantities of sodium chloride so that the salt levels were the same as those added previously to the soymilks prepared from the water presoaked beans. After measuring the conductivities and viscosities, these samples were processed at 115°C for 17 minutes after which the conductivities and viscosities were again measured. The results are set out in Table 21 and indicate that sodium chloride addition up to 0.25 percent had no effect on the viscosities of soymilks prepared from carbonate presoaked beans either in the raw state or after heat treatment.

TABLE 21. Effect of sodium chloride on the stability of proteins in soymilk (carbonate presoaked preparation).

NaCl level in sample %	Addition to raw soymilk		Addition to raw soymilk and then processed at 115°C/17 min	
	Conductivity µmhos/cm	Reduced viscosity (η_{red})	Conductivity µmhos/cm	Reduced viscosity (η_{red})
0.055	2.90×10^3	0.41	2.90×10^3	0.39
0.085	3.30×10^3	0.40	3.30×10^3	0.42
0.12	4.40×10^3	0.41	4.40×10^3	0.41
0.15	4.80×10^3	0.43	4.80×10^3	0.41
0.20	5.50×10^3	0.42	5.50×10^3	0.42
0.25	6.00×10^3	0.42	6.00×10^3	0.42
0.50	9.3×10^3	0.63	9.40×10^3	0.78

Effect of sodium chloride on the stability of proteins in soymilks (water presoaked preparation) pretreated with NaOH at pH 11.1 for various lengths of time

The results in Tables 18 and 21 reveal that the proteins in water presoaked preparation are very sensitive to NaCl whereas those in the carbonate presoaked preparation are insensitive to the same level of sodium chloride. This insensitiveness of proteins to NaCl was also observed when the water presoaked preparation was heated at 115^{o}C for 17 minutes prior to treatment with sodium chloride. This means that the alkaline pH (pH 11.1 during soaking and pH 9.7 during grinding) or/and the heating had induced changes to the structure which made the proteins less sensitive to sodium chloride. If this was the case, then the soymilk prepared from water presoaked beans if treated with alkali should give similar results.

An experiment was designed to investigate the effect of sodium chloride on the proteins in the water presoaked preparation after pretreatment with NaOH. The pH of the milk was adjusted to 11.1 and samples were taken after various intervals of time and were neutralised with 0.5 M-HCl. The sodium chloride level, conductivity and viscosity of each sample were determined. The further addition of 0.1 percent NaCl was made to each sample including a control sample which had not been pretreated with NaOH. The conductivities and viscosities were again measured. All these samples were processed at 115^{o}C for 17 minutes and conductivities and viscosities of these processed samples were measured. The results are presented in Table 22. The results indicate that after pretreatment of soymilk (water presoaked preparation) with NaOH at pH 11.1 at room temperature (17 - 20^{o}C) for $\frac{1}{2}$ an hour the proteins lose their sensitivity to

TABLE 22. Effect of sodium chloride on the stability of proteins in soymilks (water presoaked preparation) pretreated with NaOH to pH 11.1.

Time after pretreatment to pH 11.1	NaCl formation during neutralisation of pretreated samples %	Conductivity of samples µmhos/cm	Reduced viscosity (η_{red})	After addition of 0.1% more NaCl. Total NaCl in sample %	Conductivity of the samples after adding further NaCl µmhos/cm	Reduced viscosity (η_{red})	After heating at 115°C/17 min	
							Conductivity µmhos/cm	Reduced viscosity (η_{red})
Control sample	Nil	2.40×10^3	0.24	0.10	3.90×10^3	0.58	3.80×10^3	1.18
15 min	0.26	5.50×10^3	0.31	0.36	7.20×10^3	0.33	6.50×10^3	0.63
½ h	0.26	5.50×10^3	0.28	0.36	7.20×10^3	0.28	6.50×10^3	0.41
1 h	0.26	5.50×10^3	0.27	0.36	7.20×10^3	0.28	6.50×10^3	0.44
1½ h	0.26	5.50×10^3	0.28	0.36	7.20×10^3	0.28	6.50×10^3	0.43
2 h	0.26	5.50×10^3	0.27	0.36	7.20×10^3	0.28	6.50×10^3	0.43
2½ h	0.26	5.50×10^3	0.27	0.36	7.20×10^3	0.28	6.50×10^3	0.47
3 h	0.26	5.50×10^3	0.27	0.36	7.20×10^3	0.28	6.50×10^3	0.47
3½ h	0.26	5.50×10^3	0.27	0.36	7.20×10^3	0.28	6.50×10^3	0.47
4 h	0.26	5.50×10^3	0.27	0.36	7.20×10^3	0.28	6.50×10^3	0.45
9 h	0.26	5.30×10^3	0.34	0.36	6.50×10^3	0.34	6.50×10^3	0.48
24 h	0.26	5.10×10^3	0.45	0.36	6.50×10^3	0.44	6.50×10^3	0.48

sodium chloride and after 1 hour of treatment the viscosity of the sample which contained 0.26 percent NaCl, was not increased even by processing at 115°C for 17 minutes.

Effect of sodium chloride on the viscosity of soymilk (water presoaked preparation) pretreated with a low degree of heat-treatment

The results in Table 22 showed that treatment of milk prepared from water presoaked soybeans at pH 11.1 at room temperature (17 - 20°C) for ½ hour made the protein insensitive to NaCl at the concentration used. A study was made of the effect of NaCl on the viscosity of a water presoaked preparation preheated for short periods of time at relatively low temperatures. The results are given in Table 23.

TABLE 23. Effect of sodium chloride on the viscosity of soymilk (water presoaked preparation) pretreated with low degree of heat-treatment.

Processing conditions		Reduced Viscosity	Addition of NaCl	Reduced Viscosity
Temp. °C	Time min	(η_{red})	%	(η_{red})
Nil	Nil	0.24	0.10	0.55
65	5	0.25	0.10	0.26
85	3	0.25	0.10	0.25
95	3	0.26	0.10	0.27

The results in Table 23 demonstrate that heat-treatment either at 65°C for 5 minutes or 85°C for 3 minutes is sufficient to make the protein insensitive to NaCl addition.

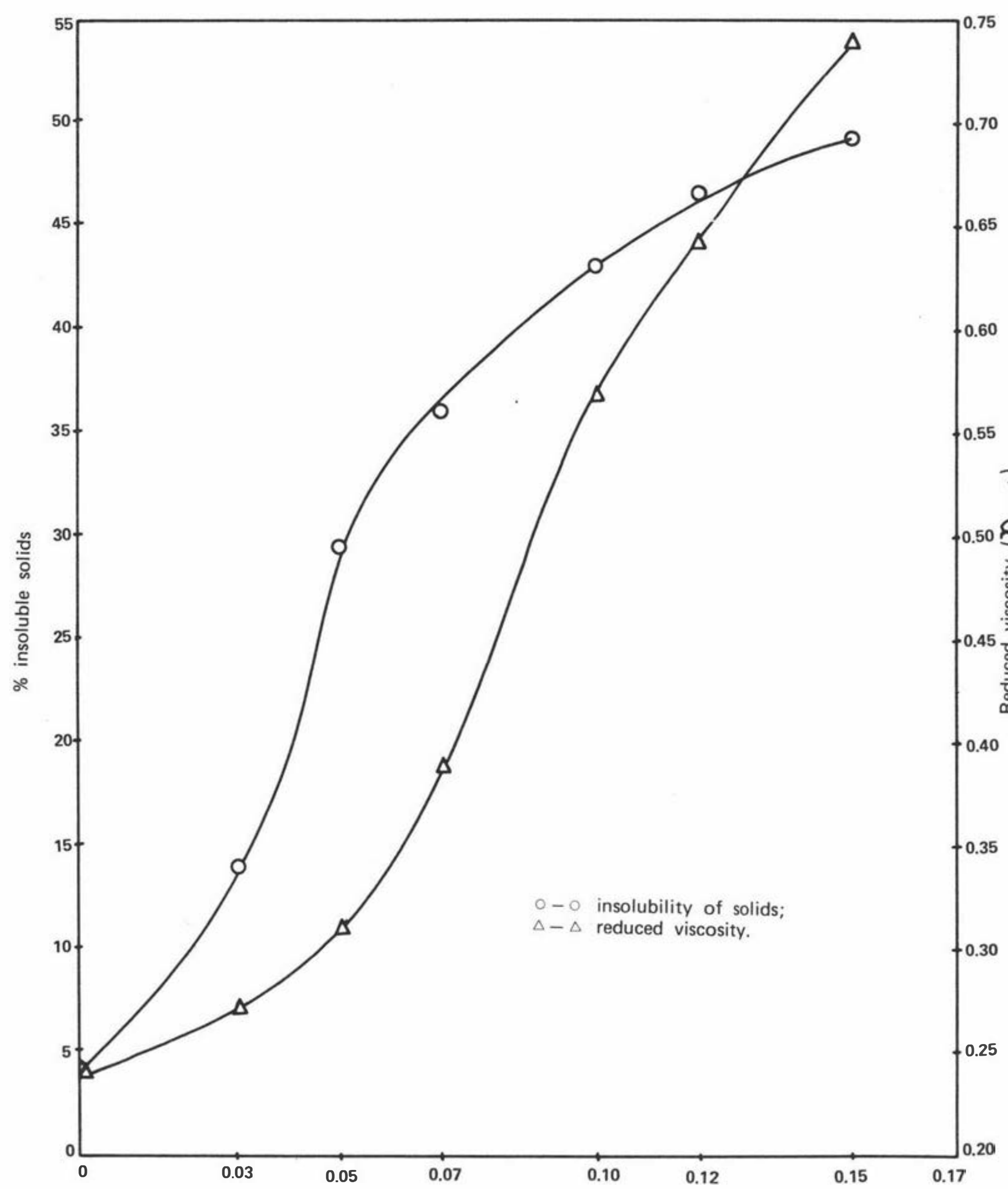

FIG.14. INCREASE IN VISCOSITY AND OF INSOLUBLE SOLIDS (38,000g) OF RAW SOYMILK (WATER PRESOAKED PREPARATION) WHEN TREATED WITH SODIUM CHLORIDE.

Increase of viscosity of raw soymilk (water presoaked preparation) with the progress of aggregation of proteins when treated with NaCl

The results are shown in Fig. 14. The results demonstrate that with the increase in concentration of sodium chloride in the system, both the viscosity and the insolubility of solids content are increased. Up to about 0.07 percent sodium chloride concentration in the sample, the increase in viscosity is not as great as the increase in the insoluble solids content, whereas at concentrations above 0.10 percent the increase in viscosity is more rapid than that of the insolubility of solids. The continuous increment of viscosity in Fig. 14 is observed because the highest concentration of NaCl used in the present experiment was 0.15 percent. At concentrations of NaCl above 0.2 percent the viscosity of the sample decreased due to the formation of visible coagulum (Fig. 13, Table 18).

7.3.4 Discussion

The native proteins in soymilk (about 3.5 percent protein) prepared from water presoaked beans are unstable when treated with the electrolytes used in this study both at room temperature (17 - 20°C) and at 115°C for 17 minutes as shown by the great increase in viscosity and at appropriate concentrations the formation of a coagulum (Tables 18 - 20). The behaviour of proteins in the water presoaked preparation toward sodium chloride (Table 18) is thus very similar to that of β-lactoglobulin as shown by Zittle and Della Monica (277). They presumed the increase in viscosity of the solution of β-lactoglobulin in the presence of sodium chloride was due to the reduction of electrostatic repulsion with a concomitant increase in cross-reaction. Working with bovine serum albumin, Zubay and Doty (279) believe that the coagulation of BSA above the isoelectric point when treated with sodium chloride is due to overcoming the repulsion caused by the charge effect by a medium of high ionic strength.

The results in Tables 18 and 19 had indicated that at the same concentration of sodium ion and under identical conductivities, sodium chloride exerts a more severe effect on the destabilisation of proteins in soymilk than sodium nitrate. Therefore, in this case the anion seems to be responsible for the aggregation process. There is no doubt that the increase in viscosity of raw soymilk treated with sodium chloride is due to aggregation as is evident from the results reported in Fig. 14. The role played by sodium chloride and sodium nitrate may be understood if one assumes that they decrease the repulsion between the electrical charges on the molecule and permit them to approach more closely. It is, however, somewhat puzzling that the anion should be more important than cation at a pH above the isoelectric point, where the protein carries a net negative charge (isoelectric point of soyprotein is 4.6 and the treatment was carried out at pH 6.8). It may be that the anion exerts its effect by being absorbed at certain critical sites which carry positive charges (despite the net negative charge of the molecule).

If salt linkages between positively charged groups on one molecule and negatively charged groups on another were important in causing aggregation, one would expect that electrolytes would inhibit aggregation, since they should inactivate the charged groups either by direct combination with them or by surrounding them with an atmosphere of gegenions (92). The fact that electrolytes promote aggregation therefore suggests that salt linkages play an insignificant role in causing aggregation.

In a study of the effect of sodium chloride on the gelation of gelatin at various pH levels, Boedtker and Doty (30) stated that little can be said with certainty of the nature of the forces involved in the crystallites believed to serve as cross-links and branch points in the aggregation. It is clear from the lack of influence of ionic strength that electrostatic forces play no dominant role. It is likely that the remaining possibilities of hydrogen bonds, dispersion forces and dipole interaction do contribute.

On changing the structure of the native proteins by heat-treatment (115°C/17 min), sodium chloride and sodium nitrate showed no effect on the destabilisation of the proteins in soymilk (Tables 18 - 19) whereas calcium nitrate is more effective in destabilising the denatured proteins than the native ones (Table 20). The cause of this insensitivity of denatured proteins to sodium chloride and sodium nitrate is not clear but the effect of calcium nitrate may be explained on the basis that the denatured protein molecule remained in solution in the extended form and the divalent positively charged calcium ion can then form an intermolecular bridge with the monovalent negatively charged carboxyl groups of the amino acids in the proteins.

The proteins in raw soymilk prepared from carbonate presoaked beans behave similarly to proteins in the heated water presoaked preparation toward sodium chloride. On the addition of up to 0.25 percent sodium chloride to the raw carbonate presoaked preparation, the viscosity of the milk is not increased. On heat-treatment at 115°C for 17 minutes, the increase in viscosity of soymilk (prepared by carbonate presoaking procedure) containing 0.25 percent sodium chloride was the same as that of the sample containing 0.055 percent sodium chloride (Table 21). This insensitiveness of either alkali or heat treated proteins toward sodium chloride is presumably due to the alteration of the protein structure. This is further supported by the fact that the proteins in the water presoaked preparation after denaturing by treatment with NaOH, exhibit the same behaviour as proteins in soymilk prepared from carbonate presoaked beans. From the results in Table 23, it appears that after even a slight change in the structure of the native protein (due to heating at 65°C/5 min), sodium chloride, at the concentration which is effective in native proteins, is now unable to destabilise the modified protein. It can be concluded that the level of sodium chloride formed during neutralisation from pH 9.7 during grinding to pH 6.7 before heat-treatment of the carbonate presoaked preparation has no effect in increasing the viscosity of the product.

CHAPTER 8

FACTORS AFFECTING CONCENTRATION OF SOYMILK AND ITS STABILITY DURING HEAT STERILISATION

8.1 Introduction

In developing countries where there is little or no dairy industry, considerable use has been made of soymilk as a protein supplement for post-weaning infants and young children who suffer from Protein-Calorie Malnutrition. Since the transport systems of these countries are generally poor, particularly in the rural areas where more than 80 percent of population are living, the soymilk must be preserved to distribute among them. Concentration by removal of water is important in reducing the transport and container cost, as well as in reducing the cost of the final drying to a dried product.

Very little information is available in the literature on the concentration of soymilk (255). It appeared from the work already reported that there are likely to be some difficulties in concentrating soymilk because of the large increase in viscosity occuring even with relatively low solids contents. Lo et al (163) postulated that this increase in viscosity of soymilk during evaporation is due to the formation of a gel-like structure. Circle et al (58) showed that the formation of a gel-like structure in a dispersion of soyproteinate during heating is due partly to sulphydryl-disulphide cross linking. Kelley and Pressey (139) reported that the viscosity of a solution of acid-precipitated soy protein is affected by reagents which influence sulphydryl and disulphide groups. On the other hand, Catsimpoolas and

Meyer (56) reported that non-covalent bonds are primarily involved in the gelation of soybean globulins.

It is well known that, as the concentration of cow's milk increases, the susceptibility to coagulation of the protein during sterilisation also increases, but forewarming and the addition of stabilisers are effective in controlling this phenomenon (260). Working with soymilk, Tan (255) reported that forewarming has an influence on the heat stability of the evaporated products. He also showed that addition of sodium citrate or potassium phosphate prior to evaporation increased the heat stability of soymilk with a solid content of 15.5 percent or less but was ineffective in soymilk with higher solids content.

8.2 Objective and Experimental Plan

The work reported in this chapter was initiated to carry out an investigation of factors affecting the viscosity of soymilk during concentration and sterilisation and of the conditions needed to produce a good quality canned soy concentrate.

It has been shown in Chapter 6 that calcium is not involved in increasing the viscosity of fluid soymilk during processing. In the concentrated product where the protein molecules are more closely packed and where the calcium content also increases there is a possibility for involvement of calcium in the aggregation process. The role calcium plays in the instability of proteins in concentrated soymilk was also studied.

