STUDIES ON METHODOLOGY IN DIETARY FIBRE ANALYSIS: A NEUTRAL DETERGENT FIBRE METHOD USING GLUCOAMYLASE

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ABSTRACT

The dietary fibre content of foods is conveniently and rapidly determined by the neutral and acid detergent methods devised originally by Van Soest and associates. A serious disadvantage of the neutral detergent method relates to the interference caused by starch during filtration when the method is applied to cereals and cereal products. In these circumstances the results of neutral detergent fibre (NDF) measurements are variable and often over-estimated.

A study of the starch-lipid reaction which takes place when cereal products are heated in Van Soest's neutral detergent solution showed that although the precipitate derived from pure wheat starch and lipid is soluble in hot water this action is often far from complete when much fibrous cereal matter is present. Much of the starch appears to be occluded in the NDF residue which then takes on a gummy-like character and tends to clog the filter.

Southgate recently recommended purified amyloglucosidase from Aspergillus niger (Boehringer) for the purpose of hydrolysing starch in cereal samples before starting the neutral detergent extraction. Present studies have been concerned with the development of this enzymatic procedure with the aim of devising improved methodology and enhancing existing knowledge of the behavioural characteristics of amyloglucosidases from A. niger and from an alternative source, Rhizopus spp.

Preliminary investigations showed that amyloglucosidase from A. niger (Boehringer) was completely effective as a starch hydrolysing agent in the pretreatment of a cereal substrate but that in order

to use the enzyme economically it was necessary to use a semi-micro version of Van Soest's neutral detergent extraction procedure. The main features of the new method are as follows: preparation of a subsample of lipid-free food sample of fine particle size; gelatinization of starch before enzyme treatment; treatment with the minimum quantity of enzyme (2 mg); extraction with neutral detergent at half the normal rate; separation of detergent solution from the residue by means of centrifugation; dehydration of the residue with acetone before filtration; special techniques for filtration, drying and weighing procedures.

A table of NDF values for various cereal products determined by the semi micro procedure is presented. The results agree, for the most part, with the results of other workers in this field, the exceptions being for cornflakes, rolled oats and puffed wheat. The coefficients of variation for the NDF values compare favourably with those of other workers.

A semi micro version of Van Soest's acid detergent method of evaluating dietary fibre was devised and is described with supporting analytical data.

Tests performed with a low cost preparation of amyloglucosidase from Rhizopus spp (Sigma) showed that the crude enzyme was capable of fully hydrolysing the starch component of cereal products before commencing the neutral detergent extraction procedure but that it also seriously reduced the NDF values. In order to establish the cause of the discrepancies two approaches were made: an attempt was made to analyse the products of enzymatic hydrolysis; and a study of the effect of enzyme concentration on the yield of neutral detergent fibre was undertaken. The former approach proved

impracticable, the latter suggested that either impurities in the crude enzyme preparation were responsible or the amyloglucosidase itself was active towards one or more components of dietary fibre. In order to determine which of the alternative explanations was correct small amounts of the crude enzyme preparation were purified by means of anion exchange chromatography using DEAE cellulose and one of two buffer systems, one based on citrate-phosphate, the other on tris-HCl. The citrate-phosphate conditions reported by Pazur and Lineback et al for the column separation of amyloglucosidase of A. niger were found to be quite unsuitable for the enzyme from Rhizopus spp. and a new set of conditions had to be determined for this enzyme.

The activity of small amounts of the purified enzyme (< 1mg) was estimated by an improvised visual method using buffered 1% wheat starch, and the effect of the enzyme on cereal fibre was determined by means of the semi micro neutral detergent procedure using 0.08-0.2 g wholemeal flour as a substrate. It was found that both crude and purified forms of the enzyme caused a loss of ca 30% NDF from wholemeal flour, from which it was concluded that amyloglucosidase from Rhizopus spp was not a suitable enzyme for use in the neutral detergent method of measuring fibre.

A literature review of the known chemistry of the amyloglucosidases of A. niger and R. delemar showed that differences in molecular structure reported by Pazur and others could account for their different electrophoretic properties. In the light of the present work it appears that another important biochemical difference between these enzymes relates to the activity of the Rhizopus enzyme towards the dietary fibre component of cereals.

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CHAPTER 1

GENERAL INTRODUCTION

Dietary fibre is defined as the indigestible matter in the diet derived from the plant cell wall (69). The definition has been extensively discussed in the literature which is reviewed in Appendix 1.

Dietary fibre has a positive physiological role to play after ingestion in the form of foods. It is presently believed that adequate dietary fibre in the diet may function as a preventive measure against numerous Western civilization diseases (18). It has long been accepted that fibre serves as a natural laxative and is necessary to promote regular bowel habits (47).

An extensive contribution to our knowledge of the role played by dietary fibre in human nutrition has been made by Burkitt and Trowell (7) based on 30 years of medical experience in East Africa. They noticed that a great number of non-infective diseases common in western man were rare in rural Africans. The largest coherent group of these diseases was and still is associated with the colon (70). It was concluded that fibre might play a part in the diseases of the colon - relating to constipation, diverticular disease, irritable colon, appendicitis, haemorrhoids, ulcerative colitis; also cancer of the large bowel (70). Other diseases considered related to fibre intake are heart disease, gallstones and obesity (66). Evidence in support of the contention that heart disease and the intake of dietary fibre are related comes from Morris et al (34) who studied a daily individual weighed dietary survey of 337 healthy middle-aged men in London and South-east

England during 1956-66. By the end of 1976 45 of them had developed clinical coronary heart disease (CHD) which showed two main relationships with diet. Men with a high energy intake (reflecting physical activity) had a lower rate of disease than the rest, and independently of this men with a high intake of dietary fibre from cereals also had a lower incidence of CHD.

Certain types of fibre, particularly pectin, have the property of binding bile salts, cholesterol and other sterols which may account for their ability to reduce blood cholesterol (48).

Fibre has water binding properties which increases the rate and volume of faecal elimination. Furthermore the satiating capabilities of fibre in food (e.g. wholemeal bread) may prevent over-ingestion of fat and sugar in the diet and reduce the potential for obesity (48).

Recently Oakenfull (36) has claimed that saponins rather than fibre lead to the reduction in blood cholesterol levels. Thus if high levels of blood cholesterol do contribute to coronary heart disease only saponin-containing fibre will reduce the risk of heart attack. The evidence is based on feeding trials with rats but also on an Italian experiment with 20 human patients carried out by Sirtori et al (53) who found that a low lipid diet containing soybean protein (a food particularly rich in saponins) considerably lowered blood cholesterol levels.

Dramatic advances have been made during the past decade or two in our understanding of the biological functions of dietary fibre in human nutrition but impressive advances have also occurred in the

analytical methodology of fibre measurement in foods - although not all authorities are yet satisfied. For example, the Institute of Food Technologists' Expert Panel on Food Safety and Nutrition (25) has complained that the results of feeding high fibre diets differ from researcher to researcher, one explanation being the relatively poor analytical methods available for establishing fibre data.

The methodology of fibre analysis has its beginnings in the early nineteenth century with the development of the Weende method for determining crude fibre in animal forages and feeds. This procedure was eventually standardised by the AOAC in 1887 (76).

Remy (46) in 1931 was the first to advocate the use of enzymes in the measurement of plant fibre in fruits, vegetables and grain. His method gave values roughly twice those obtained by the classic Weende method. Remy's enzymatic method was improved upon by Williams and Olmsted (83) and later by other workers. The basic studies of McCance and Laurence (31) in 1929 directed attention to unavailable carbohydrates in foods and led ultimately to the analytical schemes put forward by Southgate (70).

One important difficulty encountered by the pioneers of fibre analytical methodology was the lack of a generally acceptable term and definition for this type of fibre. Hipsley (22) in 1953 was the first to use the term 'dietary fibre' but 20 years were to elapse before a new definition of dietary fibre based on physiological considerations, proposed by Trowell (69), made the term widely popular (70).

Even at the present time (1979) there is still lack of agreement on

the meaning of dietary fibre and the search for a more appropriate term continues. In a recent review paper by Spiller et al (65) 14 different terms for fibre are listed which have been proposed by various authorities over many years. Spiller et al (65) favour 'plantix' so as to avoid confusion with fibre of animal origin. Yet, as Trowell (70) points out, indigestible amino polysaccharides in animal connective tissues eaten by carnivores and Eskimos (19) should be included when defining fibre. For the time being the term dietary fibre is likely to be widely used despite certain minor anomalies. One may also expect abbreviations such as NDF and ADF (for neutral detergent fibre and acid detergent fibre, respectively) to be used in appropriate circumstances to denote the methodology of fibre measurement.

A problem that concerns the fibre analyst at the present time is the difficulty experienced in measuring accurately the dietary fibre content of starch-rich foods by the rapid neutral detergent method devised by Van Soest. Even if this problem can be resolved, however, the fundamental objection remains that the neutral detergent method will underestimate total dietary fibre because the water insoluble polysaccharides are lost during extraction, these losses being quite substantial with some materials (54).

Nevertheless, any step towards the improvement of the method must be considered worthwhile. The present study reveals some of the basic problems associated with the use of starch hydrolysing enzymes when applied to the measurement of dietary fibre by Van Soest's neutral detergent method. The study also reveals how these enzymes may be exploited in a new semi micro modification of Van Soest's method. A close study of one of the enzymes investigated, amyloglucosidase

from Rhizopus spp, provides an opportunity of recording certain hitherto unreported biochemical characteristics relating to its effect on dietary fibre components.

CHAPTER 2

DEVELOPMENT OF A SEMI MICRO METHOD FOR MEASURING DIETARY FIBRE

2.1 Introduction

Van Soest's detergent fibre methods were originally designed for the measurement of dietary fibre in forages and animal feeds which usually contained high proportions of fibre, available carbohydrates (including starch) and protein. When the same methods came to be applied to human food some years later certain analytical problems became evident. Southgate (60) found that the direct application of Van Soest's methods to foods rich in fat, protein and starch created special difficulties: The protein and fat seem to produce technical problems relating to foaming during extraction and at the filtration stage. Southgate advises that it is better to extract the lipid before attempting to measure neutral detergent fibre (NDF) or acid detergent fibre (ADF) in foods rich in fats. Furthermore some starch is insoluble in neutral detergent and leads to elevated fibre values unless it is removed enzymatically.

Southgate (58) recommends the use of a starch degrading enzyme that is reasonably consistent from batch to batch and has a well defined specificity - such as the amyloglucosidase preparations from Aspergillus niger from Sigma Chemical Co, or Boehringer, Mannheim.

Although not mentioned by Southgate the high cost of purified commercial preparations of amyloglucosidase must be regarded as a serious drawback especially when applying the enzyme treatment to starchy food samples weighing 0.5 to 1g as required for Van Soest's methods. However, the problem of cost can be resolved by reducing

the size of the food sample to be analysed, although this course of action itself creates fresh problems, particularly in regard to reproducibility.

Schaller (49) used the NDF method on a variety of cereal products and compared the results with those obtained by using swine pancreatic α-amylase to remove residual starch before commencing the neutral detergent extraction. His results are given in Table 1 and indicate very substantial deviations for corn grits and cornflakes.

TABLE 1. Comparison of NDF and NDF + amylase analyses (49)

	NDF %	NDF + amylase %
Corn bran	89	89
Bagasse	79	79
Defatted alfalfa	44	44
Wheat bran	36-49	36
Heavy bran	14-47	14
Corngrits	5-11	0.6-1.0
Cornflakes	7-14	1.3

Jwuang and Zabik (26), using Schaller's enzymatic NDF procedure, analysed a wide variety of commercial breads, cereals, cookies and snacks. Their enzyme neutral detergent fibre (ENDF) value for rolled oats is given as 5.63 ± 0.28 (mean $\pm$ SD). This result is relevant to the present work on enzymatic NDF procedures using amyloglucosidase (Boehringer) for hydrolysing starch. Our result (see Table 6) was 4.97% and compares with 7.7% quoted by Southgate

and Paul (37) for total dietary fibre. McQueen and Nicholson (33) used bacterial α-amylase to hydrolyse starch before determining NDF by the conventional method of Van Soest and Wine. They found that the enzyme treatment promoted rapid filtration and gave more accurate NDF estimates and less analytical variation for samples of cereal grains and vegetables that have a high starch content.

Holloway et al (24) used both Van Soest's NDF and ADF methods to determine dietary fibre and its components in a wide range of New Zealand foods. Holloway (23) considered that the methods gave reasonable reproducibility. For example, human faeces gave a coefficient variation of 5.6% for cellulose, 18.0% for lignin and 5.1% for hemicellulose. Further studies on wholemeal flour, Weetbix and beans gave acceptable reproducibility. The results are reproduced (in part) in Table 2 with coefficients of variation (C) added.

TABLE 2. Reproducibility of neutral detergent and acid detergent methods (24).

Wholemeal flour n = 10	Mean g/100 g dry wt	S.D.	C
Neutral detergent fibre	9.76	0.23	2.3
Acid detergent fibre	2.66	0.42	15.5
Cellulose	1.76	0.65	37
Lignin	0.96	0.49	51
Weetbix n = 10			
Neutral detergent fibre	12.83	1.81	14.1
Acid detergent fibre	4.23	0.51	12.1
Cellulose	1.97	0.63	32
Lignin	2.23	0.62	28

A comparison of the coefficients of variation for the NDF content of wholemeal flour and Weetbix reveals surprising differences (2.3 v. 14.1) which suggests that serious reproducibility problems do occur when Van Soest's methods are applied to cereal products. It is not known why wholemeal flour with its high starch content should give more reproducible NDF results than Weetbix although differences in the types of starch and possibly the hemicellulose components may be implicated.

It was accepted that the difficulties in reproducibility were caused primarily by the presence of starch in the food samples. The present studies were therefore undertaken aimed at the development of a semi micro adaptation of Van Soest's neutral detergent fibre method. The main aspects of this adaptation were:

1. To consider the initial preparation of the sub-sample of food material
2. To determine the minimum concentration of enzyme required to remove all of the starch component of the food material
3. To investigate centrifugation procedures as an alternative to decantation and filtration
4. To investigate handling and weighing of minute amounts of fibre residue
5. To test the fibre residue for starch contamination.

2.2 Objective and Experimental Plan

The neutral detergent fibre method developed by Van Soest requires 0.5 to 1.0 g of dry material for each analysis. As this amount of material, when rich in starch, yields 50 to 100 mg fibre residue and up to 350-700 mg starch the cost of using purified amylo-glucosidase for starch hydrolysis is prohibitive. ($30 per 100 mg

in 1979).

The aim of the present work was to bring the cost of enzyme treatment to within acceptable limits by reducing the amount of substrate to be analysed. Initially it was decided to experiment with 0.2 g of food substrate for each determination and to investigate the problems as they developed during the analytical procedure. The stages investigated were as follows:

1. Preparation of the sub-sample with particular reference to lipid content and particle size. Note: lipid interferes with the extraction of fibre (54); particle size must be small enough to ensure satisfactory homogeneity of the sub-sample.
2. Heat treatment of the substrate in water for the purpose of gelatinizing starch.
3. Treatment with amyloglucosidase at the optimum concentration and operating conditions for the purpose of removing starch.
4. Preparation of the starch-free substrate for neutral detergent extraction using centrifugation.
5. Extraction with neutral detergent using appropriate apparatus and minimum quantities of reagents.
6. Separation of the residue by means of centrifugation and washing procedures.
7. Dehydration of the residue before filtration by addition of organic solvent (acetone).
8. Filtration by using a sintered glass crucible of suitable size and porosity.
9. Weighing procedure for 2-20 mg amounts of dried residue.