8.3 Experimental

8.3.1 Materials and Methods

Preparation of soymilk

Soymilk was prepared according to the method described previously (Chapter 2) from the beans presoaked for 24 hours either in 0.4 M sodium carbonate solution or in water, at room temperature (17 - 20°C). The prepared milk was further filtered through a sintered glass funnel no.1 followed by no.2 as described in Chapter 6.

Forewarming of soymilk

The prepared milk was heated at 90°C for 5 minutes in an open pan or at 115°C for 5 or 17 minutes in an autoclave. The heated milk was cooled immediately to room temperature.

Concentration of soymilk

Concentration was carried out using a Buchi rotary vacuum evaporator, and a water bath temperature of 65°C. An initial vacuum of 20 inches of mercury was used and then this was gradually increased up to 26 inches as the solid content increased. The milk prepared from carbonate presoaked soybeans contained dissolved CO_2 gas which was lost during the first 15 minute period of evaporation and the pH increased to about 7.8. At this point the milk was cooled and neutralised to pH 6.7 with 0.5 M HCl and the evaporation was then continued to the desired solids content. During processing the degree of concentration was approximately determined by measuring the refractive index of the sample at 20°C with an Abbe 60 Refractometer (149) but a more accurate solid content was determined according to the standard method of A.O.A.C. (13). When the sample was treated with a specific chemical reagent, the reagent was dissolved in the milk and the pH was adjusted to 6.7, if necessary, prior to evaporation.

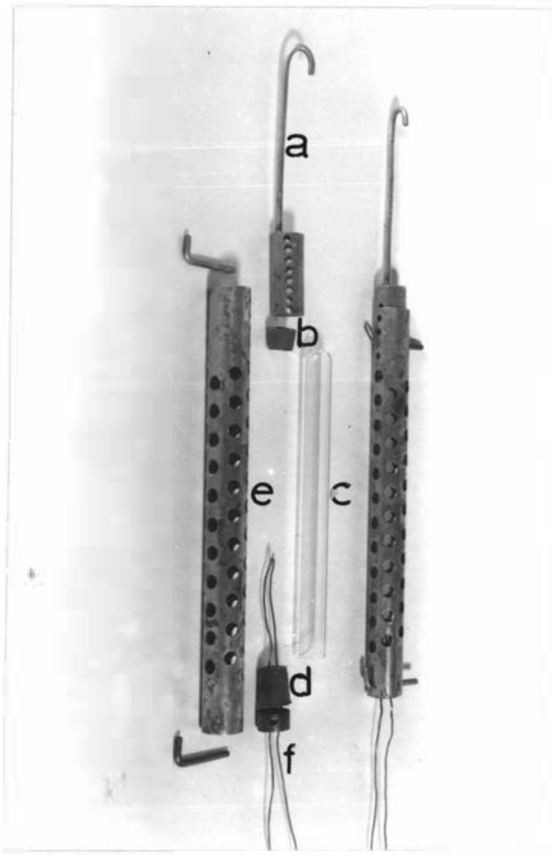

FIG.15. BRASS TEST TUBE HOLDERS FOR STUDYING HEATING EFFECTS UNDER DEVELOPED PRESSURE: (a) HANGER; (b) RUBBER BUNG; (c) THICK WALLED 20 ml TEST TUBE; (d) RUBBER CORK; (e) PERFORATED BRASS TUBE; (f) THERMOCOUPLE LEADS.

FIG.16. COMPLETE SET UP FOR STERILISATION ON A SMALL SCALE: (a) CONSTANT TEMPERATURE GLYCEROL BATH; (b) THERMOMETER; (c) BRASS TEST TUBE HOLDERS; (d) HONEYWELL TEMPERATURE RECORDER; (e) THERMOCOUPLE LEADS; (f) COOLING BATH.

Sterilisation of concentrated soymilk

A preliminary study on the effect of sterilisation of various concentrated soymilks was done on a small scale, using 6" x $\frac{5}{8}$" pyrex glass test tubes fitted with thermocouples of copper and constantan. The hot junction was placed at the geometrical centre of the tube. The tube was then placed in a brass test tube holder capable of maintaining the rubber closures continuously sealed under the pressure conditions that developed. The equipment is shown in Fig. 15. The thermocouples were connected to a Honeywell temperature recorder. The tube and its contents were immersed in a constant temperature glycerol bath at 121°C(250°F) and a continuous record was made of the changing temperature at the centre of this product (Fig. 16). The lethality applied was approximately 5.2 - 5.5 which is sufficient heat treatment for the sterilisation of soymilk (164).

The above method was used for the selection of optimum pre-treatment and concentration conditions prior to further detailed studies on the effect of sterilisation using 211 x 309 cans fitted with thermocouples at the geometrical centre (69) (Fig. 17). The can was filled with a concentrated soymilk with a standard net head space of 3/16". The can was then processed at a steam temperature of 121°C (250°F) in a horizontal static retort to a lethality of about 5.5 (Fig. 18). The change of temperature at the centre of the can was recorded by a Honeywell temperature recorder. Precise replication of concentration for each of the products presented in Table 28, was not possible since for each pre-treatment the sample had to be separately concentrated.

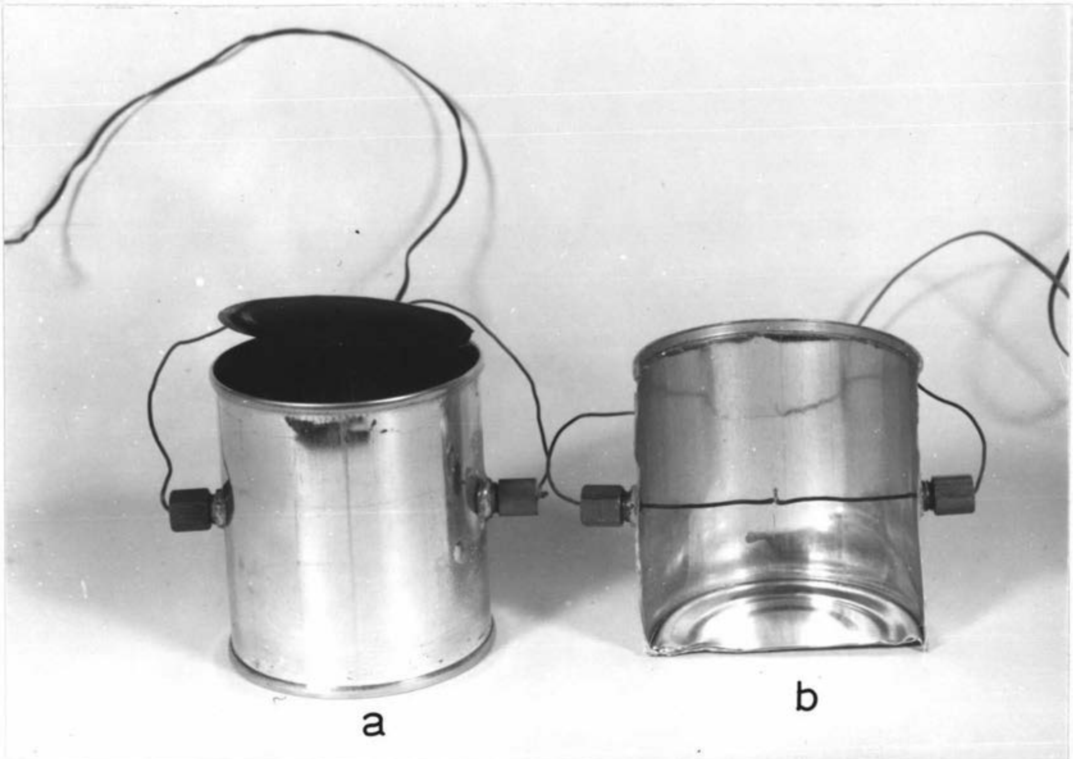

FIG.17. 211 x 309 CANS USED FOR STERILISATION: (a) CAN WITH GLAND FITTING FOR THERMOCOUPLE SEAL; (b) SECTIONED CAN SHOWING POSITION OF THERMOCOUPLE INSIDE THE CAN.

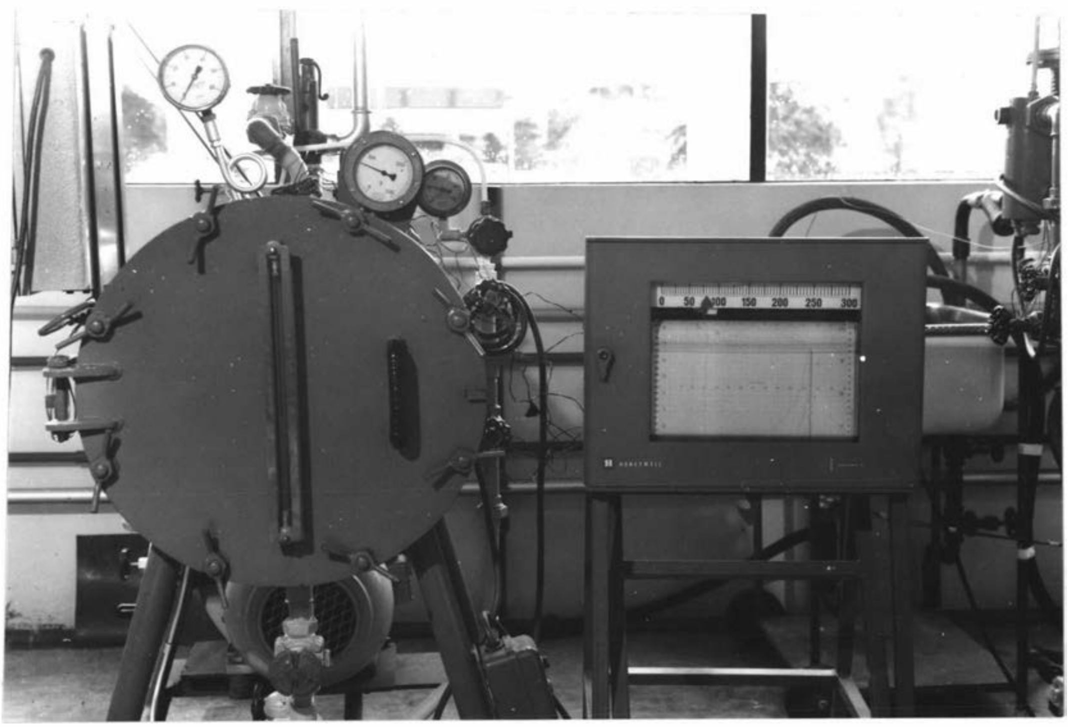

FIG.18. EXPERIMENTAL RETORT WITH TEMPERATURE RECORDER.

Viscosity measurement

The viscosity of each sample was determined at 25°C on a Brookfield Synchro-lectric viscometer (LVT model). The speed of rotation was kept constant at 6 rpm while different LVT spindles were used to cover the various viscosity ranges. This change in the size of the spindle resulted in a smaller variation in the apparent viscosity when measured on the same sample than did changes in the speed of rotation of the same spindle (163). The viscosity data are reported as apparent viscosity calculated by using the appropriate factor. It was observed during this experiment that the viscosity of the same sample is different when measured using the LVT spindle from that obtained by using the UL adapter and that is why the viscosities of soymilks prior to concentration in this chapter are higher than those reported in Chapters 6 and 7.

Determination of insolubility of solids in concentrated soymilk

Water at 60 - 70°F was added to a measured amount of concentrated soymilk so that the protein content of the reconstituted milk would be approximately 3.5 percent (equivalent to 6.7 percent total solids). The mixture was dispersed in a M.S.E. Micro Emulsifier at a speed of 1700 rpm for 2 minutes. The quantity of insoluble solids in this reconstituted milk was determined by the two methods used in Chapter 6. Results from the solubility index method would indicate whether a commercially acceptable product had been made. The second method involved precipitation at 38,000 x *g* for 20 minutes and the results from this method gave a measure of the extent of aggregation of protein occurring during heating and concentrating. In both methods the dry weight of sediment was recorded as a percentage of total solids or, where indicated in the tables, the volume of sediment per 50 ml reconstituted milk are recorded.

8.3.2 Results

Effect of concentration on the viscosity and the solubility of soymilks (Tables 24 and 25; Fig. 19 and 20)

Soymilks prepared from both water and carbonate presoaked beans, were heated at 115°C for 17 minutes to inactivate the trypsin inhibitor prior to concentration. The viscosity and the solubility of concentrated soymilks of different solids levels are given in Tables 24 and 25.

TABLE 24. Effect of concentration on the viscosity and insolubility of solids in soymilk from water presoaked beans. (After preheating 115°C/17 min).

Solid content %	Apparent viscosity cps	Insolubility of solids	
		Using solubility index method dry weight %	At 38,000 x g dry weight %
7.66	6.6	<0.01	6.00
8.76	12.0	<0.01	6.96
12.87	860	<0.01	7.11
15.15	9,100	<0.01	8.30
16.10	nd	nd	13.67
17.23	nd	nd	14.16
18.93	62,000 (Gelled)	<0.01	46.83

The viscosity increases exponentially with the increase of solids content in concentrated soymilks prepared either from water or from carbonate presoaked beans as shown in Fig. 19. The insolubility

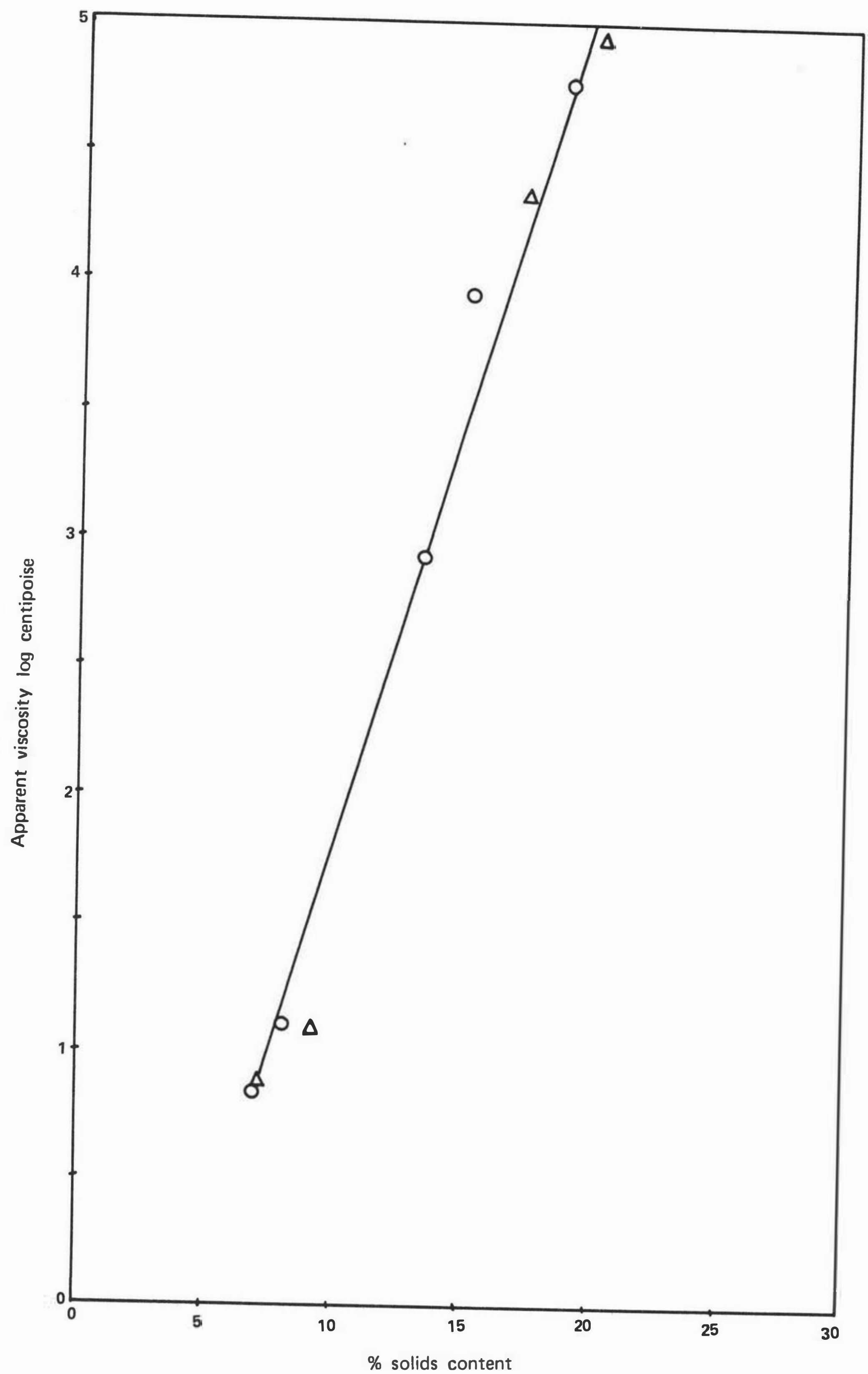

FIG.19. EFFECT OF CONCENTRATION ON THE VISCOSITY OF SOYMILKS.

Δ – Δ soymilk prepared from carbonate presoaked beans;
○ – ○ soymilk prepared from water presoaked beans.

of solids, as measured by the standard solubility index method was less than 0.01 percent of the total solids in concentrated products up to about 18 percent solid content using both presoaking procedures. Using the high speed centrifuge method the weight of sedimented solids increased with the increase of solids content, the increase being slightly higher in the milk prepared from carbonate presoaked soybeans.