In order to evaluate the proposed semi micro procedure of measuring NDF residues in starchy food materials, particularly cereals and

cereal products, Van Soest's and Southgate's methods were closely studied and compared using similar food materials.

As the starch component of a food substrate is known to cause interference in the determination of NDF (60) the reaction between starch and neutral detergent was also studied. It was hoped that a better understanding of the fibre residue contamination problem would be achieved by this means.

The studies were extended to include a semi micro adaptation of Van Soest's acid detergent method in order to ascertain whether any advantages could be gained in this way.

2.3 Experimental

2.3.1 Determination of neutral detergent fibre

2.3.1.1 Materials

All cereal products used in the analyses were purchased locally.
Neutral detergent: Prepared as directed by Van Soest and Wine (78)
Sintered glass crucibles: Capacity 70 ml, plate diam. 40 mm, porosity 1

2.3.1.2 Methods

Samples of cereal products taken for analysis were reduced to a fine state of division by means of a Philips 160w coffee grinder. Moisture determinations were carried out by drying 1-2 g sample at 102-103^{o} overnight and reweighing to constant weight. Bread samples were prepared from whole loaves according to the method of the AACC (3). Neutral detergent fibre was determined according to the method of Van Soest and Wine (78). 1.00 g sample was taken for each analysis.

2.3.1.3. Results

Considerable difficulty was experienced with the filtration and

washing of residues on the sintered glass crucibles on account of the presence of gummy substances. Attempts to wash the residues with hot water were often only partially successful. Occasional analyses were abandoned.

The results of analysis for a number of cereal products are given in Table 3. Comparable values quoted from other sources are included.

TABLE 3. Neutral detergent fibre content of cereal products

Cereal product	Neutral detergent fibre						
	range	mean	S.D.	C	Quoted from other sources: Spiller & Amen (64)	Holloway et al (24)	S.D. n=10
	g/100 g dry matter				g/100g dry matter		
All-Bran (Kelloggs)	33.1-35.4 (6)	34.4	0.78	2.3	34.0	29.7	
Weetbix (Sanitarium)	7.9-13.0 (12)	10.4	1.78	17.1		12.9	1.81
Cornflakes (Kelloggs)	4.5-10.2 (12)	7.0	1.70	24.3			
Rolled Oats (Flemings)	10.5-13.0 (4)	11.4	1.09	9.6			
Wholewheat bread	8.2-10.5 (2)	9.0			14.9		

Note: numbers in parentheses indicate number of replicates

In reference to Table 3 the standard deviation for Weetbix (1.78; n=12) is in close agreement with the findings of Holloway et al: SD = 1.81; n=10.

2.3.1.4 Discussion

Filtration problems with starch and protein-rich food materials have been discussed by Van Soest and Wine (78). They state that much gelatinous matter is likely to form from starches and protein and may tend to clog the filter. Spiller et al (65) have recently reported that a special committee from the American Association of Cereal Chemists, as well as Van Soest himself, are working to modify the neutral detergent fibre method so that it will be more applicable to high starch foods such as cereals.

The cause of the filtration problems can be largely attributed to the reaction between starch and lipid to form an insoluble complex: sodium lauryl sulphate is the source of lipid in the neutral detergent solution. The chemistry of the reaction has been discussed by Radley (45): Selective precipitation of the amylose fraction can be accomplished with virtually any polar organic compound capable of hydrogen bonding. Thus with oleic acid a crystalline complex is obtained, the precipitation occurring at elevated temperatures. The addition of 1% oleic acid to potato starch results in the production of 18% insoluble matter; the linear component (amylose) will absorb 6-7% of its weight of oleic, palmitic and lauric acid.

Confirmation of the reaction between starch and lipid can be obtained by boiling 1% wheat starch solution with Van Soest's neutral detergent. A gelatinous precipitate is formed on standing which when transferred to a sintered glass filter may easily be washed through with hot water. However, when the starch precipitate is associated with the fibrous matter present in cereal products the starch-lipid complex tends to form a gummy substance which adheres to the NDF residue and is not easily washed away and may even cause complete blockage of the filter.

A second problem associated with the separation of the NDF residue by direct filtration is that the hemicellulose fraction itself, in some cereal products at least, behaves like the starch-lipid complex in some respects by being washed through the sintered glass filter when treated with hot water. This effect is demonstrated in a test described later in this chapter (2.3.3) using Weetbix as a substrate.

Thus in order to counteract the filtration difficulties arising from the physical properties of the starch-lipid complex and of the hemicellulose fraction two important measures need to be taken: Firstly, the starch component should be entirely removed from the food sample by enzymatic hydrolysis before extraction with neutral detergent is attempted. Secondly, the NDF residue should be treated with a dehydrating organic solvent, e.g. acetone, before filtration so as to ensure that the hemicellulose fraction is wholly retained at the subsequent filtration stage. In order to be able to wash the NDF residue free from neutral detergent salts the washing process is accomplished by a repetitive process of centrifugation and withdrawal of supernatant solution. Acetone is then added to dehydrate the NDF residue, followed by normal filtration using a sintered glass crucible.

2.3.2 Studies on the starch-lipid reaction in neutral detergent

2.3.2.1 Materials

Wheat starch

Pure starch (AnalaR)

Wholemeal flour, All-Bran, Cornflakes, Rolled oats

Apparatus, equipment and reagents: as for neutral detergent method (Van Soest)

Centrifuge tubes, 50 ml, rounded ends.

2.3.2.2 Methods

Neutral detergent fibre

The method of Van Soest and Wine was used for each analysis.

0.4-1.0 g of sample was taken for each analysis.

NDF + starch-lipid complex

The method of Van Soest and Wine was modified slightly by substituting centrifugation for filtration to retain all insoluble matter. After the extraction procedure with boiling neutral detergent the suspension while still hot was transferred to 50 ml centrifuge tubes and centrifuged for 5-10 minutes (RCF value = 670). The supernatant was carefully withdrawn with the aid of a finely tapered suction tube and filter pump; the residue was then redispersed with 45 ml hot water and the centrifugation process repeated. The residue was finally transferred to a previously weighed sintered glass crucible, washed with acetone, dried at 100^{o} and weighed.

In the case of the wheat starch assay the starch-lipid residue was washed not with hot water but with cold 25% V/V ethanol - using the centrifugation procedure just described. This was done to ensure minimum loss of residue during the washing procedure. Filtration, treatment with acetone, etc., were completed as before.

Crude protein

The total nitrogen content of the residue material was determined by the semi micro Kjeldahl method. Crude protein values were calculated using the factor N x 5.8 (for wheat).

2.3.2.3 Results

The results of analysing cereal products, wheat starch and pure starch by the standard and modified neutral detergent fibre methods are shown in Table 4. No insoluble matter was formed when pure starch was

treated with boiling neutral detergent.

TABLE 4. Evaluation of residues from standard and modified neutral detergent fibre methods.

Test Product	NDF (standard method)	NDF + starch-lipid (modified method)	Starch-lipid fraction (by difference)	Starch content of product[1]	Approximate estimate of starch recovery
	g/100 g dry matter				%
All-Bran	34.4	40.0	5.6	28	20
Weetbix	10.4	21.8	11.4	69	17
Cornflakes	7.0	26.4	19.4	80	24
Rolled Oats	11.4	57.2	45.8	72	64
Wholemeal flour	11.8	51.7	39.9	74	54
Wheat starch	nil	106.1[2]	106.1	100	(102)[3]
Pure starch (AnalaR)	nil	nil	nil	100	

1 Taken from: McCance and Widdowson's The composition of foods by Paul and Southgate: reference 37.

2 the precipitate was washed with 25% V/V ethanol.

3 on ash free basis.

Crude protein values for NDF residues obtained by the two methods are shown in Table 5.

TABLE 5. Crude protein content of cereal products and NDF and NDF + starch-lipid residues.

Cereal Product	Cereal product[1]	NDF	NDF + starch-lipid	Starch-lipid fraction only[2]
	g crude protein/100 g dry material			
All-Bran	15.1	7.6	7.5	6.9
Weetbix	11.4	13.9	13.5	13.1
Cornflakes	8.6	7.6	6.2	5.7
Rolled oats	12.4	6.4	1.4	1.7

[1] Taken from: McCance and Widdowson's "The composition of foods" by Paul and Southgate.

[2] Calculated from:

$$\frac{(\text{NDF + starch-lipid} \times \text{cr. protein} - \text{NDF} \times \text{cr. protein})\ 100}{\text{starch-lipid fraction.}}$$

2.3.2.4 Discussion

(1) The NDF values shown in the first two columns of Table 4 explain in part, at least, why the neutral detergent method (Van Soest) sometimes fails to provide reproducible results. Van Soest and Wine (78) discuss the means of overcoming filtration problems by back-washing, etc., but in our view this procedure leaves much to be desired. The better approach to the difficulty would seem to be the complete removal of starch by enzymatic hydrolysis before neutral detergent extraction.

(2) Investigation of the source of variation in fibre recovery showed that when attempts were made to retain the starch-lipid fraction with the NDF residue by means of a centrifugation/washing procedure, between 17 and 64% of the original starch present in the cereal products was

recovered. These figures are only approximate as no account has been taken of the lipid part of the residue although this is assumed to be quite small.

The NDF value for wheat starch obtained by the standard neutral detergent method (Table 4, column 1) was found to be nil. This result is explained by the fact that the starch-lipid complex dissolves in hot water used for washing purposes although this effect is much less marked when cereal products are so treated because of the gummy nature of the residues.

On the other hand an apparent complete recovery of wheat starch was accomplished by using 25% V/V ethanol as a washing agent in which the starch-lipid complex is insoluble. Corrected for ash content the starch-lipid residue accounted for 102% of the original weight of wheat starch taken (Table 4, column 5). Pure starch (AnalaR), however, did not form an insoluble starch-lipid complex and therefore no recovery of starch was possible.

The crude protein values for the two kinds of NDF residue, shown in Table 5, indicate that part of the cereal protein is occluded with the starch-lipid fraction during the extraction process with neutral detergent. This effect is particularly evident for the first 3 cereals listed in Table 5, the estimated crude protein values of the starch-lipid fractions being similar to the determined values of the respective NDF residues.

If the NDF residue is contaminated with starch (after completion of the determination) it appears likely that it will also retain some protein thus compounding the errors of the neutral detergent method.

2.3.3 Determination of neutral detergent fibre by the proposed semi micro procedure.

2.3.3.1 Materials

Amyloglucosidase: supplied by Boehringer, Mannheim

Acetate buffer: 0.5M; pH 4.6

Neutral detergent: as for previous methods

Sodium sulphite: laboratory reagent

Acetone

Refluxing apparatus: Berzelius beakers, 6 x 500 ml, and condensers made from 250 ml r-b flasks

Centrifuge tubes: 6 x 15 ml cap. and 6 x 50 ml cap. (with round ends)

Sintered glass crucibles: 6 x 35 ml cap., plate diam 30 mm, porosity 1.

2.3.3.2 Methods

Preparation of Subsample

The food material to be analysed was prepared according to Southgate's method (57). Air dried food material was ground to pass 18 or 20 mesh screen. A 4-5 g sample was weighed into a 100 ml flask, extracted with 4 x 25 ml portions of boiling 85% V/V methanol and one portion of warm diethyl ether. The residue was allowed to dry overnight in a warm cupboard and then accurately weighed. The residue was ground to a fine powder with the aid of a large size mortar and pestle (previously warmed).

In the case of bread samples, which may contain extremely hard particles, the finely powdered material is examined under a binocular microscope fitted with an ocular micrometer. If many particles exceed 50 x 75 µm the sample is reground to a smaller particle size.

The moisture content of the food sample was determined by overnight drying of 1-2 g at 102-103°.

Gelatinization of starch

In order to prepare the food sample for treatment with a starch hydrolysing enzyme one of two methods was employed:

If the food material was fairly soft the starch was gelatinized by heating 0.2 g sample in 5 ml distilled water (placed in a 15 ml centrifuge tube) on a boiling waterbath. A glass filter funnel was placed in the neck of the tube to reduce evaporation losses. The suspended food material was rigorously dispersed with the aid of a glass rod and rubber policeman after about 4 minutes from the time of commencement. The rod was rinsed with a few drops of water. Gelatinization was completed in 15-20 minutes.

If the food material contained hard granules the gelatinization process was conducted in an autoclave at 121° for 1h (14). Some kinds of bread, e.g. wholemeal, may require autoclave treatment.

Enzyme treatment

After cooling the suspension containing gelatinized starch, 0.5 ml 0.5M acetate buffer pH 4.6 was added. This was followed by 0.2 ml amyloglucosidase suspension (equivalent to 2 mg) and dispersion of the contents of the tube with the aid of the glass rod and policeman. A small drop of toluene was added to each tube, caps were placed on the tubes which were then incubated at 37° overnight with occasional swerling.

Alternatively, the tubes were maintained at 55° for 3 hours in a waterbath.

Centrifugation

After completion of the enzyme treatment the tubes were centrifuged. The supernatants were withdrawn carefully, without disturbing the sediment, by means of a suction tube, and discarded.

Extraction of starch-free residues

The residues from each tube were transferred to 500 ml Berzelius beakers with 50 ml aliquots of neutral detergent. A glass bead was added to each beaker to minimize bumping followed by 0.2 g sodium sulphite to aid solution of protein.

The beakers were covered with the r-b flasks operated as condensers and heated on a hot plate to induce gentle ebullition for 1h. Under normal circumstances, because of the absence of starch, boiling proceeds smoothly and antifoam reagents are not required. The volume is kept constant at 50 ml by the addition of water as required.

Post-extraction

The 50 ml extracts containing insoluble and fibrous matter were transferred to 50 ml centrifuge tubes leaving traces of residue to be transferred later. The tubes were centrifuged and supernatants were carefully withdrawn and discarded. Each beaker was then rinsed out carefully with hot water into its respective centrifuge tube with the aid of a glass rod and policeman, making a total of ca 45 ml. Centrifuging was repeated and, as previously, the supernatants were withdrawn and discarded leaving 2-3 ml residue in each tube.

If cutin-like substances from the original food sample are present their presence may be noticed as suspended particles after the centrifuging operation. As the centrifuge slows down to a halt such particles are easily disturbed in the sediment. In these circumstances it is

advisable to reduce the centrifuge speed very gradually.

Dehydration and filtration

The fibre residue from the previous stage was dehydrated by the careful addition of 25 ml acetone to each tube. The residues were transferred to weighed sintered glass crucibles using ca 10 ml acetone per tube for this purpose. A glass rod and policeman were used to aid the transfers. The crucibles were allowed to drain without suction being applied.

After gently sucking off the last few drops of acetone the crucibles were transferred to an oven at 101-102^{o} for a minimum of 1h.