TABLE 25. Effect of concentration on the viscosity and insolubility of solids in soymilk from carbonate presoaked beans. (After preheating 115°C/17 min).

Solid content %	Apparent viscosity cps	Insolubility of solids: Using solubility index method dry weight %	Insolubility of solids: at 38,000 x g dry weight %
7.95	7.0	$<$0.01	7.16
10.12	12.5	$<$0.01	11.23
14.21	nd	nd	20.46
15.78	nd	nd	23.43
17.26	22,000	$<$0.01	46.59
20.18	96,000 (Gelled)	0.04	54.76

With the water presoak treatment, the rate of increase of sediment with the increase of total solids was slow up to about 15.5 percent total solids, above which a rapid increase in the rate was observed, but with the alkali presoaking a more uniform rate of increase was apparent (Fig. 20) but even in this case the rate increased with increasing concentration. Under the conditions used, the rates at the higher concentration were very similar for both milks.

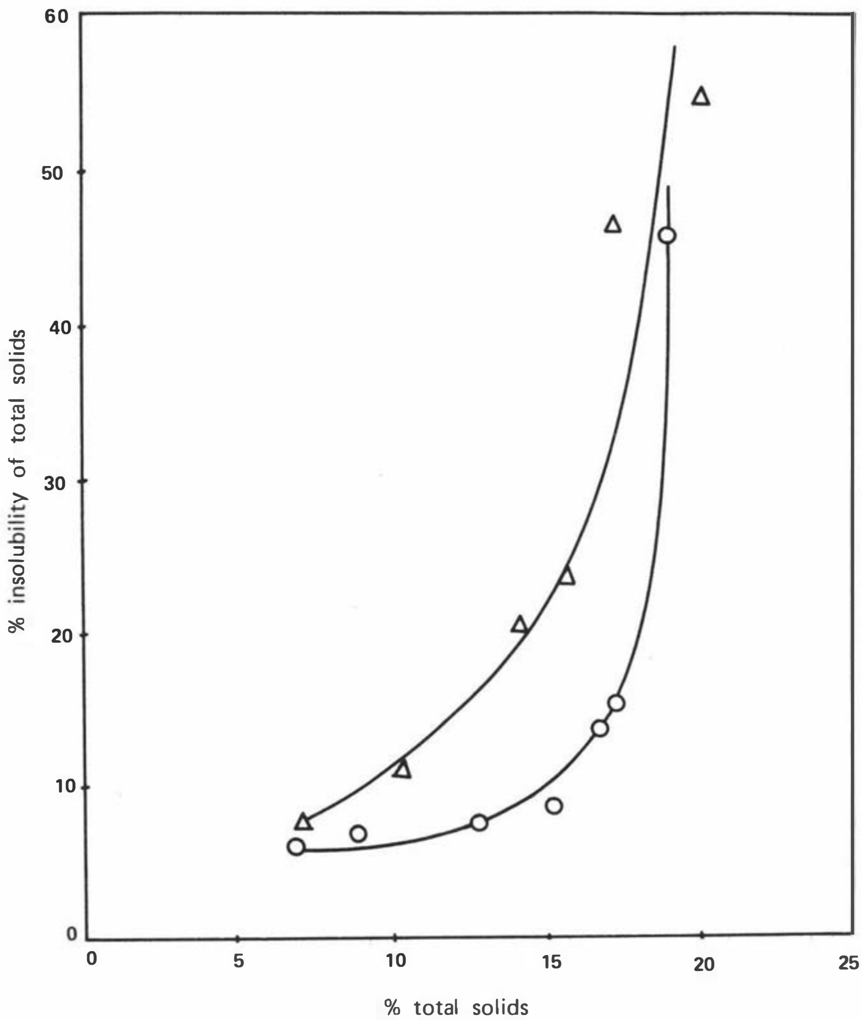

FIG.20. EFFECT OF CONCENTRATION ON THE INSOLUBILITY OF SOLIDS IN SOYMILKS.

△ – △ soymilk prepared from carbonate presoaked beans;
○ – ○ soymilk prepared from water presoaked beans.

Effect of additives on the viscosity of concentrated soymilk (Table 26)

Soymilk prepared from water presoaked beans, was first heated (115°C/17 min) to inactivate the trypsin inhibitor and then treated with several chemical reagents prior to concentration. The results are reported in Table 26.

TABLE 26. Effect of additives on the viscosity of concentrated soymilk from water presoaked beans. (After preheating 115°C/17 min). (Refer to Table 24 for controls).

Additives		Total solid content	Apparent Viscosity
Type	Concentration in dispersion %	%	cps
$(NaPO_3)_6$	0.10	15.64	22,000
$Na_3P_3O_{10}$ + NaH_2PO_4	.05 + .05	15.89	22,500
Na_2SO_3	0.10	14.58	59
Na_2SO_3	0.10	18.85	10,400
Na_2SO_3 + $Na_2P_3O_{10}$	.10 + .05		
+ NaH_2PO_4	+ .05	17.25	8,800
N-ethyl maleimide	.05	15.56	490
N-ethyl maleimide	.05	18.96	11,000

The results indicate that sodium sulphite and N-ethyl maleimide are effective in reducing the viscosity to some extent whereas sodium hexametaphosphate, trisodium polyphosphate and sodium dihydrogen phosphate raise viscosity compared with that of the untreated equivalent (see Table 24). The effect of additives on stability during sterilisation of the concentrated products using carbonate presoaked preparation is shown in Table 28.

Influence of forewarming on the sterilisation stability of concentrated soymilk (Table 27)

Forewarming has a beneficial effect on the sterilisation stability as indicated by fluidity and the insolubility of solids.

TABLE 27. Influence of forewarming on the sterilisation stability of concentrated soymilk (carbonate presoaked preparation).

Forewarming		Solid content	Lethality	Visual appearance after sterilisation	Insolubility of solids. Solubility index method. ml/50ml reconstituted milk
Temp. °C	Time min	%	F_o value		
Nil	Nil	13.58	5.2	soft gel	0.1
		14.21	5.2	soft gel	0.2
		14.70	5.4	hard gel	0.4
		18.52	5.4	hard gel	0.6
		19.58	5.3	hard gel	nd
		27.79	5.2	hard gel	nd
90	5	12.17	5.5	fluid	<0.1
		12.57	5.4	fluid	0.1
		13.02	5.4	fluid	0.2
		14.60	5.3	soft gel	0.3
115	5	13.14	5.5	fluid	<0.1
		14.33	5.5	fluid	<0.1
		15.41	5.4	fluid	0.2
		16.75	5.3	soft gel	0.2
115	17	12.24	5.5	fluid	<0.1
		13.07	5.4	fluid	<0.1
		14.76	5.4	fluid	0.2
		16.08	5.3	soft gel	0.2

This is apparent when the appearance of the sterilised products are compared. Thus the 14.70 percent concentration without forewarming gave a hard gel on sterilisation whereas the 15.41 percent concentration that had been forewarmed at 115°C/5 min was still fluid after the same sterilisation procedure. The effect of prolonged forewarming (17 minutes as against 5 minutes at 115°C) does not further improve the stability against sterilisation. The results also indicate that poor stability against sterilisation also causes an increased insoluble solids content.

Effect of pretreatment on the viscosity changes of concentrated soymilk due to sterilisation (Table 28)

The viscosity of the concentrated soymilk which has not been forewarmed is very low when compared with the sample which has been

TABLE 28. Effect of pretreatment on the viscosity changes of concentrated soymilk due to sterilisation (carbonate presoaked preparation).

Forewarming		Additive pretreatment		Solids content in milk	Apparent viscosity of the product	
Temp. °C	Time min.	Type	Concentration in dispersion %	%	Before sterilisation cps	After sterilisation cps
Nil	Nil	Nil	Nil	15.14	58	13,400
90	5	Nil	Nil	14.24	16,000	10,800
115	5	Nil	Nil	15.32	9,200	10,200
Nil	Nil	Na_2SO_3	0.10	15.52	10	14,400
115	5	Na_2SO_3	0.10	15.98	50	13,900
Nil	Nil	N-ethyl maleimide	0.05	15.38	83	13,100
115	5	N-ethyl maleimide	0.05	15.66	330	6,100
Nil	Nil	Na_2HPO_4	0.05	15.30	78	15,500
115	5	Na_2HPO_4	0.05	15.40	10,400	14,400

forewarmed at 115°C/5 min, but after sterilisation the increase in viscosity is higher in the former. However when forewarmed at 90°C for 5 minutes a very high initial viscosity was apparent and this was reduced due to sterilisation.

The results also demonstrate that although sodium sulphite is very effective in reducing the viscosity of concentrated soymilk irrespective of whether it has been forewarmed or not, its effect does not persist to the sterilised samples. N-ethyl maleimide is effective in reducing the viscosity in the sterilised product if forewarmed milk is treated prior to concentration. Disodium phosphate is found to be ineffective in increasing the sterilisation stability of soymilk.

8.3.3 Discussion

It is possible to produce concentrated milks containing up to 30 percent total solids (T.S.) when the soymilk is not preheated. The viscosity increase of these raw concentrates is still very small even at 27.8 percent T.S. This concentrate gels on sterilisation even at 13.6 percent T.S. In Table 28 the viscosity of the concentrates subject to no forewarming is seen to be less than one hundredth of the forewarmed sample but on sterilisation it is considerably higher than the forewarmed sample.

Because the heat usually applied during concentration and drying is inadequate to destroy the trypsin inhibitor in soymilk it was necessary to examine the change in viscosity and in sedimented residue with increasing concentration after heating both the water presoaked and the carbonate presoaked milks at 115^{o}/17 min to inactivate the trypsin inhibitor.

The pattern of increment of viscosity with the degree of concentration of soymilk prepared from carbonate presoaked beans is very similar to that of the water presoaked preparation and a logarithmic relationship exists between the viscosity of the concentrated soymilks and solids content. Lo et al (163) showed a similar relationship using milk prepared from water presoaked soybeans. The increase of viscosity and insolubility of solids with the degree of concentration are probably due to the progressive polymerisation of protein molecules by cross-linking leading to the formation of larger aggregates ultimately resulting in the formation of a gel-like structure. This polymerisation could be caused by secondary valence bonds (83), by salt bridges through multivalent cations (24) or by sulphydryl-disulphide inter change (131). Although increasing concentration causes a similar rate of increase in viscosity with both milks there appears to be a difference in the rates at which the amount of sediment increases. It would thus appear that the aggregation leading to sedimentation differs from the aggregation leading to the increased viscosity. It was earlier suggested that the carbonate presoaking process results in partial denaturation of the soy protein even prior to heat processing (Chapters 5 - 7). It thus appears that whereas the denaturation due to heat results in a very similar form of intermolecular reaction in the two milks as indicated by the uniform increase in viscosity, for the aggregation as measured by sedimentation it is necessary for the water presoaked milk to reach a minimum concentration of around 15 percent before sufficient reactive sites are within interaction range to form a stable aggregate, and this is less necessary with the carbonate presoaked milk because it already has more reactive sites exposed. Kelley and Pressey (139) also reported that alkaline conditions favour sulphydryl-disulphide interchange reactions.

Results reported in Tables 26 and 28 suggest that calcium in soymilk is not involved in a cross-linking reaction. Fukushima and Van Buren (97) reported that the polyvalent metal ions are not responsible for polymerisation of proteins in soymilk during drying.

The reduction in the viscosity of the concentrated soymilks which have been preheated and then treated with sodium sulphite, a disulphide bond-splitting reagent or with N-ethyl maleimide, a sulphydryl blocking reagent, confirms that sulphide-disulphide interactions are significant in the increase in viscosity on concentration. This confirms the observation by Fukushima and Van Buren (97) that disulphide bonds contribute appreciably to the formation of the protein aggregates. Reference to Table 26 indicates that the extent of the sulphite and N-ethyl maleimide reduction of viscosity is very dependent on the concentration of the milk.

The effect of sterilisation on the viscosity of sulphite treated and N-ethyl maleimide treated samples indicated in Table 28 suggests that there are quite distinct differences in the manner in which these two reagents are involved.

Although pretreatment with either sulphite at 0.1 percent or N-ethyl maleimide at 0.05 percent resulted in very considerable reductions in the viscosity of the concentrated samples prior to sterilisation it was only the N-ethyl maleimide treatment of a sample which had been previously preheated which had any significant effect on the apparent viscosity of the concentrate after sterilisation. It thus appears that N-ethyl maleimide can only increase sterilisation stability if the milk has been forewarmed first and then the reagent added prior to concentration. It does not appear to be capable of reacting during the high temperature processing stage of sterilisation. It thus appears that the -SH groups are protected from reacting with the N-ethyl maleimide in the native form of the protein but become exposed as a result of heat treatment. Under cooled conditions the N-ethyl maleimide reacts with the exposed -SH groups to give a heat stable complex which then cannot undergo a sulphydryl-disulphide polymerisation during sterilisation. On the other hand sulphite ions can affect the state of aggregation of the protein irrespective of whether the molecule has been previously heat denatured or not but the complex is unstable under sterilisation conditions.

The forewarming process is one of the most important steps upon which the heat stability of evaporated cow's milk depends (122). Using soymilk prepared from water presoaked beans, Tan (255) reported that when forewarmed under batch conditions the heat stability of the evaporated milk was maximum when heated to 80°C with no holding time. When forewarmed in a continuous "sterilisator" the maximum heat stability was obtained with a process of 90°C for 45 seconds. Tan did not use forewarming temperatures greater than 90°C in his batch processing studies.

Deysher, Webb and Holm (71) showed that cow's milk of low heat stability gave concentrated products of high viscosity on sterilisation and they also showed that variations in forewarming procedures affected the viscosity of the condensed sterilised milk through their indirect effect upon the heat stability of the protein. They also reported that increasing the temperature of forewarming above the boiling point with a limited increase of the period of exposure to the forewarming temperature increases the stability of the concentrated milk under sterilising conditions. Hunziker (122) also reported that forewarming temperatures above the boiling point produce maximum stability of concentrated cow's milk. The forewarming effect on the stability of concentrated cow's milk to heat has been attributed to changes in the salt balance particularly of calcium and phosphate ions of the milk and to modification of the serum proteins (260).

In the study reported here changes in salt balance appear unlikely to be responsible for the observed changes in viscosity on sterilisation since the addition of phosphates generally caused an increase in viscosity. The results reported here do not agree with those of Tan (255) in this respect.

The viscosity of the concentrated soymilk generally increases further during sterilisation because of the progressive unfolding and aggregation of the proteins. Modification of the form of this aggregation or unfolding is necessary if an increase in viscosity during sterilisation is to be controlled. Although some structural modification occurs in the proteins of soymilk prepared from carbonate presoaked beans, forewarming is still necessary to increase the sterilisation stability of the concentrated soymilk since, given the proper forewarming conditions, the increment of viscosity due to sterilisation is then not as high as it is with the raw concentrated milk. The results reported here indicate that the sterilisation stability of concentrated soymilk prepared from carbonate presoaked beans is optimum when forewarmed at 115°C for 5 minutes.

As no satisfactory alternative process is available for the destruction of the trypsin inhibitor it is necessary to preheat the product to 115°C/17 min prior to further processing whenever the further process does not include such a treatment as for example in producing a sweetened condensed product or a dried product. This essential preheat treatment so modifies the protein structure that a maximum concentration of about 18 percent only is possible as can be seen in Tables 24 and 25.

8.3.4 Conclusion

It is possible to produce a fluid sterilised canned soymilk concentrate based on milk prepared from carbonate presoaked beans. The concentrated milk could contain up to 15.5 percent total solids (approximately 9 percent protein) if it has been forewarmed at 115°C/5 min prior to concentration. A readily dispersible soft gel occurs at 17 percent total solids and in the light of present knowledge this represents the maximum concentration that is marketable as a sterilised concentrate.

CHAPTER 9

EVALUATION (in vitro) OF NUTRITIONAL QUALITY OF PROTEINS IN VARIOUS HEAT PROCESSED SOYMILKS

9.1 Introduction

It is known that for some processed foodstuffs, individual samples can differ widely in the nutritive value of their proteins. Processing may actually improve the nutritional value of such proteins but under some conditions may diminish it. Proper processing can destroy antitryptic factors in the leguminous proteins and enhance the biological values. Nutritional value can be reduced by excessive heat especially in the presence of carbohydrates.

These changes in nutritive value of proteins caused by over-processing may not be very important in well-fed areas of the world, where protein consumption is more than adequate, but may be very important where diets are barely adequate in protein content. Any change in quality is equivalent to a change in quantity.

The evaluation of proteins and mixed protein diets for nutrition in man and animals can be accomplished most accurately in the species for which the proteins are intended and under the circumstances in which they are used. Since this can seldom be accomplished, proteins are more conveniently tested in a variety of other species having analogous nutritional requirements, but animal tests are cumbersome, time consuming and expensive. Some of the biological methods used for the determination of nutritional quality of proteins are summarised in Appendix 9.

More recently, through our increased knowledge of amino acid requirements, better methods of amino acid analysis, and an appreciation of importance of amino acid availability, various laboratory methods have been developed which reflect more or less accurately the protein quality as applied to specific species and conditions (239).

9.2 General review of literature on the effect of processing on the nutritional quality of proteins

If food processing is defined to include all treatments of a food-stuff from the place of origin to the point of consumption, then more than 95 percent of our food is processed. Most foods are not fully acceptable and must be trimmed and cooked to make them more palatable. Most foodstuffs are not stable and must be subjected to various processing conditions including heating, freezing, drying, irradiation and chemical treatment to preserve them so that they may be stored or transported. In most cases, food processing causes a reduction of the nutritional value of a food. As a result of advances in the science of food and nutrition, the adverse effects of processing of which we were formerly unaware are now becoming apparent. Of these treatments, heat is the most important in its effect on protein quality. The effect may be either beneficial or detrimental. The beneficial effect is observed in most of the leguminous proteins which contain anti-nutritional factors especially trypsin inhibitors. Heat-treatment inactivates these and enhances the biological value (see Chapters 4 and 5), but the application of heat must be a balance between the beneficial and destructive effects.

The early work on the effect of heat on the nutritive value of many proteins has been reviewed by Rice and Beuk (230) and more recently by Liener (154). Although heat treatment may in many cases cause decreased digestibilities, the major cause of heat injury appears to be the result of an impaired assimilation of one or more essential amino acids. Thus the experiments of Greaves, Morgan and Loveen (102) indicated that the deterioration in the nutritive value of heated casein (at 120°C for 24 hours) can be compensated for by the addition of lysine to the experimental diet. Results on the sensitivity of lysine to dry heat in lysine-rich human globin were obtained by Devlin and Zittle (70). They showed that after drying in an oven at 55°C - 85°C for approximately 18 hours the availability of lysine had been impaired. Block et al (26) have shown that dry heating of purified proteins does not result in the chemical destruction of lysine because as much lysine could be isolated after acid hydrolysis of heated casein (at 150°C for 65 minutes) as from unheated. Block et al (27) reported that destruction of lysine in protein may occur during toasting of food-stuffs where the protein is in the presence of carbohydrates and fats. When a cake mixture was baked at a temperature of approximately 200°C for 15 to 20 minutes, and the cake was subsequently dried on the radiator, the PER decreased to 2.4 from its original value 3.3 - 3.5 in an unbaked mixture. If the baked cake was dried in an oven overnight at 60°C, the PER decreased further to less than 1.5. When slices of the cake were toasted in a low temperature oven at 100 - 120°C, until they had the appearance of commercial rusk (Zwieback), the PER was less than 0.7 and in some cases the animals were just able to maintain weight. However, if 0.49 percent of l(-)-lysine were added to this oven dried cake, the PER rose to 2.7; while the addition of 0.63 percent of lysine to the toasted material practically restored its initial nutritive value (PER 3.2)

Peters et al (216) studied the effect of various methods of processing oat proteins on amino acid composition and on the in vitro release of amino acids by tryptic digestion as well as on the growth performance of rats. They found that increasing the severity of heat treatment caused a progressive decrease in the ease with which lysine could be released from the protein by enzyme action. More severe heat treatment such as toasting (at 200°C for 2 minutes) and gun explosion (preheated for 5 minutes at 122°C followed by cooking at 200 lb pressure (198°C) for 2 minutes) actually caused an appreciable destruction of lysine as evidenced by the decreased amount of lysine that could be recovered by acid hydrolysis. The addition of lysine restored the nutritive value of the oat protein damaged by the toasting process. Halevy and Guggenheim (107) have shown that by autoclaving wheat gluten with glucose 50 percent of the lysine was destroyed and the amount of lysine released by pancreatin was likewise reduced by one half. These studies make it clear that lysine may become unavailable to an animal because of incomplete enzymatic digestion and/or actual destruction. The former can occur under conditions of relatively mild heat treatment which does not necessarily cause a destruction of lysine. These two effects are referred to as the "inactivation" and "destruction" of amino acids (154). The "inactivation" is defined as the difference between the amounts of an amino acid which can be measured in deproteinised hydrolysates after subjecting the raw and the heated protein to in vitro enzymatic digestion under standardised conditions whereas "destruction" is defined as the difference between the amounts of an amino acid which can be measured in the acid (or alkaline in the case of tryptophan) hydrolysates of the raw and the heated proteins.

The mechanism of the chemical changes that take place during the interaction of a protein with sugars has been studied extensively by Lea and Hannan (150 - 153) using casein and glucose as model reactants (170). They found that the most rapid chemical change observed in the system was a loss of amino groups, particularly the E-amino groups of lysine and the formation of a colourless compound, N-substituted 1-amino-1-deoxy-2-ketose in a molar ratio of 1 : 1. At this stage of reaction, lysine could be quantitatively recovered by acid hydrolysis. As the interaction between casein and glucose proceeded beyond the 1 : 1 stage, the mixture darkened and lysine could no longer be recovered by acid hydrolysis. Failure to recover lysine could be caused by the fact that the protein-sugar reaction had proceeded to a point where the protein was no longer attached to a hydrolysable glycosyl linkage (154).

Regarding the availability of lysine, it has been established that trypsin splits a protein molecule at those peptide bonds containing the carbonyl moiety of lysine or arginine provided the E-amino group of lysine or the guanido group of arginine is unsubstituted. Substitution of these groups renders the protein no longer susceptible to cleavage by trypsin.

Earlier work on the effect of processing on nutritive value was concentrated on lysine as the sole seat of damage and in certain foods and mixtures lysine appears to be the only amino acid that is damaged. Later Riesen and associates (231) investigated the liberation of the essential amino acids from raw, properly heated and over-heated soybean oil meals. They found that the amount of each of the essential amino acids liberated by acid hydrolysis of soybean oil meal was unaffected by heat treatment at 15 psi for 4 hours, except lysine, arginine and tryptophan. The liberation by pancreatic hydrolysis of these amino acids was decreased for raw and overheated meals. That there is amino acid destruction when protein is heated with certain carbohydrates has been shown conclusively by Patton et al (214). By comparing the amino acid composition of purified casein with that of casein which had been refluxed for 24 hours in 5 percent glucose solution, they showed that very significant losses of lysine, arginine and tryptophan occurred and that there were less marked losses of histidine, leucine, and isoleucine. Patton et al (215) also showed that refluxing of soy globulin in a 5 percent glucose solution for 24 hours resulted in the partial destruction of lysine, arginine, tryptophan and histidine. Refluxing in water did not decrease the total amount of essential amino acids in the protein. Evans and Butts (77), using a mixture of soybean protein and sucrose rather than a suspension of a pure protein in a glucose solution, obtained very similar results as far as destruction of lysine is concerned. In this experiment it seems that hydrolysis of the sucrose may have occurred. In subsequent work, Evans and Butts (78, 79) and Evans et al (80) reported that autoclaving at 15 psi for 4 hours of soyprotein plus sucrose destroyed more lysine, arginine and cystine than the autoclaving of soybean protein alone, and that additional amounts of these and other amino acids were made unavailable for in vitro enzyme hydrolysis. They concluded that three types of reaction are possible when soy protein is heated in the presence of sucrose:

1. Certain of the amino acids, principally lysine, aspartic and glutamic acids react with other constituents of the protein to form enzyme resistant combinations. Acid hydrolysis of these combinations will release the intact amino acids; hence they are not destroyed. This type of reaction can occur

whether or not carbohydrate is available.

2. Protein-bound methionine, cystine, histidine, threonine, glycine and aspartic acid (and perhaps other amino acids) react with sucrose, presumably after hydrolysis, to form enzyme-resistant linkages although the amino acids could be recovered unchanged after acid hydrolyses.

3. Those amino acids with unbound amino groups may react with sucrose, with resultant destruction of the amino acids. Acid hydrolysis will not regenerate the amino acids in such cases.

Recently Bender (18) reported that in some foods the sulphur amino acids suffer more damage than does the lysine. In unheated fish, for example, methionine is the limiting amino acid and remains limiting in grossly overheated fish meal. Supplementation of these poor samples with methionine does increase the N.P.U. but not up to the levels of undamaged material. Carpenter et al (48) showed that after heat treatment of herring meals a loss due to inactivation occurred in tryptophan, methionine, arginine and lysine.

9.3 Review of literature on the nutritional quality of proteins in soymilks

The nutritive value of soyprotein has been recognised for many years by both animal and human nutritionists and it is well established that the nutritional quality of properly processed soy protein is comparable to that of milk protein and the very best red meat protein (116) (see also Appendix 1). The present review is concerned with the nutritional studies on proteins in soymilks.

Fomon (85) carried out an experiment with four normal infants aged from 113 to 154 days at the beginning of the study. They were fed on experimental soybean formula as a sole source of energy for periods ranging from 36 to 72 days. The formula provided 7 percent of the calories as protein derived from soy extract made from full fat flour from the whole soybean. The mean rate of gain in weight during the interval was 21.3 g/day, a value nearly identical to that of infants of similar age receiving pooled human milk ad libitum. He also studied the relation of retention of nitrogen to intake of nitrogen with infants fed pooled human milk, those fed the soybean formula and those fed a formula providing 7 percent of calories as protein from cow milk and reported that proteins from the three different sources promote retention of nitrogen in a similar manner.

Miller (185) reviewed the work of Homma, Takahashi and Kawnzura from the Department of Pediatrics, Tokyo University School of Medicine, who fed soymilk to a large group of infants averageing in age from birth to seven months. Three babies used as controls were fed on cow's milk formulas. Their results were as follows: in all cases the soymilk was well taken by infants, no nausea and vomiting occurred. The stools were soft, dark yellow to dark green and averaged 1 to 5 in number. Body weight gain was more rapid in the soymilk fed babies than in the control, mammalian milk fed babies. Blood examination revealed neither anaemia nor low serum protein levels. The intestinal flora of these 10 babies showed moderate increases in L.bifidis and the pH of the faeces decreased until acidic. The results resembled those of breast fed babies. X-ray plates of legs and hands were normal at both the beginning and the end of the feeding period with soymilk.

Desikachar et al (68) conducted a human feeding experiment to determine the nutritive value of soymilk protein. They found that when cow's milk curd protein fed along with a poor South Indian rice diet is replaced by an equivalent amount of soymilk protein, the mixed proteins in both the cases are utilised to about the same extent. By following the nitrogen balance method on two adult subjects, they (68) reported that the average digestibility coefficient and the biological value of soymilk protein compared with egg protein are 96.6 and 94.0, respectively. Cahill et al (47) reported that the average biological value of soybean protein for maintenance in adult human subjects, using the protein of whole egg as a standard, was found to be 94.5 percent for protein in cooked whole soybean, 91.7 percent for that in cooked soy flour and 95.3 for that in a soybean milk.

DeMaeyer and VanderBorght (65) performed a comparative study of nutritive value of different protein foods on the feeding of African children and reported that the percentage of absorption, the biological value (BV) and net protein utilization (NPU) are respectively: whole eggs 97, 90, 87; cow's milk 91, 87, 79; human milk 90, 100, 90 and soymilk 95, 80, 76. They concluded that some batches of soymilk showed a nutritive value similar to that of cow's milk and if the product is carefully standardised, it might be useful in the feeding of infants when breast feeding or cow's milk are unavailable. Jones (137) noted that clinical observations have shown the infants can grow and thrive for periods of over a year with soy-milk as the sole source of proteins in their diet and that it compares favourably with mammalian milk from the standpoint of availability and biological value of its protein.

Gyorgy et al (103) using rats, determined the protein efficiency ratio (PER) of four commercial soymilk samples and found PER values ranging from 1.4 to 2.2. Three of these preparations were given as sole and first formula (with supplements of vitamins and iron) to 80 premature infants during their whole stay in the nursery and also at home after their discharge. Forty-six premature infants as control received a proprietary cow's milk formula. The soymilk with the highest PER promoted gains in weight over the whole period of observation comparable to that seen in the control group fed the cow's milk formula. No distinct difference in the figures for body length was noted in the various groups. In contrast, the serum protein values were high in the groups receiving the best soymilk or cow's milk and low in the groups fed the other two soymilk preparations. They concluded that there were distinct differences in the nutritive value of such soymilk products which was evident not only when tested on animals but also when tested on infants. These differences must primarily be due to variations in the underlying technological processes used and of these the most important seems to be the influence of exposure to heat in its intensity and/or duration. Graham (101) stated "we have evaluated a variety of soy products for infant feeding and have found important differences which must in part be attributed to the effect of processing, with a well-known soymilk giving results very similar to those of a modified cow's milk."

Hackler et al (104) investigated the effect of processing on the nutritive value of soymilk proteins when fed to weanling rats. They showed that the nutritive value of soymilk proteins was affected by both temperature and time of heating. Heating soymilk for 1 - 6 hr at 93°C had no adverse effect on protein efficiency, growth or available lysine. Heating for 32 minutes at 121°C in contrast showed that there was a definite decline in the protein efficiency ratio (PER), and an indication that available lysine was declining. The drop of available lysine was greater after soymilk had been heated 40 minutes, at 121°C. However, the highest PER value of soymilk processed at 121°C is somewhat greater than the best PER value found for soymilk processed at 93°C.

Van Buren et al (261) showed that available lysine started to decrease after 10 minutes of heating at 121°C and a high correlation was observed between the available lysine level and the biological values of the various heat processed soymilks.

Hackler and Stilling (105) carried out a further study on the effect of heat on the amino acid composition of soymilk and its correlation with the nutritive value. Their results indicated that heating of soymilk at 93°C for as long as 4 hr. had no significant effect on amino acid composition. When the soymilk was heated at 121°C, cystine and tryptophan decreased as the time of heating increased from 0 to 120 minutes. Cystine was the most susceptible to damage (decrease is immediate), whereas a decline in tryptophan was noted only with soymilk heated for 60 minutes or longer at 121°C. They put forward an explanation for the decrease of PER with the increase in time of heating at 121°C suggesting that this drop in PER was directly related to a decrease in cystine, followed by a decrease in tryptophan. It is generally recognised that the total sulphur amino acid content of soybeans limits the utilisation of its protein (188).

9.4 Objective and Experimental Plan

It appears from the literature that the variation in nutritive value of proteins in various soymilks is due to variation in the processing conditions since both under-processing and over-processing affects the utilisation of proteins in soyproducts. The under-processed products contain antinutritional factors which interfere with the utilisation of the proteins while over-processing lowers the quality by making the proteins unavailable through destruction or modification or both.

The aim of the experiment was to evaluate the comparative nutritional quality of proteins in soymilks prepared from carbonate presoaked beans and processed under various conditions but always under conditions in which the antinutritional factors were completely destroyed. Optimum heat processed soymilk prepared from water presoaked beans was taken as standard.

The determination of the real nutritional quality of an unknown protein or protein food falls in the domain of biological assay in humans and test animals. Soy protein has already been extensively tested biologically (see review above and Appendix 1) and since the carrying out of biological tests is cumbersome, time consuming and very expensive, and unsuitable for industrial process control, a number of standard in vitro methods which appear to accurately reflect the nutritional quality of protein (see review in Appendix 8) were chosen for study of their potential use in the industrial situation.

Although properly processed soymilk prepared from carbonate presoaked beans showed superiority in digestibility to that of the water presoaked preparation (Chapter 5) a conclusion on the nutritional quality of the proteins can not be drawn from this because the method used measures total free amino groups and does not distinguish between individual amino acids. The quantity of amino nitrogen measured was contributed to by both the essential and non-essential amino acids and their proportions may vary in different enzyme digests. One relatively unimportant amino acid may be rapidly and completely liberated by enzyme(s) while other essential amino acids, like lysine, may have formed new peptide linkages that are resistant to enzymatic digestion, thereby affecting the utilisation of this amino acid and the quality of the whole protein. Therefore, a further evaluation of nutritional quality of the various heat-processed soymilks prepared from carbonate presoaked beans was carried out using as a basis the amino acid composition and their availability. This data was compared with data similarly derived for a soymilk based on water presoaking and heated at 115°C for 18 minutes.

9.5 Experimental

9.5.1 Determination of amino acid composition of proteins in various heat-processed soymilks by acid hydrolysis

9.5.1.1 Materials and Methods

Preparation of soymilk samples for the evaluation of nutritional quality

Soymilks were prepared either from water presoaked or carbonate presoaked beans according to the method described in Chapter 2. Milk from water presoaked soybeans was processed at 115°C for 18 minutes. Three different types of processing were applied to the soymilk prepared from carbonate presoaked beans: (i) Processed at 115°C for 18 minutes; (ii) Processed at 98°C for 40 minutes; (iii) Preheated at 115°C for 5 minutes, then concentrated to a solids content of 15.5 percent, and sterilised in 211 x 309 cans to a F_o value of 5.5, according to the method described in Chapter 8. All four processed soymilk samples were freeze-dried and stored at -20°C until analysed.

Nitrogen determination

The nitrogen contents of the freeze-dried soymilks were determined by semi-micro Kjeldahl procedure as described previously (Chapter 3).

Preparation of hydrolysates for amino acid analysis

Hydrolysates for the determination of amino acids from freeze-dried soymilk samples were prepared according to the procedure described in Chapter 3.

Chromatography of hydrolysates

This was modified by using a modified 2 hour procedure instead of the 4 hour run as used in Chapter 3. The operating conditions have been summarised in Appendix 4.