In order to demonstrate the vital importance of treating the residues with acetone before filtration one set of residues in an aqueous condition, prepared from Weetbix, was transferred directly to sintered glass crucibles immediately after the repeat centrifuging treatment. The residues were then each washed with 25 ml hot water followed by an equal volume of acetone. The crucibles were dried and weighed in the usual way. (See Table 8 and page 24 for discussion).

Weighing procedure

Crucibles were clearly marked to assist with identification and they were always weighed in the same order. The interval between transferring a crucible from the oven to a desiccator and from the desiccator to the balance was kept at 10 minutes - sufficient to permit cooling without significant uptake of moisture.

A Mettler model H6 analytical balance with a sensitivity of $\pm$ 0.1 mg was found to be satisfactory for present requirements, but only after the lamp circuit had been modified to ensure that the lamp remained

'on' for all positions of the balance pan lift control. By keeping the lamp 'on' permanently, changes in temperature and relative humidity within the balance housing were reduced so that they did not noticeably affect the standard of weighing.

Crucible weights were generally reproducible to $\pm$ 0.1 mg. New crucibles of the sintered glass type were repeatedly heated to 500^{o}, cooled and backwashed with hot water until constant in weight.

Ashing procedure

The mineral matter content of fibre residues was determined by ashing at 400^{o} for 1h followed by 2h at 550^{o}.

Ash was removed from crucibles by backwashing with 250 ml boiling water.

The ashing procedure was carried out after every fibre determination.

Calculations

In order to calculate the percentage of neutral detergent fibre in a sample of food material on a dry matter basis the following formula was used:

$$\frac{\text{Wt of NDF residue (g)} \times f_e \times 100}{\text{Wt of sample (g)} \times f_{dm}}$$

where f_e = factor to convert fresh wt to extracted wt

f_{dm} = factor to convert fresh wt to dry wt

2.3.3.3 Results

The neutral detergent fibre content of a range of cereal products was determined by the proposed semi micro method. The results are shown in Table 6.

The amount of amyloglucosidase used for duplicate determinations was varied from 2 to 4-5 mg, the results of which appear in the first two columns of Table 6. In the majority of analyses the higher concentration of enzyme resulted in a slightly lower NDF value.

There are some discrepancies between the results obtained by the semi micro method and those quoted from the literature using Van Soest's method and Southgate's method, particularly with reference to corn-flakes and rolled oats.

An indication of the reproducibility of the semi micro procedure is given in Table 7.

The results of washing starch-free NDF residues derived from Weetbix with hot water at the filtration stage are shown in Table 8. The loss of 29.5% of NDF residue is attributed to hemicellulose which was presumably drawn through the sintered glass filter under aqueous conditions.

TABLE 6. Neutral detergent fibre content of starch-rich food products determined by semi micro method.

Food product[1]	Wt of enzyme per determination		Data from other sources:		
	2 mg	4-5 mg	Holloway et al (24)	Spiller & Amen (64)	Paul & Southgate (3
	NDF		NDF		Unavailable carbohydrate
	g/100 g dry matter				
All-Bran (Kelloggs)	34.01	33.76	29.7	34.0	27.2
Cornflakes (Kelloggs)	5.41	4.62	5.9	7.9	11.3
Puffed Wheat (Sanitarium)	6.51	6.65		8.9(Quaker)	15.8
Rolled Oats (Flemings)	4.97	4.49		10.4(Scotts)	7.7
Special K (Kelloggs)	6.51	6.50			5.6
Weetbix (Sanitarium)	12.17	11.58	12.9	13.8 (Wheaties)	13.2 (Weetabix)
Flour, wholemeal (Timaru)	11.84	11.58	10.3		11.2
Flour, white (bulk)	1.87	1.92	2.9		3.5
Flour, rice	1.04	0.91			
Flour, ryemeal (Healtheries)	13.46	13.02			
Bread, wholemeal (W.R.I.)	8.14				
Bread, white (W.R.I.)	4.13				
Bread, brown-fibre enriched (Quality Bakers)	12.23	11.90			
Bread (Hifibe - TipTop)	9.70	9.42			
Bread (Golden Grain - Q.B.)	9.52				
Potato flakes, instant (Waitaki)	3.15	3.48	18.8	4.7 (peeled)	8.7 (raw)
					16.5 (instant

[1] Source applies to first two columns of data only

TABLE 7. Reproducibility of semi micro neutral detergent fibre method.

Food product	No. of determinations n	Neutral detergent fibre range	mean	S.D.	C
		g/100 g dry matter			
All-Bran	3	33.5-33.9	33.7		
Weetbix	4	12.2-13.4	12.8	0.48	3.7
Cornflakes	4	4.6- 5.5	5.2	0.41	7.9
Rolled Oats	3	4.5- 6.7	5.4		
Bread, white	3	3.0- 3.15	3.05		
Bread, wholemeal	2	6.0- 7.0	6.5		
Wholemeal flour	10	11.22-13.56	12.30	0.3	2.4
Weetbix[1]	13			0.21	2.8
Bread[1]	8			0.26	3.3

[1] Standard deviation and coefficient of variation were calculated from n sets of tests performed in duplicate using the following formulae:

(1) $SD = \sqrt{\frac{\Sigma d^2}{2n}}$ where d = difference between duplicate determinations and n = no. of sets of duplicate determinations.

(2) $C = \frac{SD \times 100}{\text{mean value}}$ where C = coefficient of variation

TABLE 8. Effect of washing starch-free NDF residue with hot water.

Food product	Neutral detergent fibre g/100 g dry matter		Loss of NDF %
	Acetone wash[1]	Hot water wash	
Weetbix	12.2	8.6	29.5

[1] Taken from Table 6 (under 2 mg enzyme); residue was treated with acetone before filtration.

2.3.3.4 Discussion

A significant feature of the NDF results for starch-rich food materials obtained by the semi micro method, cited in Table 6, is the relationship between NDF and enzyme concentration. In most cases the higher enzyme concentration resulted in a slightly lower NDF value. It is suggested that amyloglucosidase (Boehringer) has the ability, albeit to a limited extent, of hydrolysing one or more components of the hemicellulose fraction of many cereal fibres. Other cereal fibres, e.g. those of Puffed Wheat, Special 'K' and white flour, appear not to be affected by the enzyme.

The decision to use 2 mg of amyloglucosidase (Boehringer) for the treatment of 0.2 g quantities of starch-rich food material by the semi micro neutral detergent method may be considered to be a compromise choice of enzyme concentration. Too little enzyme invites the risk of incomplete starch hydrolysis and too much may give rise to low NDF results. This was checked by microscopic examination for residual starch.

In some instances where the enzyme shows a marked tendency to attack cereal fibre, e.g. cornflakes, an alternative method of fibre analysis (e.g. Southgate's) is recommended.

In Table 6 it will be observed that there are some differences between values quoted by outside sources and those obtained for similar food products during the present work. The differences can be attributed partly to origin of food and partly to method of analysis. For example, Southgate's method for unavailable carbohydrates includes water soluble noncellulosic polysaccharides but excludes protein and mineral matter.

The reproducibility of the proposed semi micro method compares favourably with that of other methods involving the use of enzymes. Elchazly and Thomas (14) found coefficients of variation (C) for the dietary fibre values of wheat and rye, of 5.29 and 4.99, respectively. The coefficients of variation for wholemeal flour and Weetbix NDF values derived by the semi micro method were 2.4 and 2.8, respectively (Table 7). These compare favourably with C values given in Table 3 for cereal products analysed by the standard Van Soest neutral detergent procedure.

The loss of 29.5% NDF caused by hot water washing of a starch-free Weetbix residue (Table 8) can be explained by non-retention of the gelatinous residue of part, at least, of the hemicellulose fraction. It has already been reported that gelatinous starch-lipid material can be washed through a sintered glass filter of porosity 1 (see Discussion 2.3.1.4). Hemicellulose is apparently also lost in a similar way during aqueous filtration. One possible way of avoiding this loss is to use a filter of finer porosity, although this contrivance may cause other filtration problems such as blockages. A far better approach to the difficulty is to treat the residue with acetone before filtration. The effect of dehydrating the starch-free gelatinous-like residue with acetone is to produce a non-gelatinous fibrous-like material which is wholly retained on a sintered glass filter. Subsequent determination of the NDF content of many types of cereal product by the proposed semi micro technique using acetone as a dehydrating agent before filtration has shown excellent agreement between duplicates (see Table 7). This finding suggests that pretreatment with acetone prevents the loss of fine particles of fibrous residue during the filtration procedure. Using Van Soest's neutral detergent method Heller et al (21) have shown that the hemicellulose values for wheat samples

ground to pass a 60 mesh screen were 20% lower than for samples ground to pass a 20 mesh screen. Having regard to the fact that in order to produce a macroscopically homogeneous sub-sample of food material for analysis by the semi micro procedure the particle size must be very small, the acetone treatment of the residue before filtration is clearly all important.

The advantages of the proposed semi micro procedure may be summed up as follows:

1. The removal of starch by enzymatic hydrolysis before neutral detergent extraction plus the modified filtration procedure ensure the determination of NDF values with greater accuracy and improved reproducibility.
2. Considerable savings (50%) of neutral detergent reagent are achieved.
3. The use of small laboratory glass ware, e.g. sintered glass crucibles, etc., reduces expense.

Disadvantages include:

1. Longer time is required to complete the determinations (6 determinations require ca 12 man hours).
2. Amyloglucosidase (Boehringer) costs ca 60¢ per determination.
3. Extra skill is needed in handling minute amounts of fibre residue (sometimes less than 10 mg) and in weighing operations.

2.3.4 Determination of acid detergent fibre by the proposed semi micro procedure.

2.3.4.1 Materials

Acid detergent solution: 20 g cetyl trimethyl-ammonium bromide (CTAB) technical grade, is dissolved in 1 litre 1N H_2SO_4 (previously standardised).

Apparatus: Refluxing apparatus as for semi micro neutral detergent method.

Berzelius beakers, 6 x 500 ml

Centrifuge tubes, 6 x 50 ml

Sintered glass crucibles, 6 x 35 ml cap., plate diam. 30 mm, porosity 1.

2.3.4.2 Methods

Extraction with acid detergent

0.2 g of the subsample of food material prepared as for the semi micro neutral detergent method but without starch hydrolysis, was accurately weighed into a 500 ml Berzelius beaker to which 50 ml acid detergent solution was added. A glass bead was added to minimize bumping.

With the condensers in position the beakers were heated on a hot plate to maintain gentle ebullition for 1h.; the volume was kept at 50 ml by addition of water as required.

Post-extraction

The extract-suspension was transferred to a 50 ml centrifuge tube and treated in precisely the same manner as neutral detergent fibre residues at this stage (2.3.3.2) - washing the residue with hot water by the centrifugation procedure, dehydrating the washed residue with acetone, filtering, etc. The residue was dried, weighed and

ashed.

2.3.4.3 Results

ADF values for a range of cereal products, obtained by the foregoing semi micro procedure are shown in Table 9. Other values taken from the literature are included for purposes of comparison.

TABLE 9. Acid detergent fibre content of cereal products.

Cereal Product	Acid detergent fibre					
	Semi micro method			Van Soest's method(2) from Holloway et al (24)		
	g/100 g dry matter	S.D.	C	g/100 g dry matter	S.D.	C[1]
All-Bran (K)	9.8(4)	0.13	1.3	9.0		
Weetbix (San)	3.1(4)	0.21	6.8	4.23(10)	0.51	12
Cornflakes (K)	2.2(4)	0.51	23			
Rolled oats (Fl)	1.3(2)					
Bread, white:						
Square Top (Q.B.)	0.8					
Nimble (Q.B.)	1.2					
Tip Top				0.7		
Bread, brown:						
Kingmeal (Q.B.)	1.5					
Hifibe (Tip Top)	2.1					
Brown (Tip Top)				1.7		

Note: Figures in parentheses denote number of replicates

[1] Coefficients of variation calculated from literature report.

An estimate of the hemicellulose content of various cereal products can be made by subtracting ADF values from the respective NDF values shown in Tables 6 and 9 respectively. A few results derived in this way are given in Table 10 together with results from the literature for comparison.

TABLE 10. Proximate fibre analysis of cereal products.

Cereal product	Hemicellulose (NDF-ADF) Semi micro methods	Hemicellulose (NDF-ADF) Van Soest's methods[1]	Non-cellulosic polysaccharide fraction[2]
	g/100 g dry matter		
All-Bran	24.2 (71)	20.7 (70)	18.2 (67)
Weetbix	9.7 (76)	8.6 (67)	9.5 (72)
Cornflakes	3.2 (59)		7.5 (66)
Rolled oats	3.7 (74)		
White bread	2.35-3.2 (75-73)	3.5 (83)	3.35 (74)
Brown bread	2.4 (62)	3.1 (65)	6.05 (71)

Note: Figures in parentheses denote hemicellulose content as a percentage of NDF or dietary fibre.

[1] Taken from: Holloway et al (24)

[2] Taken from: Southgate et al (61)

2.3.4.4 Discussion

Acid detergent fibre (ADF) represents the sum total of crude lignin and cellulose (lignocellulose) of plant material but on occasion includes indigestible products of cooking (Maillard products) (76). In foods which contain little or no pectin such as cereal products the NDF-ADF value is a reasonably true measure of hemicellulose

content.

Van Soest (72) reported the recovery of up to 15% pentosans in acid detergent fibre. To avoid discrepancies caused by the recovery of pentosans in acid detergent fibre the proposed semi micro method could be modified to include a preliminary treatment with crude amyloglucosidase, grade II, (Sigma). Preliminary studies showed that this enzyme system possessed a high potential for degrading hemicelluloses as well as starch. For example, it was found that by using this enzyme the NDF value determined for wholemeal flour was 30% below that obtained by using amyloglucosidase (Boehringer) - the matter is discussed in full in Chapter 4.

The data presented in Table 10 show no serious discrepancies between presently found and quoted values for ADF, with the exception of cornflakes. Standard deviations for the ADF values of All Bran and Weetbix (Table 9) are quite reasonable but the coefficient of variation for cornflakes is high (C = 23%). It appears that cornflakes do not provide a satisfactory substrate for either of the semi micro methods (NDF and ADF) and should therefore be analysed by alternative methods, preferably Southgate's, to obtain true values.

Baker (6) has described a buffered acid detergent method for cereals which depends on the use of an HCl-KCl buffer solution to act as a solvent for the detergent - which is a much less aggressive reagent than 1N H_2SO_4 and is within the pH range of the human digestive medium. Excerpts from a table showing a comparison of fibre determinations by three methods is reproduced below (Table 11):

TABLE 11. Comparison of fibre determinations by three methods.

Sample	Crude fibre %	Acid detergent fibre %	Buffered acid detergent fibre %
Wheat	2.72	3.24	4.09 ± 0.18[1]
Bran	10.21	12.96	17.03 ± 0.36
Low grade flour	0.42	0.46	0.60 ± 0.02
Rolled oats (1)	1.82	1.76	4.02 ± 0.39
Rolled oats (2)	2.13	1.86	4.98 ± 0.23
Rolled oats (6)	1.48	1.94	3.18 ± 0.27
Hemicellulose (xylan)	0.7	0.8	11.8 ± 0.9

[1] Standard deviation.

Taken from: Baker, ref. 6.

Because of the high recovery of hemicellulose (xylan), by the buffered acid detergent fibre method it seems doubtful whether this method would give reliable values for the lignocellulose content of many cereal products.

2.3.5 Determination of non-available carbohydrates by Southgate's method.

2.3.5.1 Materials

Reagents and apparatus: as per modified method developed in the Dunn Nutritional Laboratory by Southgate (58).

Test materials: Weetbix and wholewheat bread

Enzymes: α-amylase and β-amylase supplied by B.D.H. Ltd.

2.3.5.2 Methods

Free sugars and starch were not determined. Otherwise, the procedure adopted was similar to that prescribed by Southgate. Starch was hydrolysed by means of a mixture of α-amylase and β-amylase. Takadiastase was not available from Parke Davis.

A 0.25 g subsample of food material (methanol extracted) was treated with 0.125 g of each enzyme in 5 ml water containing acetate buffer pH 4.6. The mixture was incubated at 37^{o} overnight.

The identification of simple sugars and uronic acid in acid hydrolysates was accomplished by thin layer chromatography using a cellulose absorbant and an elutant consisting of ethyl acetate (40 parts), pyridine (20 parts) and water (40 parts); ρ-anisidine phthalate (27) was used to develop the spots (hexose-green, pentose-red/violet; uronic acids - red/brown) (28) after heating the plates to 100^{o}.

2.3.5.3 Results

The proximate composition of dietary fibre (unavailable carbohydrates) of two cereal products was determined by Southgate's analytical procedure. The results are shown in Table 12.

TABLE 12. Proximate composition of dietary fibre of cereal products.

Cereal Product	Hemicellulose[1]			Cellulose[2]			Lignin	Total dietary fibre
	H	P	UA	H	P	UA		
	g/100 g dry matter							
Weetbix	2.8	4.2	0.3	2.4	0.2	0.2	3.7	13.8
Wholewheat bread	2.9	3.4	0.3	2.1	0.2	0.1	2.0	11.0

[1] Hemicellulose comprises H (hexoses), P (pentoses) and UA (uronic acids)

[2] Cellulose comprises H (hexose-glucose); other components comprise P (pentoses) and UA (uronic acids).

An examination of the thin layer chromatograms prepared from acid hydrolysates of the hemicellulose fractions of the two cereal products showed the presence of glucose, xylose and arabinose.

In the case of Weetbix, xylose and arabinose were present in the ratio of 5:4; total pentose content was estimated at 2.8% (dry matter basis). The colorimetric method for pentoses (orcinol/ferric chloride) indicated 4.2%.

In the case of wholewheat bread, glucose, xylose and arabinose were again the only sugars detected. The two pentoses appeared to be present in about equal concentrations, estimated at 2.2% (total). The value obtained by the colorimetric method was 3.4%.

The proximate composition of the dietary fibre of two cereal products expressed on a proportionate basis is shown in Table 13 together with

data quoted from Paul and Southgate (37) for comparative purposes:

TABLE 13. Proximate composition of dietary fibre.

Cereal Product	Hemicellulose	Cellulose	Lignin
	g/100 g dietary fibre		
Weetbix (NZ)	52.9	20.3	26.8
Weetabix (UK)	(72.2)	(18.5)	(9.3)
Wholewheat bread (NZ)	60	21.8	18.2
Brown bread (UK)	(71.0)	(26.0)	(3.0)[1]

Note: Figures in parentheses are taken from: McCance and Widdowson's "The composition of foods" by Paul and Southgate (37).

[1] 14.6 for wholemeal bread

2.3.5.4 Discussion

The measurement of dietary fibre in foods by Southgate's method was accepted as being precise and reliable. The components of unavailable carbohydrates consist of water soluble and water insoluble polysaccharides. These are hydrolysed with dilute H_2SO_4 to hexoses, pentoses and uronic acids which are determined colorimetrically. Lignin is determined as the residue insoluble in cold 72% w/w H_2SO_4. From these analyses dietary fibre is expressed in terms of cellulose, water insoluble and water soluble non-cellulosic polysaccharides and lignin; protein, mineral matter, cutin, etc., are excluded.

According to Southgate (54) the method of determining lignin is not entirely satisfactory and may give high results. He considers the best available alternative method is the one based on permanganate

oxidation devised by Van Soest and Wine (77).

The main disadvantage of Southgate's method is the length of time required to complete the determinations. If a large number of samples is to be analysed the analyst may of necessity be obliged to choose the shorter neutral detergent method. If the food samples contain much starch the proposed semi micro neutral detergent method may merit consideration.

2.4 Discussion and Conclusions

The neutral and acid detergent methods of Van Soest and associates provide a rapid and often reliable means of measuring and evaluating dietary fibre in animal forages and feeds, and also in some human foods, particularly those deficient in starch and lipid such as many fruits and vegetables. The proposed semi micro adaptation of Van Soest's methods is especially suited to cereal products and other foods rich in starch and protein. Amyloglucosidase of Aspergillus niger (Boehringer) has been shown to be a suitable starch degrading enzyme for the semi micro method but special care needs to be taken in using it. For example, cornflakes and rolled oats do not appear to be good substrates for the method; and the amount of enzyme used should be no more than is necessary to achieve hydrolysis of the starch present. Amyloglucosidase (Boehringer) quantitatively converts starch and dextrins to glucose; its effect on the hemicellulose fraction of dietary fibre is usually extremely small or nil.

Southgate (59) has discussed the effect that chemically modified starches may have when present in food samples treated with amyloglucosidase. It appears that the introduction of cross-links into starch affects the extent of enzymatic hydrolysis. Thus the

susceptibility of some modified starches to amyloglucosidase hydrolysis is much reduced.

If the above situation is encountered when using the semi micro method (NDF) it may be possible with care to detect the presence of incompletely degraded starch residues in the NDF residue by means of an iodine test (at the filtration stage). However, where cross linked starches (chemically modified) and amylopectin are concerned the iodine test is not helpful.

Southgate's schematic analytical procedure is the method of choice for the measurement of dietary fibre in all types of human food - if the necessary facilities and technical assistance are available. The proposed semi micro adaptations of Van Soest's methods are suitable for foods rich in starch and protein but are subject to the discretion of the analyst in regard to the use of amyloglucosidase.

Finally, a cheap form of amyloglucosidase derived from Rhizopus spp is supplied by Sigma Chemical Company. This enzyme preparation provides the subject of study in the following two chapters in relation to its behaviour in crude and purified form when used in the semi micro neutral detergent method of measuring dietary fibre.

CHAPTER 3

EFFECT OF FUNGAL SOURCE OF GLUCOAMYLASE ON DIETARY FIBRE MEASUREMENT

3.1 Introduction

An enzyme recommended by Southgate (58) for the measurement of starch in foods and for the removal of starch prior to the measurement of dietary fibre in foods is α-(1→4) amyloglucosidase from Aspergillus niger supplied by Boehringer or Sigma. This enzyme (syn. amyloglucosidase, glucoamylase, γ-amylase) from A. niger has been the subject of much investigation by Pazur and coworkers: Pazur and Ando (39,40); Pazur and Kleppe (42); also Lineback et al (29). The enzyme is an exo-glucosidase, splitting off single glucose units in sequence from the non-reducing ends of starch type polymers (1). It is reported to convert starch, amylose, amylopectin, amylodextrin and glycogen to glucose in yields approximating to complete conversion (39). Before a starch hydrolysing enzyme can be accepted for use in dietary fibre assay procedures it must be shown that the enzyme is without activity towards the components of dietary fibre, particularly the non-cellulosic polysaccharides of the hemicellulose fraction. Crude preparations of amyloglucosidase are not likely to satisfy this condition because of small amounts of impurities such as proteases, hemicellulases, cellulases and other carbohydrases. Commercially purified amyloglucosidase is expensive and therefore needs to be used frugally for routine analytical work.

Aspergillus niger is one fungal source of amyloglucosidase mentioned by Southgate as being suitable for dietary fibre analytical work but he clearly counsels caution by referring to its slight activity towards mannogalactans in guar gum (54). Amyloglucosidase from Rhizopus spp

has excellent starch hydrolysing properties but its suitability for dietary fibre analytical work does not appear to have been reported in the literature.

3.2. Objective and Experimental Plan

Although the subject of glucoamylase specificity is well covered in the literature especially in relation to glucosyl oligosaccharides (40) there is no mention of the activity of this group of enzymes towards the non-cellulosic polysaccharide components of dietary fibre.

The purpose of the present work was to devise a method of detecting and measuring the activity of glucoamylase of different fungal origins towards dietary fibre. Two methods of approach to this problem are possible: Firstly, the end products of enzymatic activity may be examined; secondly, the loss in weight of dietary fibre residue after enzyme action may be determined. Both methods were attempted:

1. The end products of (enzymatic) hydrolysis of non-cellulosic polysaccharides in dietary fibre comprise hexoses, pentoses and uronic acids, the proportion of pentoses being usually of the order of 70% (84). A convenient method of monitoring enzymatic degradation of non-cellulosic polysaccharides is therefore to determine the amount of pentoses present by the standard colorimetric method using orcinol/ ferric chloride reagent (58).

The acid hydrolysis products of the dietary fibre of wheat products include xylose and arabinose. Southgate (56) found that the non-cellulosic polysaccharide fraction of wholemeal flour contained 48% pentosans. However, the action of amyloglucosidase on starch causes glucose to be formed which in large excess interferes with the colorimetric measurement of pentoses. A sample of wholemeal flour

when treated with amyloglucosidase produces ca 13 times as much glucose as there is pentose present in the bound form of hemicellulose. Fortunately, amyloglucosidase hydrolyses the α-D(1→4) linkages of starch to produce β-D-glucose - an example of inversion of configuration by an exo-enzyme(82). β-D-glucose is quantitatively converted to gluconic acid by the action of glucose oxidase in the presence of catalase. Therefore by treating the amyloglucosidase hydrolysate of wholemeal flour with glucose oxidase and catalase at the appropriate pH the glucose source of interference with the colorimetric measurement of pentoses can be eliminated.

2. An alternative approach to the problem of measuring amyloglucosidase activity towards dietary fibre is simply to equate enzyme concentration with yield of NDF residue. Thus if the enzyme is inactive towards dietary fibre components the fibre residue values, as determined by the semi micro neutral detergent method, will be constant irrespective of changes in enzyme concentration. This conclusion is only valid, of course, if sufficient enzyme is present to hydrolyse all of the starch present.

3.3. Experimental

3.3.1 Measurement of products of hydrolysis

3.3.1.1 Materials

Amyloglucosidase, grade II, from Rhizopus spp: supplied by Sigma Chemical Co.

Glucose oxidase (EC 1.1.3.4) from Aspergillus niger: supplied by Sigma Chemical Co. (1385 units/ml)

Catalase: dry powdered form

Wheat starch; pure starch AnalaR

Sodium acetate buffer pH 4.6 and sodium phosphate buffer pH 5.5

Anthrone and orcinol/ferric chloride reagents:
prepared as described by Southgate (58)
Standard β-D-glucose: 500 µg/ml
Standard D-xylose: 5 µg/ml

3.3.1.2 Methods

Determination of D-xylose in presence of excess β-D-glucose.

Two sugar solutions were used:

Solution A: 4.6 µg D-xylose per ml

Solution B: 460 µg β-D glucose + 4.6 µg D-xylose per ml

Both solutions were buffered to pH 5.5 with phospate buffer. 25 ml of each solution (A and B) were transferred to 100 ml conical flasks. 50 µl glucose oxidase (70 units) and a trace of catalase were added to each flask. The flasks were placed in an incubator at 37^{o} and left for 18 hours with occasional shaking.

Glucose was determined colorimetrically in the filtrate from solution B by the anthrone method; absorbance was measured at 625 nm (58).

Xylose was determined colorimetrically in the filtrates from both solutions (A and B) by the orcinol/ferric chloride method; absorbance was measured at 665 nm (58).

Determination of pentoses in starch hydrolysates

Two starch solutions were prepared:

Solution W: 60 mg wheat starch in 50 ml water heated on a waterbath for a few minutes to complete gelatinization

Solution P: prepared as above but with 60 mg pure starch.

10 ml of each starch solution (i.e. 12 mg starch) were transferred to 15 ml centrifuge tubes. 0.5 ml 0.5M acetate buffer pH 4.6 and 0.2 ml

amyloglucosidase (Sigma) were added to each. The enzyme solution was prepared from 0.5 g powder (containing 46% insoluble carrier) in 10 ml water; the solution was centrifuged to obtain a clear supernatant. The tubes were swerled and incubated at 37° for 18h.

After cooling the tubes to 0° 4 volumes of 95% ethanol were added to the contents of each tube. After standing 3-4h the residues were separated by centrifugation; the supernatants were withdrawn and evaporated to small volume in a Rotovapor apparatus.

The two solutions (ca 10 ml each) were heated on a boiling waterbath for a few minutes (to complete inversion to β-D-glucose) and the pHs were readjusted to 5.5 with phosphate buffer.

The solutions were transferred to 50 ml flasks and each treated with 50 µl glucose oxidase and a trace of catalase (to facilitate removal of H_2O_2). The flasks were incubated at 37° for 18h with occasional agitation.

Each solution was diluted to 25 ml; glucose and pentose were determined colorimetrically as previously described.

3.3.1.3 Results

The determination of D-xylose in a solution containing considerable excess of β-D-glucose gave satisfactory results by the orcinol/ ferric chloride colorimetric method, after removal of the glucose by means of glucose oxidase. The results are shown in Table 14.

TABLE 14. Measurement of D-xylose in presence of excess β-D-glucose.

Solution identity	Composition of untreated solution		Composition after glucose oxidase treatment			
	glucose µg/ml	xylose	absorbance 620 nm	glucose µg/ml	absorbance 665 nm	xylose µg/ml
A	nil	4.6	-	nil	0.225	4.6
B	460	4.6	0.007	~ 3	0.240	5.0

Standards for absorbancy measurements:

Glucose: 24.4 µg/ml; Absorbance (620 nm) = 0.050

Xylose: 4.6 µg/ml; Absorbance (665 nm) = 0.225

The second stage of the tests with hydrolysates of starch solutions provided some disconcerting results, shown in Table 15, in that the pentose fraction of hydrolysed pure starch is much higher than expected.

TABLE 15. Glucose and pentose composition of starch hydrolysates (treated with glucose oxidase).

Identity of starch	Glucose		Pentose		as % of original starch
	Absorbance 620 nm	µg/ml	Absorbance 665 nm	µg/ml	
P (pure)	0.040	20	0.57	11.7	2.4
W (wheat)	0.035	17	0.59	12.3	2.6

Standards for absorbancy measurements:

Glucose 24.4 µg/ml; Absorbance (620 nm) = 0.050

Xylose 10 µg/ml; Absorbance (665 nm) = 0.453

As the initial concentration of the starch was 480 µg/ml (12 mg in 25 ml) it will be seen that the pentose found after hydrolysis of the starch with crude amyloglucosidase amounted to 2.4 and 2.6% of the original weights of pure starch and wheat starch, respectively.

Although a small residue of glucose was detected in each of the starch hydrolysates after glucose oxidase treatment this was too small to cause interference with the colorimetric measurement of pentoses.