9.5.1.2 Results

The amino acid contents of the various heat processed soymilks are presented in Table 29, on the basis of percentage in the crude protein. The recovery of amino acids in the soymilk analyses was from 98 - 100 percent. The data obtained from the above analysis is not discussed in detail because statistical analysis showed that there is no significant difference between the individual amino acid content in any of the soymilks analysed. The essential amino acid contents of soymilks (average of four milk samples) were compared with the reference pattern of essential amino acid requirements of the Food and Agriculture Organisation (FAO) (225), whole egg, cow's and human milks in Table 30. Although FAO do not give a requirement for arginine and histidine, the requirement of arginine proposed by Rose (233) and of histidine by the Food and Nutrition Board of the National Academy of Science (74) for both human infants and albino rats are also included in the table. Table 30 shows that the percentage of sulphur amino acids in soymilk is lower than that of cow's milk but both milks are deficient compared with the FAO provisional pattern. The essential amino acid index (EAAI) as modified by Mitchell (189) calculated for the heat processed soymilk samples, human and cow's milks are also given in Table 30. The EAAI of soymilk is the lowest among the three milks.

TABLE 29. Amino acid composition of proteins in various heat-processed soymilks.

	g amino acid/100 g protein			
	Milk from beans pre-soaked in water, processed at 115°C for 18 minutes	Milk from carbonate presoaked beans		
		Processed at 115°C for 18 minutes	Processed at 98°C for 40 minutes	Concentrated, sterilised *
Lysine	6.08 ± 0.04	6.38 ± 0.02	6.12 ± 0.23	6.10 ± 0.02
Histidine	2.02 ± 0.00	2.09 ± 0.00	2.15 ± 0.04	2.15 ± 0.04
Arginine	7.96 ± 0.08	7.65 ± 0.06	7.81 ± 0.10	7.56 ± 0.35
Aspartic acid	12.09 ± 0.09	12.78 ± 0.34	12.64 ± 0.03	12.15 ± 0.24
Threonine	3.77 ± 0.03	3.81 ± 0.00	3.88 ± 0.01	3.79 ± 0.03
Serine	4.52 ± 0.02	4.78 ± 0.06	4.83 ± 0.02	4.67 ± 0.04
Glutamic acid	20.98 ± 0.09	21.15 ± 0.00	20.81 ± 0.86	20.57 ± 0.04
Proline	4.30 ± 0.07	4.48 ± 0.05	4.55 ± 0.09	4.43 ± 0.09
Glycine	3.79 ± 0.02	3.84 ± 0.02	3.89 ± 0.05	4.05 ± 0.46
Alanine	3.55 ± 0.00	3.70 ± 0.02	3.70 ± 0.02	3.41 ± 0.00
½ cystine	1.49 ± 0.14	1.39 ± 0.01	1.30 ± 0.02	1.35 ± 0.04
Valine	4.90 ± 0.01	4.50 ± 0.70	5.24 ± 0.06	5.12 ± 0.17
Methionine	1.13 ± 0.01	1.16 ± 0.01	1.15 ± 0.12	1.14 ± 0.06
Isoleucine	4.57 ± 0.01	4.70 ± 0.03	4.66 ± 0.06	4.49 ± 0.04
Leucine	7.63 ± 0.01	7.89 ± 0.09	7.86 ± 0.08	7.63 ± 0.12
Tyrosine	3.70 ± 0.04	3.66 ± 0.09	3.72 ± 0.10	3.41 ± 0.03
Phenylalanine	4.73 ± 0.11	4.82 ± 0.07	4.83 ± 0.06	4.52 ± 0.01
Tryptophan	1.42 ± 0.00	1.40 ± 0.00	1.38 ± 0.05	1.53 ± 0.01

* Preheated at 115°C for 5 minutes, concentrated to 15.5% solids content, sterilised to a F_o value of 5.5.

TABLE 30. Comparison of the pattern of the essential amino acids in soymilk with that of FAO provisional pattern, egg, cow's and human milks.[a]

Amino Acid	FAO Provisional pattern	Soy-milk	Human milk	Cow's milk	Hen's egg
Isoleucine	4.2	4.61	6.4	6.4	6.6
Leucine	4.8	7.78	8.9	9.9	8.8
Lysine	4.2	6.17	6.3	7.8	6.4
Total "aromatic" amino acids	5.6	8.35	10.1	10.0	10.0
Phenylalanine	2.8	4.73	4.6	4.9	5.8
Tyrosine	2.8	3.62	5.5	5.1	4.2
Total sulphur-containing amino acids	4.2	2.53	4.3	3.3	5.5
Cystine	2.0	1.35	2.1	0.9	2.4
Methionine	2.2	1.15	2.2	2.4	3.1
Threonine	2.8	2.81	4.6	4.6	5.1
Tryptophan	1.4	1.43	1.6	1.4	1.6
Valine	4.2	4.94	6.6	6.9	7.3
Arginine	2.0[b]	7.75	-	3.2	6.4
Histidine	2.4[c]	2.10	2.2	2.3	2.6
EAAI	-	77.62	92.9	89.74	100

[a]FAO Pattern of amino acid requirements (Ref.226)

[b]Amino acid requirements suggested by Rose (Ref.233)

[c]Requirement of albino rat or human infant (Ref.74)

9.5.2 Determination of available essential amino acids by enzymatic methods

9.5.2.1 Materials

Soymilk samples - same as those used in experiment 9.5.1

Pepsin - 1 : 60,000 (2x crystalised) Sigma Chemical Company, U.S.A.

Trypsin - 1 : 9,000 BAEE per mg, Miles Seravac, Grade IV, Maidenhead, England

Pancreatin - The British Drug House Ltd, England

Erepsin - ex hog intestine (2451h), Koch-Light Laboratories Ltd, England

Resin-'Dowex' 2-x8, 20-50 mesh (Cl), The British Drug House Ltd, England

9.5.2.2 Methods

The method of Ford and Salter (87) with slight change was used for enzymatic digestion and of Akeson and Stahmann (2) for the estimation of amino acids. As carried out in the present study, a sample of freeze-dried soymilk representing 15 mg protein was weighed into a conical flask of 50 ml capacity, and to this 15 ml of 0.05 M-HCl containing 4 mg of pepsin was added. The final pH of the mixture was 1.6. The flask and contents were incubated at 37^{o}C for 24 hours with frequent swirling. After pepsin digestion, the pH of the digest was brought to about 8 with 0.5 M-NaOH and 0.5 g of $NaHCO_3$ was added to bring the pH to 8.2. 2 mg of crystalline trypsin and 8 mg of pancreatin were added to the digest. 0.5 ml of toluene were added as preservative and incubation was continued for a further period of

24 hours with frequent swirling. At the end of the second digestion period, 1 ml of 0.02 M phosphate buffer of pH 7.6 containing 25 mg of erepsin was added. Incubation was continued for another period of 24 hours with frequent swirling.

At the end of the digestion period, 100 ml of 1 percent picric acid solution was added to each flask to precipitate the undigested proteins and larger peptides. The precipitate was removed by centrifugation at 1530 x g for 30 minutes followed by filtration through Whatman No.52 filter paper. The filtrate was passed through a column (0.9" x 25" resin bed) containing the anion exchange resin (about 15 gm) in chloride form to remove the picric acid. This was followed by rinsing the column with three small volumes about 25 ml for each wash) of 0.02 M-HCl. The total solution after passing through the column was concentrated to a slurry using a rotary vacuum evaporator at a bath temperature of 40°C. The sample was then diluted to 50 ml with pH 2.2 sodium citrate buffer. Amino acid analysis of the sample was made by the ion exchange method as described in Chapter 3, using the 2 hour procedure as in 9.5.1.

9.5.2.3 Results

The results are reported in Table 31. The table shows that the release of most of the essential amino acids by enzymes is lower in soymilk prepared from water presoaked beans than with any of the carbonate presoaked preparations. The overall effect of the three different types of processing as applied to the soymilk prepared from carbonate presoaked beans showed no significant differences. The better recovery of lysine and leucine from the carbonate presoaked milk is highly significant and that of histidine is just significant. The size and shape of the cystine peak in the

TABLE 31. In vitro availability of essential amino acids (g amino acid/100 g protein)

Amino Acid	Water presoaked preparation, processed at 115°C/18 min	Carbonate presoaked preparation		
		processed at 115°C/18 min	processed at 98°C/40 min	concentrated and sterilised [a]
Lysine	3.25 (52.67)	3.92 (63.53)***	3.61 (58.51)***	3.84 (62.24)***
Histidine	0.56 (26.67)	0.77 (36.67)*	0.60 (28.57)*	0.66 (31.43)*
Arginine n.s.	6.57 (84.83)	6.70 (86.51)	6.52 (84.18)	6.79 (87.67)
Threonine n.s.	0.49 (12.85)	0.49 (12.85)	0.49 (12.85)	0.48 (12.59)
Cystine	Trace	Trace	Trace	Trace
Valine n.s.	0.85 (17.21)	1.01 (20.45)	1.17 (23.68)	1.02 (20.65)
Methionine n.s.	0.45 (39.30)	0.50 (43.67)	0.47 (41.05)	0.49 (42.79)
Isoleucine n.s.	1.02 (22.15)	1.12 (24.32)	1.18 (25.62)	1.06 (23.02)
Leucine	4.78 (61.46)	5.63 (72.39)**	5.64 (72.52)**	5.71 (73.42)**
Tyrosine n.s.	2.27 (62.66)	2.40 (66.25)	2.29 (63.22)	2.44 (67.36)
Phenylalanine n.s.	3.30 (69.84)	3.80 (80.42)	3.74 (79.15)	3.88 (82.12)
Total	23.54	26.34	25.71	26.37

The figures in parentheses give the percentages of the total released for each amino acid.

[a] Preheated at 115°C for 5 minutes, concentrated to solids content 15.5%, sterilised to a F_0 value 5.5

*, **, *** Significant at $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively

n.s. Alongside an amino acid means that the release of that amino acid is not significantly different in any of the soymilks. The level of significance indicated is based on a comparison with the values for the water presoaked milk.

chromatogram rendered it impossible to calculate its quantity accurately and it is, therefore, recorded in the table as "trace". Tryptophan was destroyed during the picric acid procedure and so was not determined in the enzyme hydrolysates (2).

9.5.3 Determination of available lysine of various heat-processed soymilks by FDNB "difference" procedure

9.5.3.1 Materials

Soymilk samples - same as those used in experiment 9.5.1.

9.5.3.2 Methods

Available lysine contents in various heat-processed soymilks were determined according to the method developed by Roach et al (232).

Determination of "total" lysine contents in soymilks

The lysine contents recovered from the acid hydrolysates of soymilks in 9.5.1 and reported in Table 29 were taken as the total lysine values in the present experiment.

Determination of "residual" lysine contents in soymilks

1.0 g of each freeze-dried soymilk sample was taken in a ground joint round-bottomed flask of 150 ml capacity and to this was added 8 ml of 8 percent ($^{W}/_{V}$) sodium bicarbonate solution. The flasks were shaken gently to disperse the material and then allowed to stand for at least 10 minutes. 0.3 ml of 1-fluoro-2,4-dinitrobenzene (FDNB), previously dissolved in 12 ml of ethanol was added to each flask, which was then stoppered and shaken gently on a Griffin flask shaker for 2 hours. Ethanol was then evaporated off on a steam bath. 25 ml of 8 M-HCl were introduced into each flask and refluxed for 24 hours. At the end of the hydrolysis period each condenser was washed down with 50 ml of distilled water. The hydrolysates were allowed to stand for more than an hour and then filtered through Whatman No.52 filter paper with water washing and the filtrate was made up to 200 ml. 0.5 ml of the hydrolysate was applied to the column of the amino acid analyser for the separation of residual lysine under the same conditions as used in experiment 9.5.1. The residual lysine content as measured in this experiment was subtracted from the total lysine content of the same sample and the difference is the available lysine value of the sample.

9.5.3.3 Results

The results are given in Table 32. The results show that the lysine availability in soymilk prepared from water presoaked beans is slightly lower than that in any of the three samples prepared from carbonate presoaked soybeans. Statistical analysis of the results shows that there is no significant difference among the lysine availabilities in the milk samples analysed. The available lysine values found are about 90 - 91 percent of the total lysine recovered from the acid hydrolysates.

TABLE 32. Available lysine content in various heat-processed soymilks (g amino acid/100 g protein).

Samples	Processing Conditions		Available lysine	Significance of difference from T
	Temp. °C	Time min		
Water presoaked preparation	115	18	5.57[T] (90.28)	
Carbonate presoaked preparation	115	18	5.65 (91.57)	n.s.
Carbonate presoaked preparation	98	40	5.63 (91.25)	n.s.
Carbonate presoaked preparation (canned)*	121	F_0 = 5.5	5.64 (91.41)	n.s.

* Preheated at 115°C for 5 minutes, concentrated to 15.5 percent solids content, sterilised at 120°C to a F_0 value of 5.5.

The figures in parentheses give the lysine recovery as a percentage of the total lysine recovered from acid hydrolysates.

n.s. - not significant.

9.5.4 Determination of pepsin digestibility of proteins in various heat-processed soymilk using low pepsin strength

9.5.4.1 Materials

Soymilk samples - same as those used in experiment 9.5.1.

Pepsin - 1 : 2,500, The British Drug House Ltd, England.

9.5.4.2 Methods

The method of A.O.A.C. (13) as modified by Lovern et al (165) was used through out these studies. 1.0 g of each freeze-dried soymilk sample was taken in a conical flask of 250 ml capacity. To this was added 150 ml of freshly prepared pepsin - HCl solution (0.0008 percent of 1 : 2500 pepsin in 0.075 M-HCl) prewarmed to 45°C.

The samples were incubated at 45°C for 16 hours in a constant shaking incubator (Gallenkamp Orbital incubator) at 150 rev/minute. To a similar sample in a second flask acid alone was added. After incubation, about 1.0 g of filter aid (standard super-cell) was added to each flask and the contents were filtered with suction through Whatman No.52 filter paper. The residue in the Buchner funnel was washed three times with warm water, then the filter paper and residue were transferred to a Kjeldahl flask and the nitrogen content was determined as described previously (Chapter 3).

Calculation

$$\% \text{ "Corrected pepsin digestibility"} = \frac{D - S}{100 - S} \times 100$$

where D = % total nitrogen solubilised by acid + pepsin

S = % total nitrogen solubilised by acid alone

9.5.4.3 Results

The results are presented in Table 33. The table shows that the susceptibility of proteins to pepsin attack in three differently processed soymilk samples prepared from carbonate presoaked beans is about the same whereas the digestibility of proteins in the water presoaked preparation is significantly lower ($P < .01$) than that in any of the carbonate presoaked preparations.

If these corrected pepsin digestibilities of the various heat-processed soymilks (Table 33) are plotted against available lysine values (Table 32), they reveal a significant correlation ($r = .913$, $P < .001$) as shown in Fig. 21.

TABLE 33. Pepsin digestibility of various heat-processed soymilks.

Samples	Processing Conditions		Corrected pepsin digestibility %	Significance of difference from X	Significance of difference from Y
	Temp. °C	Time min			
Water presoaked preparation	115	18	74.31 X		
Carbonate presoaked preparation	115	18	80.29 Y	***	
Carbonate presoaked preparation	98	40	79.14	***	n.s.
Carbonate presoaked preparation, preheated at 115°C for 5 mins, concentrate to total solids 15.5%, sterilised	121	F_0 = 5.5	78.71	***	n.s.

*** significant at $P < .001$

n.s. - not significant

9.6 Discussion

The preprocessing conditions, either presoaking in water or carbonate solution, and the processing conditions, either heating at 115°C for 18 minutes, or 98°C for 40 minutes or 121°C for a F_0 value of 5.5, used in the present study showed no significant effect on the amino acid composition of proteins in soymilks. Although the amino acid composition of proteins is influenced by the varietal differences and climatic conditions in which soybean grows (197), yet the amino acid compositions of heat-processed soymilks in the present study (Table 29) agree quite well with the results of the recent reports of

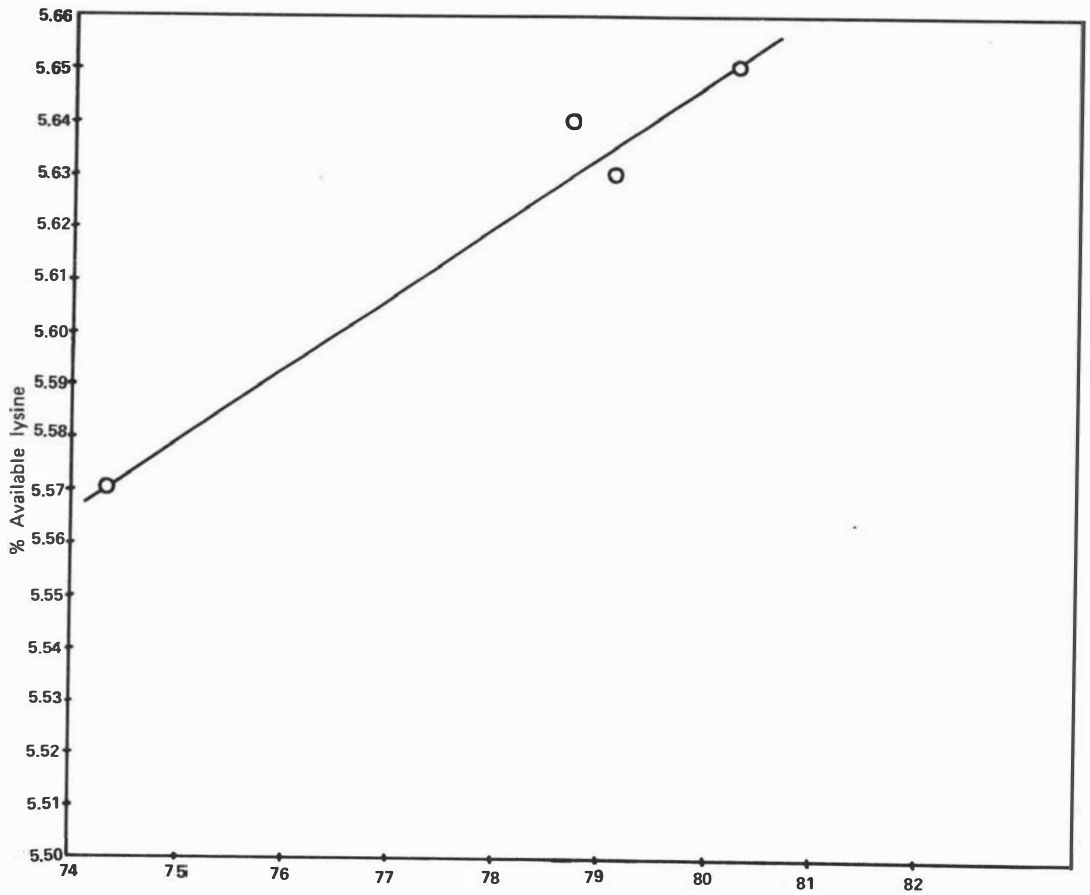

FIG.21. CORRELATION BETWEEN CORRECTED PEPSIN DIGESTIBILITIES AND AVAILABLE LYSINE VALUES OF PROTEINS IN VARIOUS HEAT–PROCESSED SOYMILKS.