3.3.1.4 Discussion

Schryver and Thomas (50) and Ling and Nanji (30) reported that cereal starches were found to contain up to 4% of insoluble floc which was identified as hemicellulose. The net yield of hemicellulose from wheat starch was given as 1.35%. These findings provide an explanation for the residue of 2.4-2.6% of pentoses found for pure and wheat starches, respectively, during the previous experiment.

The relatively large amount of pentose residue found in the starch hydrolysates makes it highly improbable that the method of measuring pentoses in food hydrolysates would have any practicable value in assessing the activity of amyloglucosidase towards hemicelluloses in dietary fibre determinations.

For example, if it is assumed for the sake of argument that 5% of the hemicellulose fraction of wheat dietary fibre is hydrolysed by amyloglucosidase, the hydrolysate would be expected to contain 0.5 mg of pentose from the hemicellulose contained in 0.2 g food sample. But according to the above findings the wheat starch itself would contribute 3.6 mg pentose. This latter quantity makes it virtually impossible to measure 0.5 mg additions with any degree of

certainty.

3.3.2. Method of equating enzyme concentration with neutral detergent fibre residue.

3.3.2.1 Materials

Amyloglucosidase, grade II, from Rhizopus spp. (Sigma)

Amyloglucosidase from A. niger (Boehringer)

Clarase 900: Supplied by Miles Laboratories

Weetbix: ground to pass 18-25 mesh screen

Wholemeal flour: extracted with 85% v/v methanol as prescribed by Southgate (58); ground to a fine powder.

3.3.2.2 Methods

The semi micro neutral detergent method described in Chapter 2 was used for all NDF determinations.

3.3.2.3 Results

The NDF residue weights from 3 treatments of Weetbix samples using a different starch hydrolysing enzyme for each treatment were obtained by the semi micro procedure. The results are given in Table 16.

TABLE 16. Effect of different amylolytic enzymes on the determination of neutral detergent fibre in Weetbix

Enzyme	Wt of enzyme per determination	NDF residue weight[1] (mean values)
	mg	g
Amyloglucosidase from *Rhizopus spp.* (Sigma)[2]	100	0.0120
Amyloglucosidase from *A. niger* (Boehringer)	5	0.0214
Clarase 900	4	0.0192

[1] derived from 0.2 g substrate

[2] contained 80% by weight of insoluble carrier

A comparison of Weetbix NDF residue data corresponding to treatments with crude amyloglucosidase (Sigma) and purified amyloglucosidase (Boehringer) (Table 16) shows that the crude enzyme preparation severely attacked the fibre component. Additional supporting evidence to this effect is furnished by the data provided in Table 17 which relates crude enzyme concentration to Weetbix NDF residue weights:

TABLE 17. Effect of crude amyloglucosidase from Rhizopus spp (Sigma) on Weetbix NDF assays

Wt of crude enzyme preparation per determination	NDF residue wt (from 0.2 g Weetbix) (mean values)[2]	SD of procedure 0.0004 g
mg	g	
5[1]	0.0169	
10	0.0156	
25	0.0147	
50	0.0134	
80	0.0131	
120	0.0122	

1 5 mg dry solids has approximately the same activity as 1 mg amyloglucosidase (Boehringer)

2 The corresponding NDF (Boehringer) value for residue weight was 0.0246g.

With reference to the results given in Table 17 even the low enzyme addition of 5 mg (46% insoluble carrier in a fresh sample of reagent) caused an appreciable loss of fibre component. At 120 mg addition rate the loss of fibre component amounted to ca 50%.

The data given in Table 18 relates the effect of increasing concentrations of purified amyloglucosidase (Boehringer) on NDF assays with wholemeal flour as a substrate. The results show a slight fall in NDF value (6%) over a wide range of increasing enzyme concentration. The difference in results caused by the change in time/temperature conditions is scarcely significant. The results suggest that 2 mg enzyme (Boehringer) adequately removes interfering starch but over

this level no significant change in NDF occurs which is in very real contrast with the data for the Sigma enzyme (Table 17).

TABLE 18. Effect of amyloglucosidase from A. niger (Boehringer) on NDF assays with wholemeal flour

Wt of enzyme[1] per determination	Time/temp conditions for enzyme treatment	NDF residue wt from 0.2g substrate (meal values)	SD of procedure
mg		g	g
1.0	18h/37°	0.0244	± 0.0006
1.4	18h/37°	0.0237	
1.75	18h/37°	0.0227	
2.5	18h/37°	0.0230	
3.75	18h/37°	0.0226	
5.0	18h/37°	0.0230	
0.75	3h/55°	0.0240	
1.75	3h/55°	0.0236	
3.5	3h/55°	0.0229	

[1] Maker's declaration: 10 ml suspension contains 100 mg enzyme

Figure 1 shows curves which illustrate the effect on residue weights of increasing the enzyme concentration for crude amyloglucosidase from Rhizopus spp and purified amyloglucosidase from A. niger.

3.3.2.4 Discussion

The pattern of behaviour of the crude amyloglucosidase from Rhizopus spp on Weetbix NDF residue recovery is what would be expected if the cause of the enzymatic attack on the fibre were due to enzyme impurities such as hemicellulase. This aspect of the present studies on amyloglucosidase from Rhizopus spp is examined in Chapter 4.

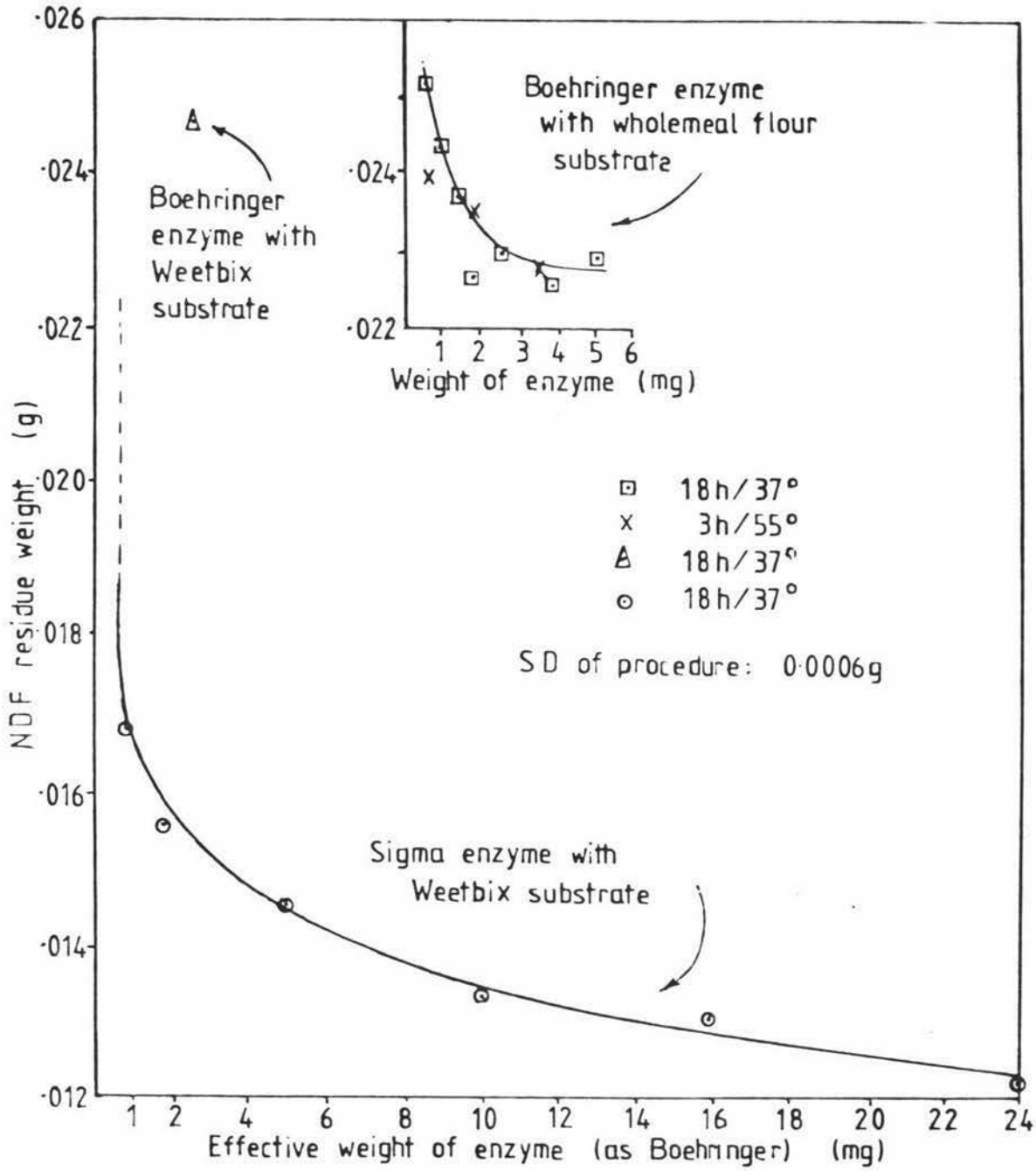

FIG. 1 EFFECT OF CRUDE AMYLOGLUCOSIDASE FROM *RHIZOPUS ssp* (SIGMA) AND OF PURIFIED AMYLOGLUCOSIDASE FROM *ASPERGILLUS niger* (BOEHRINGER) ON NDF ASSAYS OF CEREAL PRODUCTS

The results shown in Table 19 relate the effect of increasing concentrations of purified amyloglucosidase from A. niger (Boehringer) on NDF assays with various cereal products as substrates. With most of the cereal products it is evident that increased enzyme concentration does result in slightly lower NDF values.

A close examination of Figure 1 indicates that no advantage is to be gained by taking more than 2 mg of this enzyme for each NDF determination using 0.2 g substrate. On the other hand, care must be taken if less than 2 mg of enzyme is taken because of the risk with some cereal products of incomplete hydrolysis of the starch component. In the latter event the starch contamination of the NDF residue would give results which are too high. Having regard to all the circumstances of the situation the 2 mg level of enzyme per determination appears to offer the best compromise solution.

TABLE 19. Effect of amyloglucosidase from A. niger (Boehringer) on NDF assays with various cereal products[1]

Cereal product	Wt of enzyme per determination	NDF residue wt from 0.2g substrate (mean values)	SD of procedure
	mg	g	g
Weetbix	2.5	0.0247	± 0.0004
	5.0	0.0235	
Cornflakes	2.0	0.0112) 2	± 0.0008
	4.0	0.0105)	
Ryemeal flour	2.0	0.0275	
	4.0	0.0266	
Rice flour	2.0	0.0019	
	4.0	0.0017	
All-Bran	2.0	0.0402	
	4.0	0.0399	
Puffed Wheat	2.0	0.0130	
	4.0	0.0133	
White flour (bulk)	2.0	0.0039	
	4.0	0.0037	
Special 'K'	2.0	0.0155	
	4.0	0.0155	
Premium bread (Q.B.)	2.0	0.0264	
	4.0	0.0257	

1 related to the results in Table 6

2 traces of starch present

CHAPTER 4

PURIFICATION AND EVALUATION OF GLUCOAMYLASE FROM RHIZOPUS SPP.

4.1 Introduction

Glucoamylase, α-D-(1→4) glucan glucohydrolase (EC 3.2.1.3), plays an important role in the evaluation of cereal products by the proposed semi micro neutral detergent procedure described in Chapter 2. This enzyme is derived from a variety of fungal organisms including species of Aspergillus, Rhizopus and Endomyces. The glucoamylases from A. niger and R. delemar have been the subject of intensive studies by Pazur and co-workers (39,40,42,44); also by Lineback et al (29).

Southgate (50; Appendix 2) has reported that amyloglucosidase from A. niger supplied by Boehringer or Sigma is satisfactory for the purpose of estimating starch or of hydrolysing starch in the first stage of his method of determining unavailable carbohydrates in foods.

Because of the high cost of supplies of purified preparations of amyloglucosidase from A. niger from commercial sources the present studies have been directed towards the search for a cheap source of amyloglucosidase starting material which could be purified in the laboratory. The Sigma Chemical Co. supply amyloglucosidase from Rhizopus spp at low cost and this material was used in the first experiments. Pazur and Kleppe (42) and Lineback et al (29) used Diazyme, a product of the Takamine Laboratory, Miles Laboratories Inc., New Jersey, U.S.A., as their starting material for amyloglucosidase from A. niger. This material is not available in New Zealand at the present time.

The purification procedures used to prepare pure samples of fungal amyloglucosidases is well documented in the literature a review of which is given below:

4.2 Review of Literature on Ion Exchange Chromatography of Fungal Glucoamylases and their Properties.

(a) Glucoamylase from A. niger

Pazur and Ando (39) isolated two isoglucoamylases from Aspergillus niger from a dialysed extract of diazyme by means of column chromatography using DEAE cellulose and citrate-phosphate buffers. The starting buffer was 0.05M citrate-phosphate at pH 8.0 and separation of glucoamylases and other constituents was accomplished by a step-wise elution procedure using 0.05M citrate phosphate buffers firstly at pH 6.0 and then at pH 4.0. Pazur and Ando (41) identified the carbohydrases in 4 main fractions of the eluate: transglucosylase was found in the fraction at pH 8.0, an amylase in the fraction at pH 7.0 and two glucoamylases in fractions at pH 6.0 and pH 4.8.

Pazur and Kleppe (42) obtained electrophoretic and ultra centrifugal patterns from the purified amyloglucosidase preparations which indicated a high degree of homogeneity. The molecular weight was given as 97,000 $\pm$ 5%. These workers showed that the amyloglucosidase was capable of hydrolysing the α-D-(1$\rightarrow$4), α-D-(1$\rightarrow$6) and α-D-(1$\rightarrow$3) glucosidic linkages in oligosaccharides and considered it logical to expect these bonds in branched polysaccharides of the starch type to be hydrolysed by the enzyme also. It was further demonstrated that the amyloglucosidase acted as a typical α-D-glucosidase in that a single glucosyl unit was liberated from the non-reducing end of the substrate.

Lineback et al (29) repeated the chromatographic purification procedure described by Pazur and Ando, with slight modifications. The starting buffer strength was reduced to 0.025M (citrate-phosphate at pH 8.0). Elution of the enzyme was accomplished by both a step wise procedure and by using a linear gradient of decreasing pH. The first protein peak contained glucosyl transferase and α-D-glucosidase. The second and third peaks contained glucoamylase II and glucoamylase I, respectively. The purified glucoamylases were found to possess a high degree of purity as indicated by paper electrophoresis, sedimentation velocity and disc-gel electrophoresis. Among the properties investigated the two enzymes differed only in electrophoretic mobility, iso-electric point and to a slight extent, temperature stability. The purification procedure provided approximately a 2.5-fold increase in the activity of both glucoamylases.

Fleming and Stone (15) used culture filtrates of A. niger as starting material for the purification of amyloglucosidase. After lead acetate precipitation and propan-2-ol fractionation the filtered extracts were submitted to DEAE cellulose chromatography in a linear gradient of NaCl in 5mM tris-HCl buffer, pH 7.5. Several α-glucosidase components were eluted between 0.05 and 0.12M chloride, and two amyloglucosidase components between 0.15 and 0.18M chloride, and between 0.21 and 0.29M chloride. The last component contained 90% of the total amyloglucosidase activity of the preparation.

The monosaccharides present in amyloglucosidase from A. niger were found after acid hydrolysis to be mannose, galactose and glucose, confirming the findings of Pazur et al (43).

(b) Glucoamylases of Rhizopus delemar

Pazur and Okada (44) described the preparation of a highly purified

single glucoamylase from R. delemar by successive filtration and chromatography on Duolite A-2 resin (supplied by Diamond Alkali Co., Redwood City, Cal.) and Amberlite XE-64 resin (a product of Rohm and Haas, Phil.) These workers reported that the electrophoretic mobility of the R. delemar enzyme was markedly different from that of the major isoenzyme of the glucoamylase from A. niger.

The starting material, a glucoamylase preparation from Rhizopus delemar, was obtained from Miles Chemical Co., Elkhart, Indiana. The optical density of a filtered solution prepared from 5 g of starting material in 50 ml water and diluted 250-fold was 0.227 at 280 nm. α-amylase and glucanosyl transferase were found present in the crude enzyme preparation.

Pazur and Okado found that the glucoamylase from R. delemar, like that from A. niger, contained 13% carbohydrate. However, whereas the enzyme from A. niger contained D-mannose, D-glucose and D-galactose, the enzyme from R. delemar contained primarily D-mannose and 2-amino-2-deoxy-D-glucose. Migration rates on paper chromatograms indicated that the carbohydrate residues of acid hydrolysates of the enzymes were attached as polymeric segments to the polypeptide chain of the enzymes. It was estimated that the hexosamine comprised about 25% of the total carbohydrate in R. delemar glucoamylase.

Pazur (38) claims that the function of the carbohydrate residues appears to be as stabilizers of the tri-dimensional structure of the molecule.

Glucoamylase activity was determined quantitatively by an arseno-molybdate reducing method using 0.6% starch solution buffered to pH 5.2 with 0.02M acetate buffer at 30^{o}.

In summarizing their findings Pazur and Okada claimed that the action pattern of the glucoamylase from R. delemar on starch and malto-oligosaccharides was similar to that for the glucoamylase from A. niger. The two enzymes are stated to be of the same molecular size but to differ markedly in their electrophoretic properties.

(c) Substrate specificity of the glucoamylases

The substrate specificity of the glucoamylases was studied by Abdullah et al (1) who concluded that: 1. The rate of hydrolysis of non-reducing α-(1 → 4) bonds increases with the molecular weight of the substrate. 2. The rate of hydrolysis of α-(1 → 6) bonds increases in the series isomaltose to isomaltopentaose but there is a marked difference as to whether the next bond in sequence is (1 → 4) or (1 → 6). 3. The rate of hydrolysis of α-(1 → 3) bonds is low and increases only slightly as the size of the molecule increases. 4. (1 → 6) branch linkages of amylopectin are likely to be hydrolysed rapidly by the enzyme.

At the 4th Ciba Medal Lecture Whelan (81) explained that a non-crystalline preparation of amyloglucosidase from A. niger (found later to contain an endoenzyme, α-amylase) caused quantitative conversion of glycogens and amylopectins into starch, yet when a crystalline amyloglucosidase from R. niveus (which proved to be free of α-amylase) was used for the same purpose, conversions in many cases were far from complete - but became so on addition of α-amylase. A table providing data in relation to these findings is reproduced below (Table 20).

Whelan explained that an α-(1 → 6) bond is rapidly hydrolysed by

amyloglucosidase when the next bond in sequence is α-(1 → 4). However, when two α-(1 → 6) bonds occur in sequence the result is very different: The first α-(1 → 6) bond to be encountered by the enzyme may be hydrolysed only very slowly, accounting for incomplete conversion of the polysaccharide. A small proportion of such structural features could block the further hydrolysis of large sections of a branched macromolecule.

TABLE 20. Incomplete hydrolysis of starch fractions and glycogens by Rhizopus niveus amyloglucosidase

Substrate	Glucose released by enzymic hydrolysis (expressed as % of glucose released by acid)		
	Rhizopus niveus amyloglucosidase	Rhizopus niveus amyloglucosidase + α-amylase	Aspergillus niger amyloglucosidase
Potato amylose	90.1	101.0	97.0
Waxy-maize starch	97.6	103.0	100.0
Floridean starch	77.8	97.5	90.8
Cat liver glycogen	82.2	98.8	93.1
Human muscle glycogen	87.8	99.4	96.3
Skate liver glycogen	89.3	99.3	95.3

Taken from: Whelan, W.J. (ref. 81, p.616).

4.3 Objective and Experimental Plan

The effect of crude amyloglucosidase from Rhizopus spp on cereal fibre when used in the semi micro procedure for measuring neutral detergent fibre has been discussed in Chapter 3. The aim of the

present work was to determine the effect of a purified form of this enzyme on neutral detergent fibre measurements in order to evaluate the Rhizopus source of amyloglucosidase. If it could be established that the purified enzyme did not attack cereal fibre the Rhizopus source might then be considered as a possible cheap alternative to the Aspergillus niger source normally obtained from chemical suppliers.

The conditions relating to buffer ionic strength and pH reported by Pazur and Ando (39) and Lineback et al (29) to effect strong adsorption of amyloglucosidase from A. niger onto DEAE cellulose were not effective with the amyloglucosidase from R. spp. Modified separation procedures therefore had to be developed. Two major elution procedures were investigated as follows:

The crude preparation of amyloglucosidase from Rhizopus spp (Sigma) was purified by modified versions of the ion exchange chromatographic procedures, firstly, of Pazur and Ando (39) and Lineback et al (29), and secondly, of Fleming and Stone (15). Fractions of eluate containing purified amyloglucosidase were tested by the semi micro neutral detergent method using wholemeal flour and Weetbix as substrates.

The activity of fractions containing the purified enzyme was estimated by visual means using wheat starch solution as a substrate.

4.4 Experimental

4.4.1 Ion exchange chromatography of glucoamylase using DEAE cellulose, citrate-phosphate buffers and pH gradient elution.

4.4.1.1 Materials

Amyloglucosidase, grade II from Rhizopus spp: Sigma Chemical Co.

Amyloglucosidase from A. niger: Boehringer, Mannheim.

DEAE cellulose, medium mesh, 0.86 meq/g: Sigma Chemical Co.

Centrifuge tubes: 110 x 16 mm; 15 ml cap.

Citrate-phosphate buffers

1. 0.025M, pH 8.35: 30.2 ml 0.4M Na_2HPO_4 + 0.1 ml 1M citric acid in 250 ml water (previously boiled to expel CO_2)
2. 0.0025M, pH 8.35: 24.2 ml 0.4M Na_2HPO_4 + 0.1 ml 1M citric acid in 2 litres water (boiled)
3. 0.0035M, pH 8.35: 8.47 ml 0.4M Na_2HPO_4 + 0.035 ml 1M citric acid in 500 ml water (boiled)
4. 0.005M, pH 6.0: 7.85 ml 0.4M Na_2HPO_4 + 0.99 ml 0.1M citric acid in 500 ml water (boiled)
5. 0.005M, pH 4.0: 4.8 ml 0.4M Na_2HPO_4 + 1.64 ml 0.1M citric acid in 500 ml water (boiled)

All reagents were AnalaR grade.

4.4.1.2 Methods

Initial tests to determine optimum conditions for column chromatography of amyloglucosidase from Rhizopus spp.

DEAE cellulose was prepared for column chromatography by the method of Lineback et al (29) as far as and including precycling and removal of fines. A 10 ml portion of slurry containing ca 0.5 g dry weight of DEAE cellulose was equilibrated with one of a series of buffers

to be tested, each with a different molar strength and pH, in the range 0.05M-0.0025M, pH 8.0-8.35. 2 ml 0.25% amyloglucosidase (Sigma) in an appropriate buffer was added and well stirred. After standing for 10 minutes the slurry was filtered. 0.1 ml filtrate was added to 5 ml 1% wheat starch (freshly prepared) buffered to pH 4.6 by addition of 0.5 ml 0.5M acetate buffer. A 15 ml cap. centrifuge tube was used as a container. The contents were well mixed and the tube was placed in a waterbath at 37^{o}. The time taken for the solution to clear was noted and also when flocculation occurred.

At the same time as the test run a second tube was prepared as a control. 2 ml 0.25% enzyme solution was added to 10 ml similar buffer and mixed. 0.1 ml of the diluted enzyme was added to 5 ml buffered 1% wheat starch and transferred to the waterbath at 37^{o}. Times of clearing and flocculation were noted.

The tests were repeated with other buffer solutions of the series until maximum absorption of the enzyme by the adsorbant was observed - corresponding to the longest time of clearing of the starch solution.

Dialysis of the enzyme solution before chromatography was found to be unnecessary.

Gel filtration of the enzyme solution before ion exchange chromatography was also tested but found not to improve the subsequent chromatographic separation process. The investigation was therefore discontinued. Further information relating to the results of gel filtration tests with crude amyloglucosidase (Sigma) are recorded in Appendix 3.

Preparation of the column

DEAE cellulose (15 g) was prepared for chromatography as described by Lineback et al (29) taking particular care to remove fines. The ion exchange material was washed, filtered and suspended in 0.025M citrate-phosphate buffer, pH 8.35. Addition of 0.5 ml 1M citric acid was required to restore the pH to 8.35.

The equilibrated ion exchanger was de-aerated (filter pump), poured into a glass column 360 x 25 mm and finally equilibrated to pH 8.35 by washing the bed with 3 litres 0.0025M citrate-phosphate buffer, pH 8.35 (conveniently performed overnight).

Column chromatography of crude amyloglucosidase from Rhizopus spp

A clear solution of amyloglucosidase, grade II (Sigma) was prepared by mixing 0.5 g powder in 10 ml starting buffer followed by centrifugation and collection of the supernatant. The enzyme was allowed to drain into the top portion of the bed and was followed by 250 ml starting buffer (0.0025M, pH 8.35); 500 ml 0.0035M, pH 8.35 buffer; 500 ml 0.005M, pH 6.0 buffer; and 500 ml 0.005M, pH 4.0 buffer. The rate of flow was maintained at 50 ml/h. Fractions of the eluate (each 10-12 ml) were collected automatically by means of a fraction collector. The fractionation was performed at room temperature (ca 18^{o}).

Measurement of protein component of fractions

Optical density measurements were made at 280 nm using a 1 cm cuvette and an Hitachi spectrophotometer model 101. Fractions were diluted if necessary to avoid absorbance readings in excess of 1.0. In peak areas the absorbance of fractions was also measured at 260 nm.

Preliminary measurement of enzyme activity of fractions

Standards: 5 ml 1% wheat starch solution (boiled 2 min.) + 0.5 ml acetate buffer pH 4.6 were placed in each of 4 centrifuge tubes 110 x 16 mm (round ends) and treated with 10, 20, 40 and 100 µg quantities of amyloglucosidase from A. niger (Boehringer). 0.2 ml suspension of the Boehringer preparation was diluted to 10 ml with distilled water and 0.05 to 0.5 ml aliquots were taken to provide the above amounts of enzyme (10-100 µg).

The tubes were immediately placed in a waterbath at 37° and kept under observation for 20 hours. Without disturbing the flocculated residues the heights of floc from the base of each tube were measured after various intervals of time as shown in Table 21.

TABLE 21. Enzyme activity standards: Wheat starch (1%) and amyloglucosidase (Boehringer)

Enzyme added	Floc height in tubes (110 x 16 mm) after		
µg	1.8h	3.2h	20h
	cm		
10	2.1	-	1.1
20	1.95	1.55	1.1
40	1.8	1.5	1.0
100	1.3	1.25	0.5

Fractions: A set of tubes containing freshly prepared 1% wheat starch buffered to pH 4.6, as described above for the standards, was treated with 0.1-0.2 ml aliquots of the fractions to be tested (fractions showing high absorbance readings at 280 nm). The floc heights were measured at suitable intervals after placing the tubes

in a waterbath at 37°.

A comparison of the test results with those of the standards provided an indication of amylolytic activity of fractions in terms of amyloglucosidase (Boehringer) activity.

Concentration of purified enzyme by ethanol precipitation

Selected fractions shown to possess high amylolytic activity were combined and cooled to 0°. By adding 4 volumes of cold 95% ethanol and allowing the solution to stand for a period in the refrigerator the protein component settled out as a flocculent precipitate. The protein residue was separated by a combination of decantation and centrifugation methods and finally dissolved in 1-2 ml 0.05M citrate phosphate buffer pH 8.0. The concentrated enzyme solution was reserved for tests performed with wholemeal flour as the substrate and using the semi micro neutral detergent method of measuring fibre, as described below:

Evaluation of purified preparations of amyloglucosidase

1. Measurement of amylolytic activity

Standards:

(i) 0.200 g wholemeal flour (methanol extracted) was weighed into each of a series of tubes (110 x 16 mm) followed by 5 ml water and the starch gelatinization treatment previously described. The pH was adjusted by addition of 0.5 ml acetate buffer, pH 4.6, to each tube. Amyloglucosidase (Boehringer) was then added to each tube at increasing rates, in the range 0.25-2.5 mg. The determination of NDF was continued in the normal way as previously described, except that the residue heights in the tubes were measured at suitable intervals during the 18 hour incubation period at 37°. The results of these measurements are shown in Table 22.

TABLE 22. Enzyme activity standards: Wholemeal flour (0.2 g) and amyloglucosidase (Boehringer)

Enzyme added mg	Time Elapsed	Residue height in tubes (110 x 16 mm) after (h): 0.75	1	1.5	1.75	2	2.25	2.75	3.2	4.5	7	24h
		cm										
0.25		2.75		2.35				2.2		2.0	1.8	1.4
0.6		2.4		1					1.85	1.65	1.55	1.1
1.4		2.3				1.75		1.5	1.45	1.3	1.2	1.1
2.5			2.2		1.7	1.6	1.55		1.4	1.3	1.2	1.1

(ii) Instead of 0.2 g wholemeal flour, 0.080 g was taken for the second set of standards. The water was reduced to 2 ml per tube and the buffer addition to 0.2 ml. The addition of amyloglucosidase (Boehringer) was reduced proportionately. Otherwise, the treatment and measurements were similar to the first set of standards. The results are shown in Table 23.

TABLE 23. Enzyme activity standards: Wholemeal flour (0.08 g) and amyloglucosidase (Boehringer)

Enzyme added[1] mg	Time Elapsed	Residue height in tubes (110 x 16 mm) after (h): 1	1.5	2	3	4	4.5	6.5	24h
		cm							
0.25		1.3		1.15	1.0		0.9	0.8	0.65
0.5			1.1	1.0	0.9	0.75			0.6
1.0			1.1	0.95	0.8		0.75	0.7	0.55
2.0		0.95		0.75	0.7		0.7	0.7	0.55
5.0		0.7		0.65	0.6		0.6	0.6	0.55

[1] For ease of comparison the enzyme addition is given at the 0.2 g wholemeal flour rate.

Combined fractions: In order to obtain an approximate indication of the amylolytic activity of combined fractions of purified amyloglucosidase from Rhizopus spp the semi micro neutral detergent fibre procedure was performed as described above for the standards, except that a suitable aliquot of the concentrated enzyme solution was used in place of amyloglucosidase (Boehringer). For relatively strong solutions of enzyme the 0.2 g level of wholemeal flour was chosen; for weaker solutions the 0.08 g level was chosen.