Hackler and Stillings (105) on soymilk proteins and of Evans and Bandemar (81) and Tkachuk and Irvine (258) on soybean proteins.

There is an indication that the sulphur-containing amino acids in the FAO Provisional Pattern (225) is too high (Table 30) and several authors (225) have suggested reducing the amount to 3.04 - 3.52 g/100g protein (%N x 6.25). Even so the soymilk proteins are deficient in sulphur amino acids (Table 30). The Essential Amino Acid Index (EAAI) of soymilk in the present study is about the same as reported by Hackler and Stillings (105) for the optimum heat-processed soymilks.

The results of the amino acid availability as determined by the enzymatic method (Table 31) show that some of the amino acids are very poorly released in all samples and most of the amino acids were not released in quantities comparable with those in acid hydrolysates (Table 29). Furthermore, the lysine availability reported in Table 32 as determined by the FDNB-procedure using acid hydrolyses was much higher than the value reported in Table 31. Therefore, there has been incomplete hydrolysis by the enzymes used and in the specified time period, the results reported in Table 31 are not the absolute values of available amino acids using these enzymes. Complete in vitro enzymatic hydrolysis is very difficult to achieve (20, 180). Thus the difference between the quantity of the same amino acid recovered from enzyme hydrolysates (Table 31) should not be considered as unavailable amino acid content. However, the release of essential amino acids from the various heat-processed soymilks is comparable and will indicate the relative nutritive value of proteins. Bender (20) stated "enzymatic hydrolyses do not go to completion but a comparison of the amino acids liberated from processed and unprocessed foods gave a measure either of nutritive value of the proteins or of amino acid availability that agree very well with biological measures."

Very recently during a critical discussion of the results reported by Ford and Salter (87) of available amino acids measured by the microbiological method, Mauron (175) noted that the microbiological test after prolonged digestion with enzymes gives higher values for amino acids than the values measured by the Moore and Stein technique. Despite this difference Mauron considered the replacing of the microbiological test with an enzymatic in vitro digestion followed by measuring only the free amino acids released using the Moore and Stein technique was satisfactory. It would greatly facilitate the determination of amino acid availability in laboratories not equipped for microbiological tests but having at their disposal an amino acid analyser. One draw back of the exclusively enzymatic procedure persists, however, for it yields only relative and not absolute values.

Although the recovery of some of the amino acids in the present study is very low it is interesting to note that their relative release in all samples is of the same order. We could not calculate the cystine content in the digest due to the very shallow peak in the chromatogram. The chromatogram of the original method of Akeson and Stahmann (2) did not show a cystine peak either.

We feel that using the enzymatic digestion procedure followed by measuring the released amino acids by ion-exchange chromatography may be a very useful technique for the evaluation of the nutritional quality of protein foods but further research is necessary to improve the method.

However, the statistical analysis of the results reported in Table 31 shows that there is no significant difference in essential amino acid availability in the three differently processed samples prepared from carbonate presoaked beans whereas the quantities of lysine, histidine and leucine released from the water presoaked preparation are significantly lower than those in any of the three samples prepared from carbonate presoaked soybeans. The average percentage of digestibility calculated from the release of essential amino acids in the various samples shows the same trends as were found with pepsin and trypsin digestibility studies, with higher recoveries from carbonate presoaked preparations irrespective of the heat processing (see Chapter 5).

The available lysine value determined by the FDNB-procedure in the water presoaked preparation (Table 32) is very similar to the value reported by Van Buren et al (261) and Hackler et al (104) of soymilk prepared from water presoaked beans. Although the FDNB available lysine value in soymilk prepared by water presoaking method is lower than the values found in the samples prepared from the carbonate presoaked beans, the difference is not statistically significant.

The FDNB available lysine value is a measure of lysine in intact protein whose E-amino group is free to react with FDNB and Carpenter and his associates proposed that only the lysine molecule with reactive E-amino group is nutritionally available (49). The reduction of availability of lysine is due largely to the reaction of its E-amino group with other reactive groups under conditions of moist heat to form a linkage that resists hydrolysis with enzymes (230). In the present study, under identical heat processing conditions, for example 115°C for 18 minutes, the available lysine in milk prepared from water presoaked beans is 5.57 percent compared to 5.65 percent in carbonate presoaked preparation. The possibility of the lower availability of lysine in water presoaked preparation being due largely to the reaction with other constituents in this case is negligible. The slightly lower availability of lysine in milk prepared by the water presoaking method than that in the carbonate presoaked preparation is probably due to the degree of denaturation (unfolding) of the protein molecule which is higher in the latter case due to the combined action of alkali and heat thus enabling more E-amino groups to become available to react with FDNB. This is supported by the fact that in the native β-lactoglobulin, 12 E-amino groups of lysine react with FDNB and 3 in native ovalbumin, whereas in the denatured proteins 31 and 9 E-amino groups react respectively (220). Solomons and Irving (248) reported that in intact soft-tissue collagen only about two thirds of E-amino groups reacted with FDNB, but the concentration of reactive E-amino groups of hard-tissue collagens increased from very low levels almost to the theoretical value on complete decalcification.

Since soyprotein is compactly folded and a high intensity of heat treatment is necessary to unfold the protein molecules completely (see Chapter 5), it is likely that some E-amino groups may be within the protein molecule and thus may be unable to react with FDNB, unless they are exposed by suitable treatment (262). Hackler et al (104) and Van Buren et al (261) also found that the optimum heat-processed soymilks using higher temperature (121°C) gave higher values of available lysine than that in milk processed at lower temperature (93°C).

The use of the pepsin test as modified by Lovern et al (165) is highly successful in the quality assessment of fish meal and the digestibility (as measured by soluble nitrogen) is highly correlated with the available lysine (165, 206) and NPU (207) of the variously heat processed fish meals. We have tested the method in the present study to find whether it is applicable to soyprotein products in which the antitryptic factor has previously been destroyed by heat-treatment. Our results also show a highly significant correlation ($r = 0.913$, $P < 0.001$) between the corrected pepsin digestibilities and available lysine values as shown in Fig. 21. The corrected pepsin digestibility of proteins in soymilk prepared from water presoaked beans is significantly lower than that of proteins in any of the three carbonate presoaked preparations. A difference in pepsin digestibility of proteins in optimum processed soymilks prepared either from water or carbonate presoaked beans could not be found in the studies reported in Chapter 5. This is due to the fact that the concentration of pepsin used in Chapter 5 was too high (0.125%) and at high pepsin strength the method becomes insensitive and cannot distinguish between the slightly different protein qualities (166).

The low strength pepsin method is very sensitive and easy to handle, but is limited to the detection of over-processing. Under-processing in soybean products or in most of the leguminous proteins, which contain antitryptic factors, cannot be detected by the pepsin digestibility test because of the lack of action of the trypsin inhibitor on pepsin (226). Therefore, a combination of the determination of trypsin inhibitor activity with the pepsin test may be the most useful for quality assessment and process control of products based on legume proteins. Similarly the FDNB procedure can only measure over-processing. The FDNB method of Carpenter (50) has a further limitation in its application to plant protein products which contain large quantities of carbohydrates because of the instability of E-DNP-lysine during acid hydrolysis in the presence of carbohydrate. This defect can be overcome by using the "difference" procedure of Roach et al (232) but considerable work is involved unless one uses an automatic amino acid analyser.

The higher digestibility by enzyme or enzyme combinations of proteins in carbonate presoaked preparations found throughout this study is possibly due to the alteration of the structure of protein by the action of alkali or of the alkali plus heat favouring enzymic attack. It illustrates clearly the need for more extensive study of the effect of processing factors on the nutritive value of our protein foods.

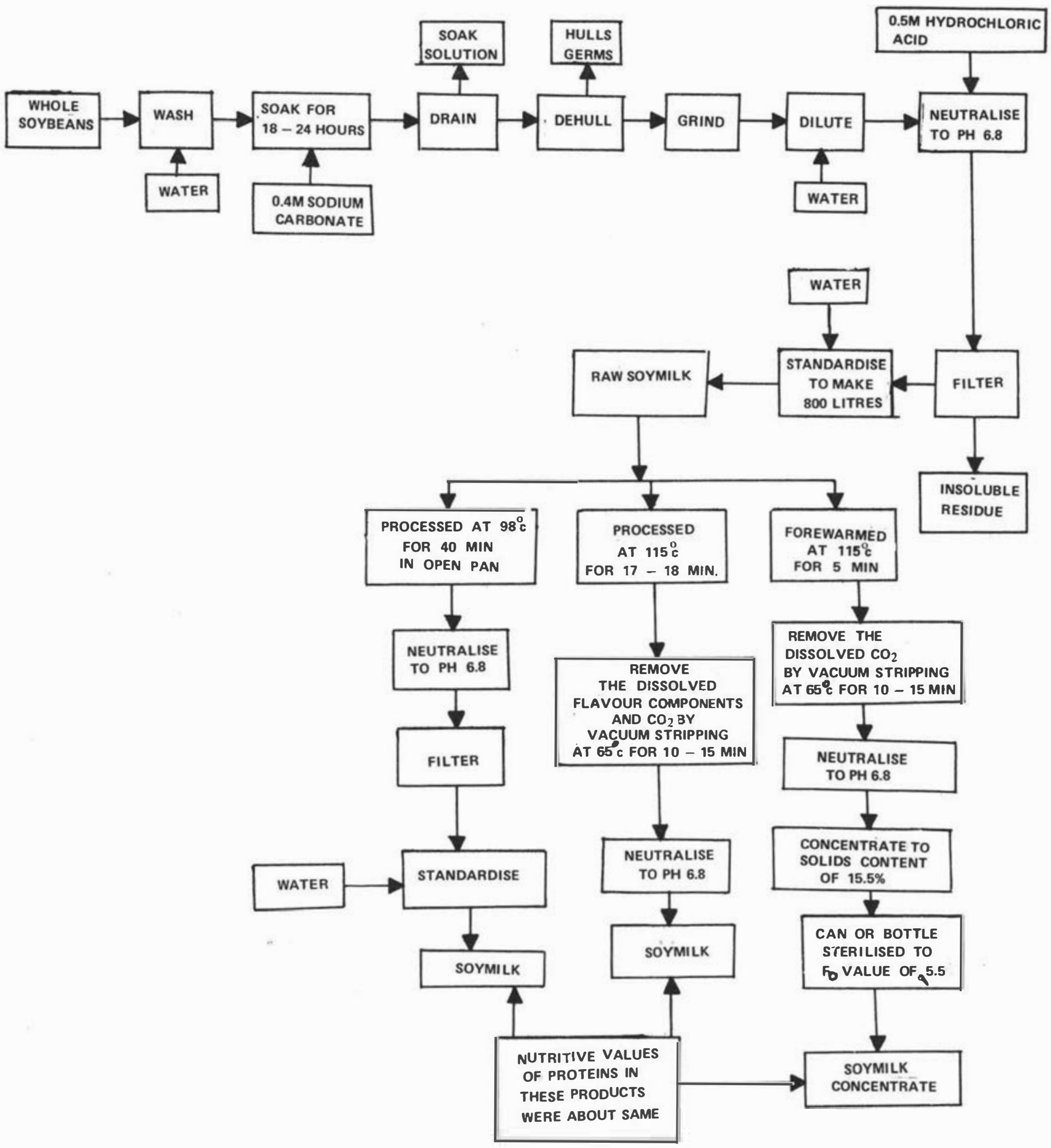

FIG.22. FLOW SHEET FOR THE PREPARATION AND PROCESSING OF SOYMILKS.

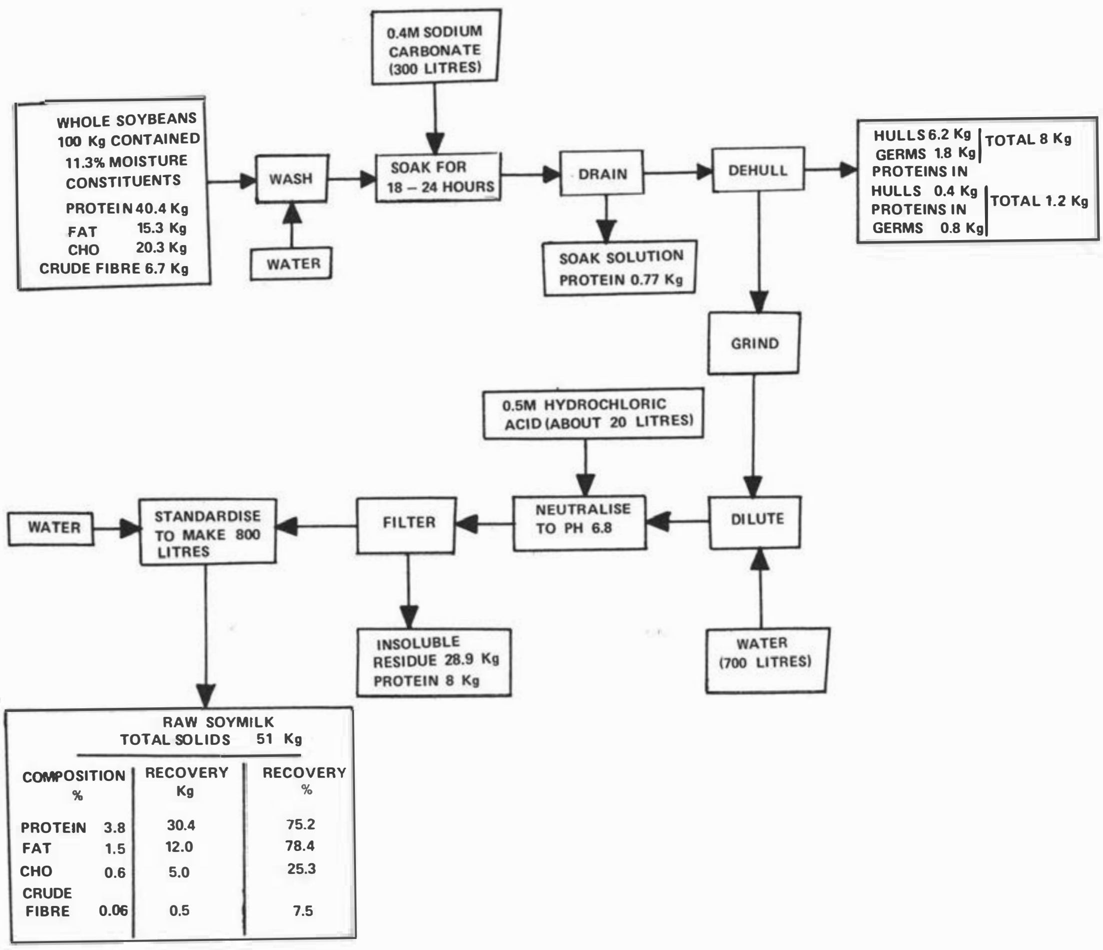

FIG.23. MASS BALANCE SHEET FOR THE PREPARATION OF SOYMILK.

APPENDIX 1

THE POTENTIAL OF USING SOYBEANS AS A PROTEIN SUPPLEMENT

The soybean has been grown in the Orient for many centuries. The first mention of it that is known, dates back to the reign of the Chinese Emperor, Sheng-Nung, who ruled 2838 years before the Christian era. The soybean is repeatedly mentioned in later records and was considered to be both the most important cultivated legume, and essential to the existence of the Chinese civilisation. Being a sacred food of China, the seeds were sown with great ceremony. The oriental people have come to understand by experience, rather than by scientific deduction, that soybean is essential to livelihood and cannot, with safety, be omitted from their diet. A Chinese legend has it that one of the invisible gods is placed in the soybean plant to provide food to serve all the needs of the family. Soybean is sometimes referred to as the "poor man's meat" in China, while the general use of soymilk has earned it the title of the "Cow of China". American authorities have spoken of it as "Gold from the Soil", "Universal Protective factor in food". It has also been graced with such highly significant names as the "Magic Plant", the "Miracle Bean", etc. (147).