By comparing the residue heights obtained after known intervals of time with those found for measured amounts of amyloglucosidase (Boehringer) the strength of the enzyme concentrates was roughly estimated.

2. Measurement of enzymatic activity towards dietary fibre

The semi micro neutral detergent fibre assays commenced above at the 0.2 and 0.08 g levels of wholemeal flour with concentrates of amyloglucosidase from Rhizopus spp were continued to completion in order to monitor the effects of the enzyme on dietary fibre components of the substrate. The residues were tested for the presence of unhydrolysed starch by placing a small drop (5 µl) of 0.05N iodine in potassium iodide solution onto the dried residue and examining the stain under a binocular microscope. Residues which produced a blue stain were discounted.

4.4.1.3 Results

Elution diagrams

The first part of the development pattern (pre-elution) shown in Fig. 2 was found to contain a large proportion of protein impurities derived from the crude amyloglucosidase from Rhizopus spp. Absorbance measurements at 280 nm showed that fractions 14-19 contained much of

the protein originally present in the enzyme sample. However, the amylolytic activity of the peak fractions, as determined by the wheat starch method (Tables 21 and 24) indicated that only about 6% of the protein present had amyloglucosidase activity. The pre-step elution stage of the chromatography therefore appeared to bring about a useful degree of purification.

TABLE 24. Measurement of amylolytic activity of fractions using 1% wheat starch solution

Fraction number (0.2 ml taken)	Floc heights (110 x 16 mm tubes) after			
	0.4h	0.8h	1.4h	18h
	cm			
14	-	-	-	1.4
16	3.0	1.9	1.4	0.6
18	-	-	2.9	1.4
20	-	-	-	2.3
22	-	-	-	2.6
Control (no addition)	-	-	-	2.9

The elution pattern shown in Fig. 3 contained 4 protein peaks all of which were found to exhibit strong amylolytic activity (Table 25). The estimated equivalent amyloglucosidase (Boehringer) content of peak fractions (10 ml) is given in Table 26.

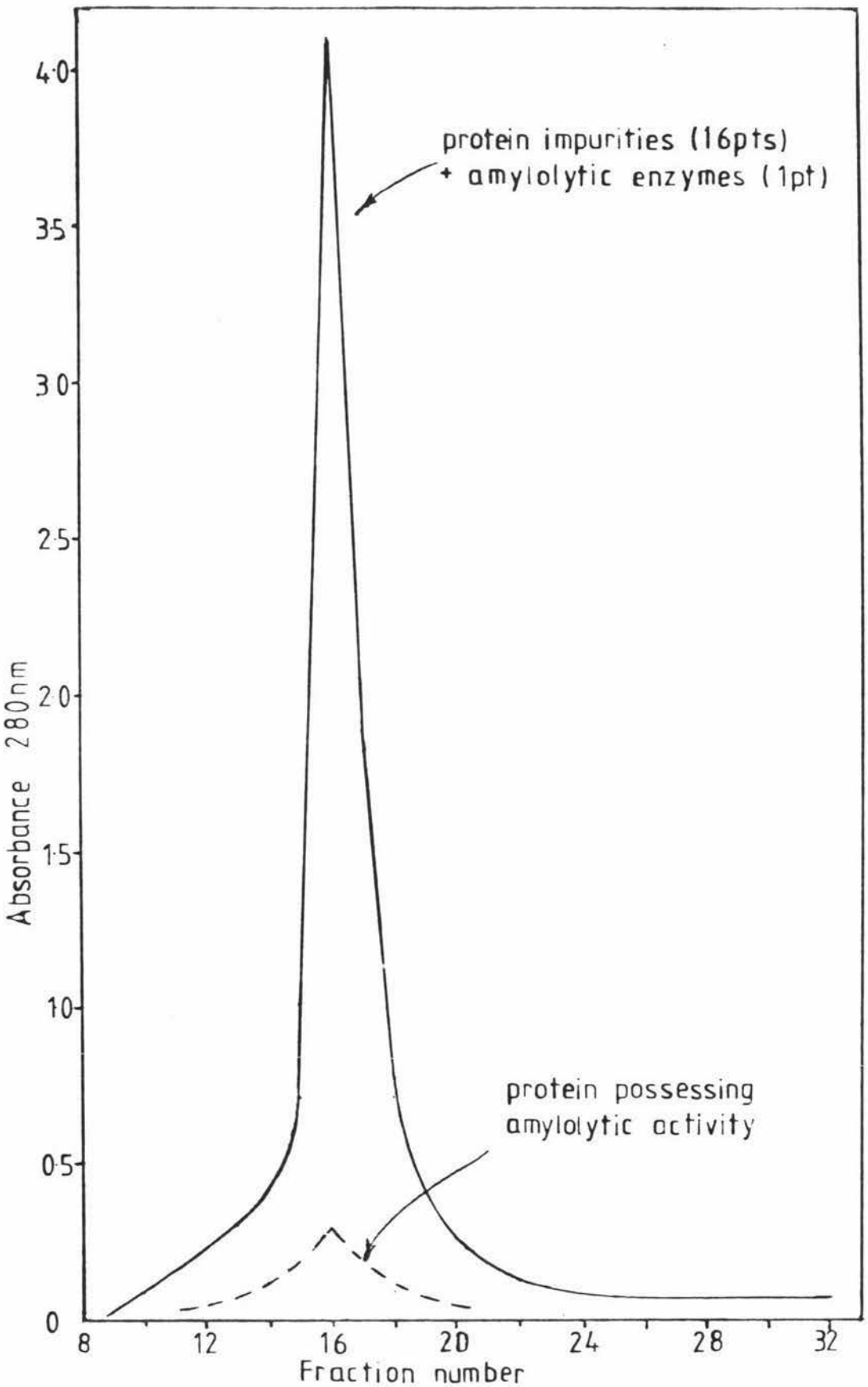

FIG. 2 DEVELOPMENT PROFILE OF CRUDE AMYLOGLUCOSIDASE (SIGMA) ON DEAE CELLULOSE

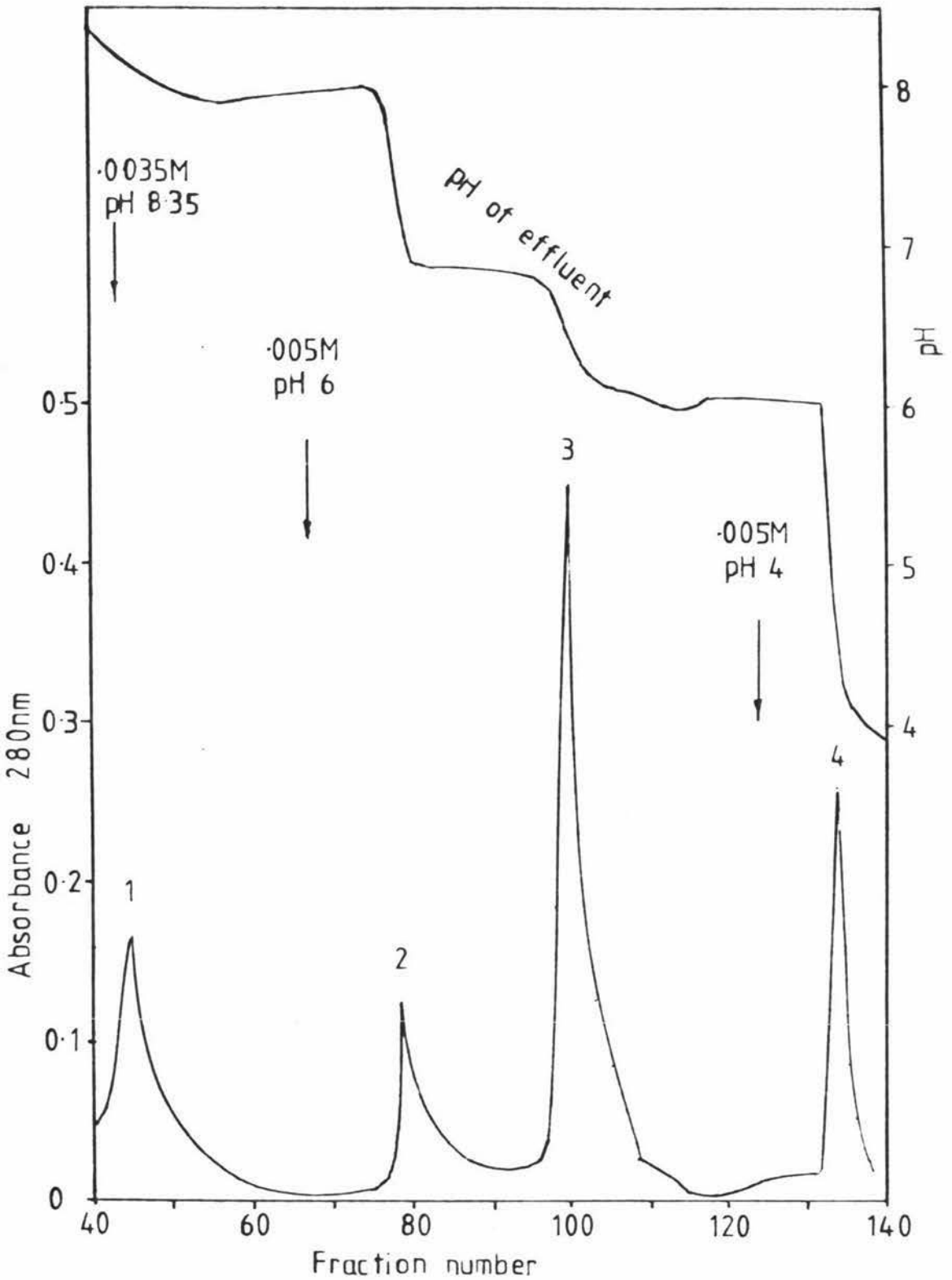

FIG. 3 ELUTION PROFILE OF CRUDE AMYLOGLUCOSIDASE (SIGMA) ON DEAE CELLULOSE

TABLE 25. Measurement of amylolytic activity of fractions using 1% wheat starch

Fraction number (0.2 ml taken)	Floc heights (110 x 16 mm tubes) after (h)								
	0.25	0.5	0.6	0.9	1.3	2.0	3.2	6	24h
	cm								
45	2.8	2.1	-	1.8	-	-	1.5	-	1.3
79	-	-	-	2.6	-	-	1.6	-	1.45
98	-	-	-	-	-	-	-	-	1.6
100 (0.1 ml)	-	3.5	-	2.4	-	-	1.6	-	1.35
103	-	-	-	-	-	-	-	-	1.6
134	3.0	-	1.75	-	1.4	1.3	-	1.2	-

TABLE 26. Estimated amyloglucosidase content of peak fractions (10 ml)

Peak number (Fig. 3)	Fraction number	Amyloglucosidase (Boehringer) equivalent (est.) (from standards - Table 21)
		mg
1	45	2
2	79	1.5
3	100	4
4	134	5

The 3rd and 4th peaks shown in Fig. 3 have the characteristic shape of those obtained for highly purified amyloglucosidase as exemplified by the patterns shown by Lineback et al (29), reproduced in Fig 4:

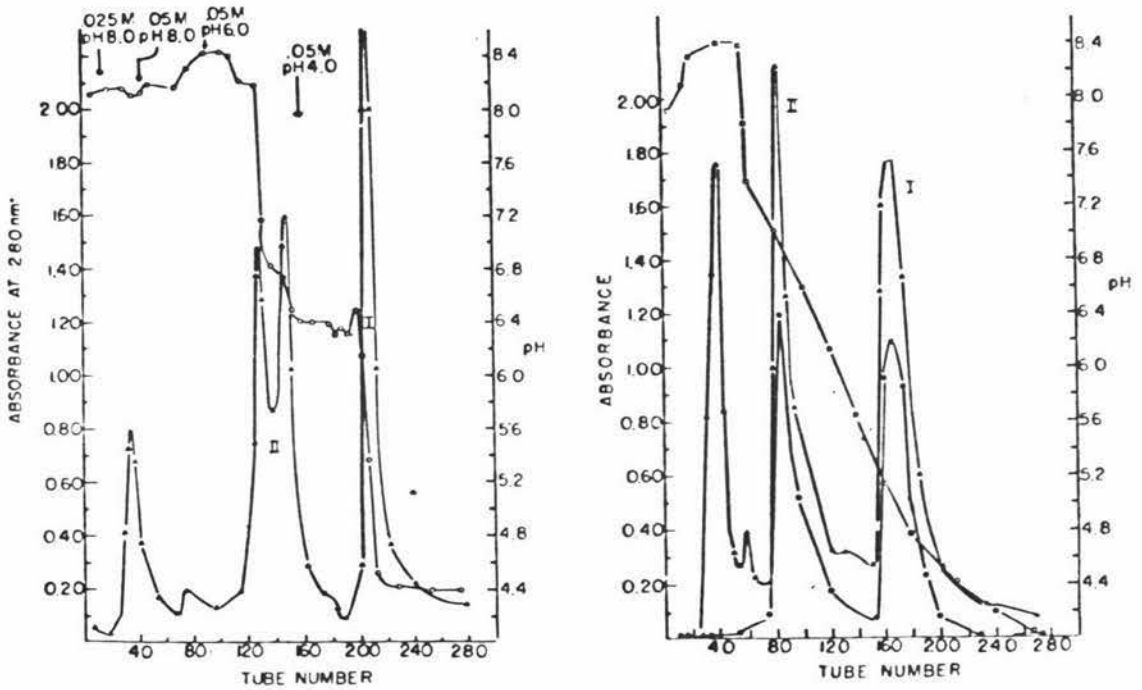

FIG. 4 CHROMATOGRAPHY OF CRUDE ENZYME (DIAZYME) ON DEAE CELLULOSE

Taken from : Lineback et al, ref 29

The first peak (Fig. 3) of the elution pattern was found to contain two enzyme components, one of which appeared to possess the characteristics of α-amylase, and the other of amyloglucosidase.

α-amylase tests: Fractions 43-46 were combined and treated with 4 volumes cold 95% ethanol as described under Methods. After standing in a refrigerator at 0^{o} for 24h large needle shaped crystals were found attached to the wall of the glass cylinder (containing the solution) and mixed with flocculated protein deposit. Most of the crystalline material was separated from the amorphous protein matter by a process of decantation and centrifugation.

To test for amylolytic activity a few crystals, previously washed with 80% V/V ethanol were added to a tube containing 2 ml 1% starch solution buffered to pH 4.6. The latter was maintained at 37^{o} for 24h and then divided into two portions: One portion was analysed directly for reducing sugars by means of Fehlings solution. The second was treated with glucose oxidase and catalase for a few hours at 37^{o} and subsequently analysed for reducing sugars.

It was found on analysis that 74% of the starch present in the first portion was converted to reducing sugar, expressed as maltose, and 70% in the second portion. As β-D-glucose was apparently not present in the hydrolysate it was concluded that the crystalline enzyme was α-amylase. This was supported by the observation that addition of the enzyme to strong starch solutions caused a rapid reduction in viscosity - a characteristic of α-amylase but not of amyloglucosidase (82).