Botanically the soybean was for a long time named *Glycine hispida*, but according to the international botanical nomenclature it is now cited as *Glycine max*. In China it is known as "Shi-yu" or "Tatou". The Japanese named them "Soja" after "Saju", a dark rich sauce made from the bean.

According to Morse (198) the production of soybeans, which for many centuries was confined to the countries of Asia, spread rapidly after World War I to the western world and since World War II, practically all leading nations have become more and more interested in the culture and production of the crop. It is estimated that there are more than 3,000 distinct varieties of the soybean. During the past half-century no less than 2,500 different types have been introduced into the United States for experimental purposes, and much valuable knowledge has been gained concerning the adaptability of varieties to soil and climatic conditions.

Agricultural experimental stations throughout the world have become engaged in the development of the varieties of soybeans suited to their soil and climatic conditions through introduction, selection and hybridisation. Successful results have been obtained and the crop has become an important factor in many nations' agriculture (198).

Soybeans are the world's largest oilseed crop, totalling as much as 35 million metric tons annually and account for about 40 percent of the total (187). Soybean production in the United States has had phenomenal growth during the past four decades. In 1926 the total production of soybeans was less than 5 million bushels. The 1968 crop exceeded a billion bushels representing a 200-fold increase (268).

Weight for weight the soybean contains twice as much protein as lean beef, four times as much as eggs, wheat and other cereals, five times as much as bread and yet it is a cheaply-grown food. The yield of soyprotein per unit of area is very high compared with other foods. Table A-1 shows the average output of protein per acre of land and per 100 hours of labour. 100 hours of labour will produce only 45 pounds of protein from cattle, against 2,821 pounds from soybeans. If man's approximate yearly need of protein is 53 pounds, this amount can be secured from soybeans in only 2 hours of labour (147).

TABLE A-1. Average output of protein per unit of resources*

Products	Pounds per acre of lands	Pounds per 100 h of labour
Milk, whole	39	89
Chickens	25	74
Eggs	26	56
Hogs	18	58
Beef cattle	7	45
Wheat, whole flour	90	1,002
Wheat, white flour	56	621
Corn, corn meal	57	211
Dry beans	150	576
Peanuts	116	200
Soybeans	339	2,821

* Taken from: Lager, M. (ref. 147)

Soybeans are one of the basic food materials which have survived the severe test of milleniums on a large scale natural experiment. As an economic source of exploitable dietary elements soybean probably has no peer, being rich in protein and fat, in minerals and in many of the vitamins. The composition of soybean is dependent on the variety as well as on climatic and soil conditions (198). The averaged composition of ten varieties of soybeans grown in five different locations in America in five different crop years are given in Table A-2.

TABLE A-2. Chemical composition of soybeans (on a moisture-free basis)*

Component	Lowest %	Highest %	Average %
Ash	3.67	5.90	4.99
Oil, crude	14.95	22.95	19.63
Crude fibre	4.34	7.60	5.52
Crude protein (N x 6.25)	36.62	53.19	42.78
Sugars (total as sucrose)	2.70	11.97	7.97
Phosphorus	0.419	0.822	0.659
Potassium	1.29	2.17	1.67
Calcium	0.169	0.470	0.275

* Taken from: Morse, W.J. (ref. 197)

Among plant derived foods, soybeans are unique in their nutritional value because of their high percentage of protein and fat. Not only is the protein high in quantity but also it is of good quality, being practically the same in food value as animal protein (see Table A-3).

TABLE A-3. Nutritive value of soyprotein in comparison with that of animal proteins[a,b].

Food Products	True Digestibility	Biological Value	PER
Egg	100	94	3.80
Beef	100	76	3.20
Soybean (optimally heated)	96	75	2.34[d]
Milk (whole)	95	90	2.34[d]
Fish[c]	89	88	2.50

a. Taken from: Mitchell, M.M. (In ref. 197, p.383)

b. Taken from: Rice, E.E. and Beuk, J.F. (ref. 230)

c. Taken from: Yanez, E., Ballester, D. and Donoso, G. Effect of drying temperature on quality of fish protein. J.Sci.Fd.Agric. 21, 426 (1970)

d. Taken from: Jones, D.B. and Divine, J.P. Soybean and other proteins and flours, Soybean Digest. 2(11),10 (1942)

Brock and Autret (44) compared the composition of the soy-proteins with that of animal proteins, both in the form of milk, (Table A-4).

TABLE A-4. Comparison of the essential amino acid contents in cow's milk with those of soymilk*

	Cow's milk g/litre	Soymilk g/litre
Arginine	1.26	2.82
Histidine	0.91	1.01
Lysine	2.625	2.40
Tryptophan	0.525	0.50
Phenylalanine	1.785	1.95
Methionine	0.840	0.56
Threonine	1.505	1.57
Leucine	3.434	2.93
Isoleucine	2.625	2.09
Valine	2.52	2.00

* Taken from: F.A.O. Nutritional Studies No.8 (ref. 44)

The authors conclude that the amino acid content of soymilk is comparable to that of cow's milk, except in methionine. The methionine content of soymilk content is, however, double that of European human milk and three times that of African human milk.

Table A-5 compares the nutritive value of soyprotein with that of the proteins from two other major oil-seeds and clearly demonstrates its higher nutritional quality.

TABLE A-5. Comparative nutritive value of proteins in three major oil-seeds.

Essential amino acid content[a]	Cottonseed (AXTE) %	Peanut (Spanish) %	Soybean (Harasoy) %
Arginine	10.6	11.6	7.5
Histidine	2.7	2.3	2.6
Isoleucine	3.0	3.4	4.2
Leucine	5.4	6.6	8.0
Lysine	4.2	3.6	6.5
Methionine	1.0	0.9	1.0
Methionine + cystine	2.4	2.0	2.2
Phenylalanine	4.6	4.9	4.9
Threonine	3.3	2.5	3.7
Tryptophan	1.5	1.7	1.8
Valine	4.2	4.1	4.6
Protein nutritive value (PNV)[a] $= \frac{\text{wt. gain} \times 100}{\text{wt. gain of rats fed casein}}$	-	44	99
Protein efficiency ratio (PER)[b]	2.1	1.7	2.4
Biological value[c]	58	56	75

a. Taken from: Evans, R.J. and Bandemer, S.L. (ref. 81)

b. Taken from: Swaminathan, M. Availability of plant proteins, In: Newer methods of nutritional biochemistry 3, 197 (1967)

c. Taken from: Mitchell, H.H. In: reference 197, p.383

Soybeans contain in nearly optimum proportions the amino acids essential for the nutrition of man and animals, and are sufficiently complete to sustain life for an extended period of time. Because of their high yield and tolerance of a wide range of cultivation and climate conditions they represent a very valuable source of protein food.

APPENDIX 2

COMPARISON OF COMPOSITION OF SOYMILK WITH COW MILK*

Constituent	Cow Milk	Soymilks (probably oriental method)				Modern U.S. Soymilk reconstituted		
						For Infants	All Purpose	Canned
Water %	87.30	92.00	90.00	89.25	92.50	88.00	87.97	88.60
Protein %	3.42	3.70	4.95	3.15	3.02	2.79	3.80	3.03
Fat %	3.67	2.00	2.97	3.10	2.13	2.22	3.50	3.48
Carbohydrate %	4.78	1.80	1.34	3.02	0.03	6.05	5.10	4.53
Other substances %	-	-	-	1.02	1.88	-	-	-
Ash	0.73	0.50	0.44	0.45	0.41	.094	0.35	0.36
Vitamin A	1660[a]	-	-	-	-	3000[a]	-	2000[b]
Thiamine mg %	350	-	-	-	-	120[a]	-	500[b]
Riboflavin mg %	1660	-	-	-	-	450	-	1000
Vitamin D	-	-	-	-	-	-	400[a]	500[b]

* Taken from: Burnett, R.S. (ref. 46)

[a] International units;

[b] United State Pharmacopoeia requirement.

APPENDIX 3

COMPOSITIONS OF SODIUM CITRATE BUFFERS USED IN THE CHROMATOGRAPHIC SEPARATION OF AMINO ACIDS BY THE MODIFIED MOORE AND STEIN TECHNIQUE

	4-hour procedure			2-hour procedure		
	Short column	Long column		Short column	Long column	
pH	5.28 ± 0.02	3.25 ± 0.01	4.26 ± 0.02	5.36 ± 0.01	3.49 ± 0.01	4.40 ± 0.01
Sodium concentration	0.35 N	0.20 N	0.20 N	0.35 N	0.20 N	0.20 N
Sodium citrate. H_2O	1372.6 g	784 g	784 g	1373 g	784 g	784 g
Concentrate HCl	260 ml	503 ml	335 ml	256 ml	468 ml	323 ml
Thiodiglycol (TG)	None	100 ml	100 ml	None	100 ml	100 ml
Pentachlorophenol	4 ml	4 ml	4 ml	4 ml	4 ml	4 ml
Final volume	40 litres	40 litres	40 litres	40 litres	40 litres	40 litres

APPENDIX 4

OPERATING CONDITIONS FOR CHROMATOGRAPHIC SEPARATION OF THE AMINO ACIDS BY THE MODIFIED MOORE & STEIN TECHNIQUE

	Analysis of Amino Acids			
	Accelerated 4-hour procedure		Accelerated 2-hour procedure	
	Basic	Acidic and Neutral	Basic	Acidic and Neutral
Column size	23 x 0.9 cm	69 x 0.9 cm	23 x 0.9 cm	69 x 0.9 cm
<u>Packing</u>				
Resin type	PA 35	PA 28	PA 35	UR 30
Height of resin column	5.5 cm	56 cm	5.5 cm	56 cm
Resin dilution buffer	pH 5.28(0.35 N)	pH 3.25(0.20 N)	pH 5.36(0.35 N)	pH 3.49 (0.2 N)
Buffer flow rate	68 ml/hour	68 ml/hour	70 ml/hour	70 ml/hour
<u>Analysis</u>				
Duration of run	48 min	195 min	48 min	115 min
Flow rate of buffer	68 ml/hour	68 ml/hour	70 ml/hour	70 ml/hour
Flow rate of ninhydrin	35 ml/hour	35 ml/hour	35 ml/hour	35 ml/hour
Buffer change	None	95 min	None	30 min
Operating temperature	$50^{o}C$	$50^{o}C$	$53.7^{o}C$	$53.7^{o}C$
<u>Regeneration</u>				
Cleaning (0.2 N NaOH)	approx. 3 ml	approx. 15 ml	approx. 3 ml	approx. 15 ml
Equilibrium buffer	approx. 40 ml	approx. 70 ml	approx. 40 ml	approx. 70 ml
Equilibrium buffer pH	5.34 ± 0.01	3.49 ± 0.01	5.34 ± 0.01	3.49 ± 0.01

APPENDIX 5

REVIEW OF LITERATURE ON THE BIOLOGICAL PROPERTIES OF SOYBEAN TRYPSIN INHIBITOR

Bielorai and Bondi (21) reported that the intestinal contents of chickens fed raw soybean meal or animal protein feeds supplemented with trypsin inhibitor contained a great deal of undigested protein which was absent when the chicks were fed on animal protein or cotton-seed meal devoid of trypsin inhibitor. There is evidence that inhibition of proteolysis occurs in the upper part of the intestine of young chicks (9) and that overcoming of this inhibition depends on the age of the chicks and on the length of the period of feeding with raw soybean meal (204). Almquist and Merritt (3, 4) found that the growth inhibition of chicks was almost fully apparent when as little as one-fourth of the dietary protein was furnished in the form of the raw soybean meal and that crude trypsin was capable of reversing this growth inhibition. Melnick et al (178), on the basis of observations on the in vitro release of amino acids from soybean protein by the enzyme pancreatin, suggested that methionine of raw soybean protein was liberated more slowly by the proteolytic enzymes of the intestines than the other essential amino acids so that it was not available for mutual supplementation. Subsequent reports (121, 123, 155, 231), however, did not support such a conclusion, since heat treatment was found to increase the enzymatic release of other amino acids to the same proportionate extent as methionine. Almquist and Merritt (5, 6) have questioned the necessity of postulating a specific interference with the enzymatic release of methionine to explain the methionine deficiency provoked by raw soybean meal. They believe that the action of the inhibitor is a general interference with digestion so that a substantial amount of the most limiting amino acid of soybean protein, methionine (7) is excreted unabsorbed, thus precipitating a methionine deficiency. In confirmation of this concept, these authors have shown, with chicks, that the addition of the trypsin inhibitor in the form of raw soybean meal to rations containing marginal levels of lysine, arginine, isoleucine, or tryptophan caused these rations to become markedly deficient in these particular amino acids. The result of these studies suggested that this effect is due to interference with the enzymes pancreatin and trypsin leading to reduced digestion and absorption of nitrogenous material from food containing the inhibitor in its active form.

There are, however, several experimental observations which did not agree with this concept. Thus active antitryptic preparations have been shown to retard the growth of rats (67, 156) and mice (266) when incorporated into rations containing predigested protein. Westfall et al (266) with concentrates of soybean trypsin inhibitor and Klose et al (141) with an inhibitor preparation from the limabean were able to produce growth inhibition equivalent to that with the whole meal even when adequately supplemented with hydrolysed casein. Hill et al (117) were unable to prevent growth depression in chicks fed raw soybean when the diet was fortified with adequate levels of essential amino acids. Consequently, it was felt that the trypsin inhibitor acted to inhibit growth other than through its depressant effect on intestinal proteolysis.

Recent work has focused attention on the effect of trypsin inhibitor on the pancreas. In 1948, Chernick et al (57) reported that chicks fed raw soybean meal developed hypertrophic pancreases which contained abnormally high concentrations of trypsinogen. It was suggested that these changes were the result of a reaction to the soybean antitrypsin. These initial observations have been extended by Lyman and Lepkovsky (168) and Haines and Lyman (106). Lyman (169) and Booth et al (31) suggested that trypsin inhibitors might produce growth retarding effects through stimulating increased enzyme secretion, with some of the endogenous nitrogen being incompletely reabsorbed and ultimately lost to the animal. The high cystine content in the undigested enzyme secretion presumably causes depletion of methionine by its conversion to cystine to meet the requirements of the pancreas to synthesize the secreted enzymes. Besides, earlier reports of Melnick et al (178), Barnes et al (16) and Kwong and Barnes (146) have also reported that there is a factor in unheated soybean which interferes with the utilisation or metabolism of the methionine and cystine. Borchers (32, 34) has presented evidence which indicates that the metabolism of methionine, threonine and valine may be impaired by some factor present in raw soybean meal. Khaymbashi and Lyman (140) indicated that the mechanism of growth depression appears to be due to a loss of the critical amino acids, methionine (via cystine), threonine and valine, caused by the soybean trypsin inhibitor's ability to stimulate the pancreas to discharge excessive quantities of endogenous protein into the intestinal tract. Although much of this endogenous nitrogen may be reabsorbed, bacterial degradation, especially of those amino acids most limiting for growth, would prevent their normal reabsorption and subsequent utilisation in structural protein

There is evidence also to indicate that trypsin inhibitors may have some effect on the fat absorption in chicks (202).

The method of interference in metabolism by the trypsin inhibitor is thus not clear and some of the confusion may be consequent upon the presence of some other anti-nutritive factor.

APPENDIX 6

PREPARATION OF HAEMOGLOBIN SUBSTRATE

A solution is made up containing 8 ml of 1 M sodium hydroxide, 72 ml of water, 36 g of urea, and 10 ml of 22 percent haemoglobin (22 g haemoglobin per 100 ml solution). This alkaline solution is kept at 25°C for 30 - 60 minutes to denature the haemoglobin and is then mixed with a solution containing 10 ml of 1 M potassium dihydrogen phosphate and 4 g urea. The final pH is 7.5. 1 mg merthiolate or thiomersalate is added to each 50 ml of haemoglobin solution as a preservative.

APPENDIX 7

THE CONVERSION OF RESISTANCE TO CONDUCTIVITY

The measured resistance using the Philips resistance meter and conductivity cell is converted to conductivity as follows:

(a) the measured resistance (ohm) is multiplied by the reciprocal of the cell constant (the cell constant marked on some cells may not be in a form appropriate for this calculation) to obtain the resistivity (ohm centimetre).

(b) the electrical conductivity in micro mhos per centimetre = $\frac{10^6}{\text{Resistivity in ohm centimetre}}$

The figure marked on the cell used was the reciprocal of the cell constant. This was established by measuring the resistance of standard solutions of potassium chloride (0.02 M) and sodium chloride (0.1%). The cell constant was determined as follows:

Measured resistance of 0.1 percent sodium chloride at $20^{\circ}C$ using the cell in question = 0.41×10^3 ohm

Conductivity of 0.1 percent sodium chloride at $20^{\circ}C$ = 1.75×10^{-3} ohm^{-1} $centimetre^{-1}$ (264).

Since cell constant = Resistance x conductivity

cell constant = 0.41×10^3 ohm x 1.75×10^{-3} ohm^{-1} cm^{-1}

= 0.72 cm^{-1}

APPENDIX 8

REVIEW OF LITERATURE ON THE IN VITRO METHODS FOR MEASURING THE NUTRITIONAL QUALITY OF PROTEINS

a. Prediction of protein quality based on analyses of amino acid composition

The fundamental studies of Osborne and Mendel (209) led to a realisation that the nutritive value of proteins is dependent upon their content of the amino acids required for growth, maintenance and other metabolic functions. Proteins are composed of 22 or more different amino acids. Eight to twelve of these, depending on the species and physiological state of the animal must be supplied in the diet because of the inability of the body tissues to synthesise these in adequate amounts and so they are called essential amino acids (188). The effectiveness of any protein as a source of these metabolic requirements is obviously limited by its amino acid composition. Thus zein of corn, being devoid of tryptophan and lysine, and gluten of wheat which lacks tryptophan, completely fail to meet the dietary requirements of test animals. Other proteins which contain all the essential amino acids but have relatively small quantities of some, do not satisfy the metabolic needs unless they are consumed in abnormally large quantities. This fundamental relationship between nutritive value and amino acid composition has proved the basis for a number of methods of evaluating protein foods. Block and Mitchell (28) and Mitchell and Block (188) developed a procedure for determining the chemical score of proteins based on their amino acid composition relative to egg protein which is almost completely utilised by human subjects or growing rats. The chemical score was determined by comparing the essential amino acid present in least amount with the amount present in the reference protein (egg protein). They reasoned that the amount of protein synthesised would be limited by the essential amino acid most deficient in the food. The theory might be tenable but at the same time, it assumes that the reference protein has an amino acid composition identical to that required by the organism. Since preparing this review a paper, of Cresta et al (280) has come to our notice in which they recommend that the value of the chemical score method can be considerably improved by varying the reference protein used according to whether the biological value or the protein efficiency ratio was being used as a basis for the chemical score correlation.

The "chemical scores" method was improved by Oser (210) who has developed a method, called Essential Amino Acid Index (EAAI) based on the contribution of all the essential amino acids rather than the one in greatest deficit, as is used in chemical score. He argued that the probability that all the amino acids are available at the site of synthesis is a function of their concentration product. He included histidine and arginine in addition to the list of essential amino acids recommended by the joint FAO/WHO expert group (226) in the calculation of EAAI because they could not be synthesised at a rate sufficient for the young adult male (211). Oser (211) claimed EAA Indexes provide an especially useful short cut approach for predicting the nutritional value of proteins and for estimating the mutual supplementary effect of different proteins on each other.

Later Mitchell (189) modified the EAAI by dropping arginine from the list of "essentials" and including tyrosine with phenylalanine. He claimed closer correlation with biological value.

In a discussion on the determination of the nutritive value of proteins by chemical analysis, Bender (19) has stated that the nutritive value of a protein can be forecast from chemical composition. It is necessary to know the amino acid composition with some degree of accuracy. The obstacle to the use of chemical values is the question of availability. Chemical analysis normally measures the quantity of amino acids present after acid (alkaline in the case of tryptophan) hydrolysis and this is often not the same as the quantity biologically available to the animal.

Menden and Cremer (180) noted that it is widely known that the amount of a nutrient in a food determined by analysis does not necessarily represent the amount of that nutrient which is utilised when consumed as food. Meeting the protein requirement is directly dependent on the amino acid composition of food proteins and on the release and absorption of amino acids at a proper time during digestion. These are important facts in estimating the quality of a protein. Consideration of these requirements suggests a possibility for the evaluation of changes of protein quality in the laboratory as follows:

1) Determine the amino acid composition after acid and alkaline hydrolysis and estimate the "potential nutritional value" of the proteins; and then

2) Estimate the presumably available amino acids from proteins by using enzymatic hydrolysis and compare this data with the results from the chemical hydrolysis.

b. Enzymatic method for measuring protein quality

i) Amino Acid Availability

It is recognised that the nutritive value of a protein depends not only on the pattern of its component amino acids but also on their physiological availability. The concept of "available" as distinct from "total" amino acid present in a protein is used to differentiate between amino acids modified or damaged in some way during denaturation, with the subsequent loss of nutritive value, and those which remained nutritionally available to the metabolic process. The denaturing process could be brought about by processing, which causes amino acids to decompose or to react chemically with other compounds (230).

Several authors have tried to measure amino acid availability using in vitro enzymatic methods (180). These methods were not intended to establish analytical procedure, but to demonstrate the fact that the nutritional quality of proteins may be quite different, even though the amino acid composition is the same; these differences arise from changes in the ability of enzymatic digestion to liberate the amino acids.

In vitro analytical procedures for determining amino acid availability based on enzymatic release of amino acids from proteins have been developed by Sheffner et al (240), Mauron et al (174) and Ford and Salter (87). Sheffner et al (240) studied the relationship between the pattern of amino acids released by digestive enzymes and the biological value of food proteins. An amino acid index was devised which combined the pattern of essential amino acids released by in vitro pepsin digestion with the amino acid pattern of the remainder of the protein to produce an integrated index - the pepsin digest residue (PDR) amino acid index. The results obtained with the new index were highly correlated with net utilisation values of the proteins studied, including those which were heat-processed with various degrees of severity. The work involved was considerable since 10 amino acids had to be determined in an acid hydrolysate as well as in a pepsin digest by the use of microbiological techniques. Therefore, this method did not appear suitable for evaluation of large numbers of food proteins. The procedure was modified by Akeson and Stahmann (2) making use of pepsin plus pancreatin - the pepsin pancreatin digest (PPD) index. The amino acid content was determined by ion exchange chromatography using an automatic amino acid analyser. Menden and Cremer (180) prefer enzymatic hydrolysis with pancreatin only.

The procedure of Mauron et al (174) is concerned with the measurement of only three essential amino acids - lysine, methionine and tryptophan - the amino acids which are most likely to be limiting in foodstuffs. Tryptophan and methionine are determined colorimetrically, lysine with a specific decarboxylase and total amino nitrogen gasometrically, using a protein hydrolysate obtained by using pepsin digestion followed by pancreatin digestion.

Ford and Salter (87) measured the availability of several essential amino acids in various protein products by digesting with a combination of enzymes. They used microbiological techniques to measure the amino acids.

ii) <u>Pepsin digestibility method at low pepsin concentrations</u>

In principle the complete evaluation of a protein should consist of the determination of the content and availability of all the essential amino acids, but under some circumstances this may not always be possible. Therefore, a single parameter to predict the nutritional quality of a protein would be very helpful. One such parameter is the pepsin digestibility at low pepsin concentration (206).

In 1956, the Association of Official Agricultural Chemists set up a collaborative study of pepsin digestibility of proteins as an index of protein quality. It soon became apparent that the official method was not an accurate measure of the protein quality because the results did not agree with those from animal feeding tests (247, 257).

In 1961, Kastell (138) indicated that reduction of pepsin strength in the tests gave better results. Subsequent to this information, pepsin digestibility with greatly reduced levels of pepsin was extensively studied at Torry Research Station, Aberdeen, The Fishing Industry Research Institute, South Africa, and the College Park Laboratories of U.S. Bureau of Commercial Fisheries for assessing the quality of fish meals by correlating the digestibilities with the corresponding biological values. At Torry Research Station, the corrected pepsin digestibility of a number of fish meals, using 0.0002 percent pepsin of 1 : 10,000 strength instead of 0.2 percent as in A.O.A.C. method, have been shown to be highly correlated with the available lysine values (165). In American and South African methods, the pepsin level used was 0.002 percent, the pepsin used in the South African method had an activity of 1 : 2,700 whereas the American method used a pepsin strength of 1 : 10,000. Pepsin digestibility by the American method was correlated with chick growth tests (10) and that of the South African method with available lysine (73).

c. Microbiological methods for the evaluation of protein quality

Many investigators have used the microbiological assay of enzymatic digests and attempted to correlate the results with the corresponding values of biological assay. Halevy and Grossowicz (108) hydrolysed a variety of proteins for 48 hours with pancreatin and determined the quantities of the hydrolysate needed to promote half-maximum growth in their test cultures. Only about 40 percent of the proteins was hydrolysed during the enzymatic digestion, but the results of the tests were broadly similar to those of the rat growth tests. Horn et al (119, 120) evaluated the nutritional quality of a number of cottonseed meals by measuring the increase in growth of Leuconostoc mesenteroides P-60. The meals were preincubated for successive 24 hour periods with pepsin, trypsin and a preparation of pig-gut mucosa. This method gave good correlation with the biological value of meals which had been subjected to various degrees of heat processing.

A bacteriological method for determining protein digestion coefficients is presented by Mertz et al (181). This method combines the proteolytic action of pepsin with that of the bacterium Pseudomonas aeruginosa. In most cases, the bacteriological digestion coefficients of the foods and feeds tested agree within 10 percent of the protein digestion coefficients obtained in feeding trial with rats.

A method of assay was developed by Ford (88) in which the proteolytic organism Streptococcus zymogenes, NCDO 592, was used to provide an estimation of protein quality and the values obtained for a variety of food proteins were found to correlate closely with those obtained in biological tests with rats. Using this organism, Ford (89, 90) assessed the availability of 7 essential amino acids in food proteins. Although the organism can hydrolyse the test protein the process is relatively slow and the assay is improved if the protein is predigested either with papain or with pepsin. It is stated, however, that the values obtained for available methionine vary with the degree of predigestion and that the most appropriate concentration of papain to use is that which gives results that agree best with available amino acids found with higher animals. This means that the conditions of assay must be re-tested with each type of protein and that the results obtained have only relative and not absolute values (20).

Tests of protein quality with the protozoan Tetrahymena pyriformis simulate more closely the circumstances of the biological test. The ability of Tetrahymena pyriformis W to utilise a wide variety of intact proteins, and the relative nutritive values of the various protein sources used, were generally similar to those found for growing rats (82, 234, 254). Sheffner (239) recently noted that many aspects of the nutritive requirements of T. pyriformis W are still unknown, and its use for the study of protein quality in natural materials must be viewed cautiously. Furthermore, culturing and measuring growth of the protozoan are complex procedures subject to many variables. He concluded that the procedure may not be suited for the routine assay of proteins for nutritional quality.

d. Estimation of protein quality by dye binding

It has been known for sometime that reactions occur between proteins and different kinds of dyes (91). Frolich (94) studied a number of dyes for reaction with proteins in various heat processed soybean meals and showed that heat processing increased the absorptive property of meals for dyes containing a phthalein group. Phenolphthalein was most useful in distinguishing between under-heated, properly-heated and overheated meals. It has, however, the disadvantage of not being stable when made alkaline in presence of substances present in soybean oil meal. Cresol red is much more stable, but the absorption of dye reflected heat treatments in under-heated to properly-heated meals. Overheated meals could not be measured.

Fraenkel-Conrat and Cooper (91) showed that the acid dye, orange G (1-phenylazo-2-naphthol-6,8 disulphonic acid sodium salt) combined stoichiometrically with basic protein groups, the free amino groups, the imidazole groups of histidine, and the guanidyl group of arginine, provided they are in a free or dissociated state. Moran et al (195) used this reaction of the dye, orange G, with basic amino groups to determine the availability of free amino, imidazole and guanidyl groups of proteins and showed the dye binding capacity of soybean meals heated for varying periods of time was closely related with the growth of chicks fed the meals. Ascareth and Gestetner (12) also found that dye binding capacity of soybean meal was a good measure of its nutritional value. On the other hand using Moran's procedure, Morrison (196) has not found that the dye binding capacity of Canadian foods was related to their protein value. Van Buren et al (261) attempted to relate heat damage to the ability of soymilk protein to bind the dye, orange G. The extent to which the dye was bound decreased as heat damage increased, but the differences were rather small. Under heating conditions where 20 to 30 percent of the available lysine was lost, the dye-binding ability of the soymilk decreased by 5 to 7 percent. Due to this lack of sensitivity the authors concluded that measurement of the binding of orange G, while it may be useful with soybean meal, is not a suitable method for use in the quality control of soymilk.

Bender (20) noted that some proteins show a decrease and others an increase in dye binding capacity with processing damage and therefore the method cannot be used to compare the qualities of different types of proteins after processing damage.

e. <u>Available lysine value (FDNB - procedure)</u>

When lysine is bound intramolecularly or by carbohydrate it becomes unavailable and this results in a diminution in biological value of the protein so that total lysine estimations are of little value. Carpenter and associates (45, 49) proposed that it is only the lysine molecule with a reactive E-amino group that is nutritionally available. On the basis of this hypothesis, a method was developed for the determination of "available" lysine in foods by using the Sanger reaction between free amino groups and fluorodinitro-benzene (FDNB) and the method is known as "FDNB - procedure" (50). Treatment of the finely ground protein products with fluorodinitro-benzene yields dinitrophenylated proteins which are then hydrolysed with acid. Interfering substances are removed from the hydrolysate by ether extraction. The E-dinitrophenyl lysine (E-DNP-lysine) is measured colorimetrically.

Results obtained with the Carpenter procedure for a series of fish, whale and meat products indicated a close correlation with the corresponding results from chick feeding tests (51). Determination of available lysine values using the FDNB-procedure were also made on ground nut, cottonseed and soybean meals but the results did not correlate so well with the corresponding test on chickens (40). Difficulties are encountered in using Carpenter's procedure for measuring available lysine in products rich in carbohydrate due to lower recoveries of E-DNP-lysine which is destroyed during acid hydrolysis in the presence of carbohydrate and also there is formation of other yellow products which are not easily separated from the hydrolysate (232).

A number of modifications of Carpenter's procedure were proposed by several authors. A group of workers have used column chromatography to separate DNP-lysine from possible interfering colours (229).

Carpenter (50) has introduced a second reagent, methoxy-carbonyl chloride, to react specifically with DNP-lysine in the ether washed hydrolysate, the product is then extractable with ether which leaves a blank whose extinction can also be read and the difference between the direct reading and the reading on the treated sample is taken as measuring the E-DNP-lysine which is itself, a measure of available lysine.

The destructive effect on DNP-lysine by carbohydrate may be partially diminished in some materials by increasing the volume of acid for hydrolysis (180). Carpenter (52) noted, however, that no hydrolysis conditions examined are effective in fully stabilising the DNP-lysine.

There are some other minor limitations in the Carpenter method, in particular, it also estimates hydroxy-lysine and ornithine as lysine and it does not estimate N-terminal lysine or free lysine (172) and therefore lysine supplemented foods cannot be evaluated.

Considering the various limitations in using Carpenter's procedure especially in plant protein products which are rich in carbohydrate, Roach et al (232) developed a method called the "difference-procedure", which measures the "available" lysine as the difference between the total lysine obtained after acid hydrolysis and the "bound" lysine which is the non-complexed lysine present in the acid hydrolysate of the same material after it had been pretreated with FDNB. Since lysine itself is relatively little affected by the conditions of acid-hydrolysis, there should be no recovery problem with this method and in addition any free lysine in the original material would be included in the determination. In a discussion of the various methods being considered as improvements of Carpenter's procedure, Carpenter (53) himself stated "if one has the automatic equipment it may prove more satisfactory to use the "difference" procedure rather than bother with the recovery problems which have to be faced however one determines DNP-lysine".

APPENDIX 9

SOME OF THE BIOLOGICAL METHODS USED FOR THE DETERMINATION OF THE NUTRITIONAL VALUE OF PROTEINS (239)

1. Protein efficiency ratio (PER):- The protein efficiency ratio (PER) is the gain in weight of a growing animal divided by its protein intake. It is a measure of protein quality particularly in relation to growth requirements when determined under specific conditions. However, as pointed out by Block and Mitchell (28) the PER is not a true efficiency ratio because not all the protein is used for growth, only that consumed above maintenance (29). Also, Mitchell has criticised the PER because it varies with the food intake (190). The PER is used mainly in feeding experiments with small animals and has been used in study on infants.

2. Biological value (BV):- The biological value is determined by nitrogen balance and is defined by the ratio nitrogen retained : nitrogen absorbed (191). This expression of protein quality measures the percentage of absorbed nitrogen retained for growth and maintenance with its main emphasis on maintenance, but it does not include a correction for incomplete absorption. The protein must be fed at or below the level needed for maintenance in order to achieve maximum efficiency of utilisation. Generally this level is 9 to 10 percent of the diet ($^{w}/_{w}$).

3. Net protein utilisation (NPU):- The net protein utilisation (NPU) expresses in a single index both the digestibility and BV of a protein. It is the product of the coefficient of digest-

ibility and the BV, and therefore represents the proportion of food nitrogen retained, i.e., nitrogen retained : nitrogen intake. The efficiency with which a protein is utilised is diminished if the caloric intake is too low or if the protein is fed in excess. For comparison of the quality of proteins, the NPU is measured under standardised conditions, with protein supplied at or below maintenance levels in a diet providing adequate calories (NPUst). The NPU operative (NPUop) refers to the utilisation of a protein under those conditions in which it is actually eaten.

4. Net dietary protein value (NDpV):- The efficiency and concentration may be combined in a single index, called the net dietary protein value (217). This expression is the product of protein concentration and NPU (determined at the same protein level). The term NDpCal% (218) is used when in the latter expression protein concentration is expressed as a percentage of calories in the diet.

5. Gross protein value (GPV):- Growth results have also been assessed in terms of the contribution which a protein makes when used as a supplement to a standardised diet. For example, the gross protein value for chicks measures the value of a protein source as a supplement for cereals (54).

APPENDIX 10

LIST OF PAPERS

1. KHALEQUE, A., BANNATYNE, W.R. and WALLACE, G.M. Studies on the processing and properties of soymilk. 1. Effect of preprocessing conditions on the flavour and compositions of soymilks, J.Sci.Fd.Agric. 21 (11), 579 (1970) (Reprint attached)

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