Optical density measurements of fractions

The optical densities of fractions 98-102 and 133-135 were measured at 280 nm and 260 nm, the results of which are shown in Table 27.

TABLE 27. Ratio of absorbances at 280 and 260 nm of various fractions

Fraction number	Absorbance 280 nm	Absorbance 260 nm	Ratio A_{280}/A_{260}
98	0.189	0.155	1.22
99	0.342	0.280	1.22
100	0.448	0.380	1.18
101	0.230	0.190	1.21
102	0.163	0.134	1.22
133	0.198	0.140	1.41
134	0.260	0.200	1.30
135	0.084	0.065	1.29

The ratios of the absorbances measured at 280 nm and 260 nm given in Table 27 are practically constant for fractions 98-102 which correspond to the 3rd peak of the elution pattern. The near constant ratio strongly suggests that the protein component of each fraction is identical, a further indication of high purification achieved by column chromatography.

The relationship between protein component and absorbance values for two wavelengths is explained by Clark and Switzer (8): Tyrosine and tryptophane residues of proteins exhibit an U.V. absorbance of 275 and 280 nm, respectively. Because the combined levels of these amino acids are generally constant in many proteins the concentration of protein (in pure solution) is generally proportional to the absorbance at 280 nm. Pure proteins have a 280 nm/260 nm ratio of ca 1.75, variations being due to differences caused by number and type of aromatic amino acids present. Nucleic acids have a 280 nm/

260 nm ratio of 0.5.

Evaluation of purified preparations of amyloglucosidase

In order to carry out semi micro neutral detergent fibre determinations it is necessary to restrict the volume of starch hydrolysing enzyme to 0.5 ml or less per determination. Therefore in order to test the enzyme activity of fractions it was necessary to adopt a concentration technique using 95% ethanol as described under Methods. Fractions from the 1st, 3rd and 4th peaks were separately combined and concentrated to small volume. The concentrates were then used in place of amyloglucosidase (Boehringer) in order to carry out the determination of NDF in wholemeal flour (0.2 g or 0.08 g) by the semi micro method. By measuring the residue heights in the tubes as previously described (Methods) an estimate was obtained for the amount of enzyme added per determination, in terms of amyloglucosidase (Boehringer).

Standard curves were prepared for amyloglucosidase (Boehringer) shown in Figs. 5 and 6 using data supplied in Tables 22 and 23. Curves for various fractions are included in Figs. 5 and 6 to facilitate the estimation of enzyme strength.

The results of measuring wheat flour residue heights in the tubes corresponding to treatments with various aliquots of concentrated fractions containing purified amyloglucosidase from Rhizopus spp are shown in Table 28.

TABLE 28. Measurement of enzymatic activity of concentrated fractions using wholemeal flour

Fraction numbers	Vol. of concentrate per determination ml	Wt of whole meal flour g	Residue heights in tubes after (h) Time elapsed: 0.75	1.0	2.5	3.2	4.5	5	20h
			cm						
F.43-46	0.6	0.08			0.9			0.8	0.65
	0.9	0.08			0.75			0.7	0.65
F.98-104	0.3	0.2	3.35			2.1	1.8		1.25
	0.6	0.2	3.35			1.9	1.55		1.25
F.133-134	0.4	0.08		1.3					0.7

The results of NDF determinations on wholemeal flour using various fraction concentrates to hydrolyse the starch component are given in Table 29. Also included in the table are estimates for the amount of purified enzyme added to the substrate (calculated at the 0.2 g wholemeal flour rate) and the calculated loss of hemicellulose component resulting from enzymatic attack on the cereal dietary fibre.

It will be seen that the concentrates prepared from fractions 43-46 and 133-134 caused almost as much loss of hemicellulose from the cereal dietary fibre as the unpurified (crude) enzyme - the concentrations being similar.

On the other hand, the concentrate from fractions 98-104 appeared to be about half as active at degrading the hemicellulose component as the previous two, again for similar enzyme concentrations.

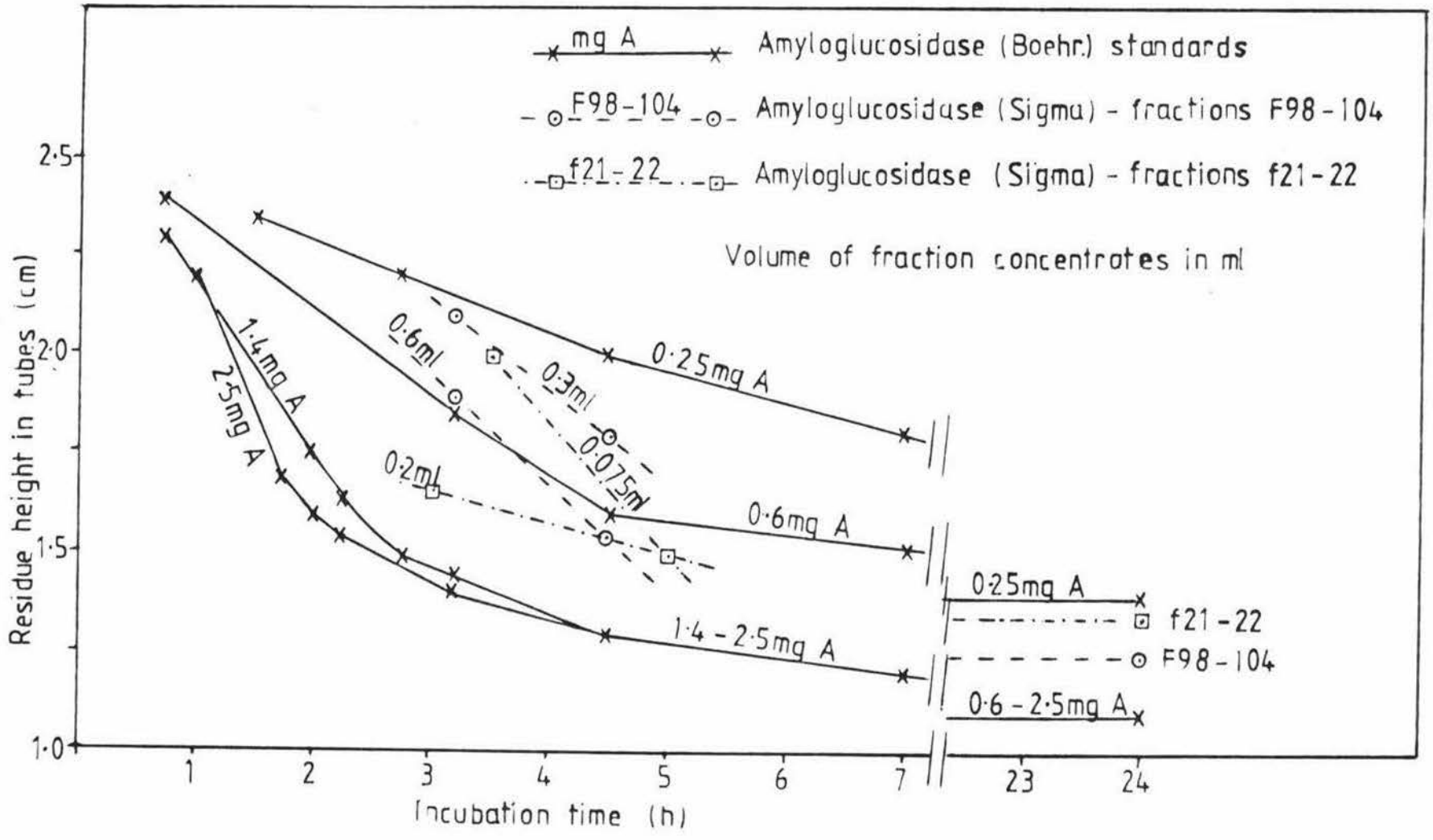

FIG. 5 AMYLOGLUCOSIDASE FROM *A. niger* (BOEHRINGER) ACTIVITY STANDARD CURVES AND AMYLOGLUCOSIDASE FROM *RHIZOPUS spp* (SIGMA) ACTIVITY CURVES WITH WHOLEMEAL FLOUR (0.2g PER DETERMINATION)

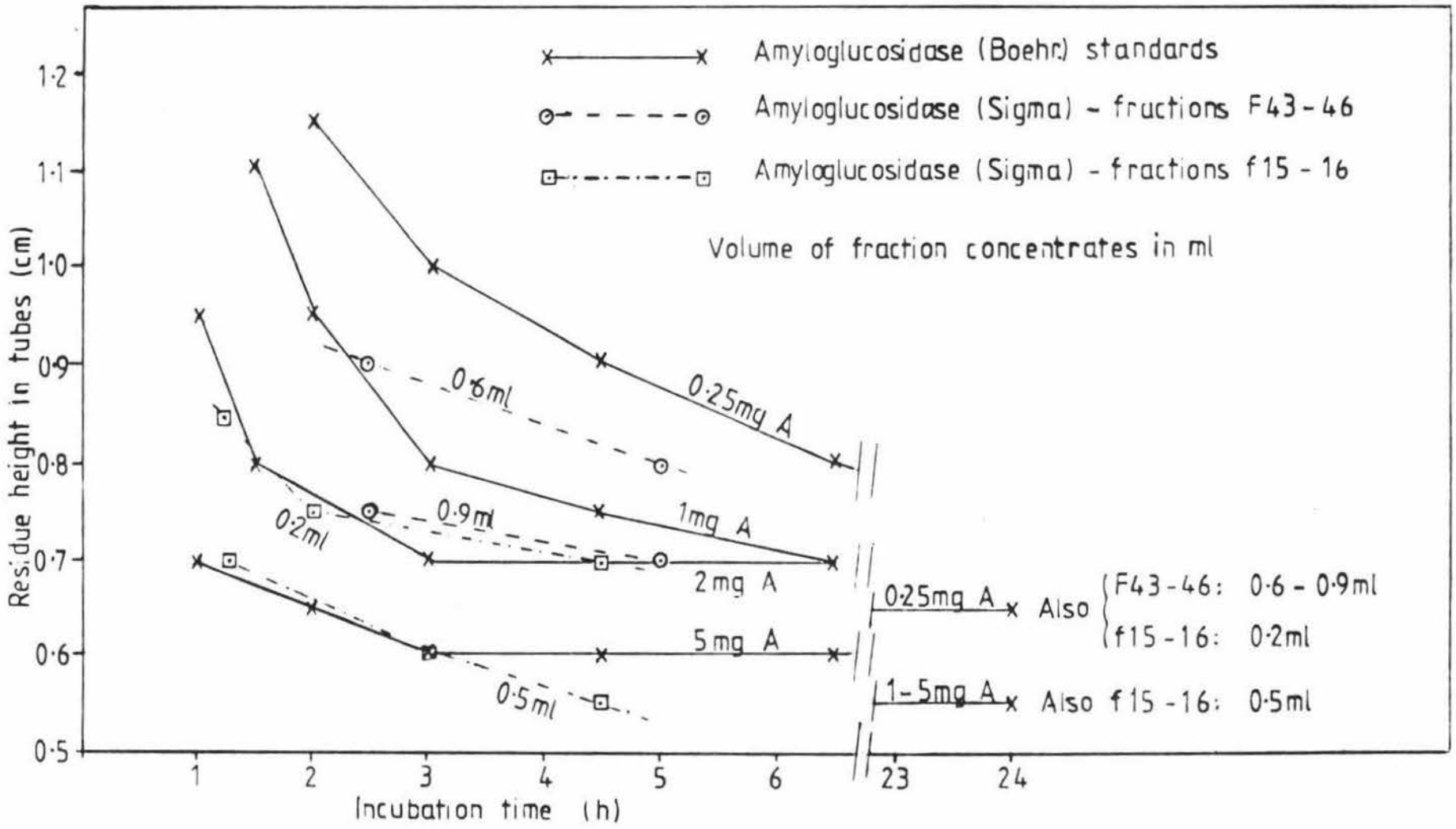

FIG. 6 AMYLOGLUCOSIDASE FROM *A. niger* (BOEHRINGER) ACTIVITY STANDARD CURVES AND AMYLOGLUCOSIDASE FROM *RHIZOPUS spp* (SIGMA) ACTIVITY CURVES WITH WHOLEMEAL FLOUR (0.08g PER DETERMINATION)

TABLE 29. Evaluation of fraction concentrates containing purified amyloglucosidase from Rhizopus spp

Peak number	Fraction numbers of concentrates	Volume of concentrate per determination	Estimated amount of enzyme added[1]	NDF residue	Calculated loss of hemicellulose fraction[2]
		ml	mg	g/100g dry matter	%
1	F.43-46	0.6	1	9.2	34
		0.9	1.5	8.7	40
3	F.98-104	0.3	0.5	10.6	16
		0.6	1	10.1	22
4	F.133-134[4]	0.4	(1)[3]	8.4	44
Controls:					
Crude amyloglucosidase (Sigma)		10 mg (powder)	~ 2	8.5	43
Amyloglucosidase (Boehringer)		0.2 ml	2	11.8	nil

1 Estimated from graphs in Figs. 5 and 6.

2 Hemicellulose content of dietary fibre of wholemeal flour taken as 65%: ref. Southgate (57)

3 Very approximate value because of incomplete data

4 The concentrate was prepared from precipitated amyloglucosidase after separating α-amylase crystals.

The concentrate of fractions 98-104 was tested by the semi micro neutral detergent method using Weetbix as a substrate. Tests with amyloglucosidase (Boehringer) as the starch hydrolysing enzyme with Weetbix indicated that 0.9 ml of the fraction concentrate contained ca 1.5 mg amyloglucosidase. This amount when tested with Weetbix (0.2 g) produced a value of 9.8% for

NDF content, compared to 12.8% when amyloglucosidase (Boehringer) was used. Thus ca 1.5 mg purified enzyme caused a loss of 31% of hemicellulose component, assuming Weetbix dietary fibre contains 72% of hemicellulose fraction (Southgate et al: ref. 61).

4.4.2 Ion exchange chromatography of glucoamylase using DEAE cellulose, tris-HCl buffers and salt gradient elution I.

4.4.2.1 Materials

Enzymes, ion exchange material, glass tubes, etc., as for chromatography with citrate-phosphate buffers.

Tris-HCl buffers

1. 0.1M, pH 8.5: 6.05 g tris + 14.7 ml 1N HCl in 500 ml water (previously boiled)
2. 0.1M, pH 7.05: 500 ml 0.1M tris-HCl pH 8.5 + 31.2 ml 1N HCl
3. 0.1M, pH 7.05 for linear salt gradient:
   250 ml 0.1M tris-HCl, pH 7.05 + 14.8 ml N HCl + 11.7 g NaCl

Reagents: AnalaR grade.

4.4.2.2 Methods

Initial tests to determine optimum conditions for column chromatography. These tests were performed as described under previous Methods except that tris-HCl buffers in the range 0.05-1.0M, pH 8.0-8.5 were used.

Preparation of column

The column was prepared as previously described except that 0.1M tris-HCl buffer, pH 8.5 was used for equilibration purposes.

Determination of chloride in eluate

A suitable aliquot of the fraction containing chloride ions was titrated

with 0.05N $AgNO_3$ using potassium chromate as an indicator.

Column chromatography of crude glucoamylase

A solution of amyloglucosidase, grade II from Rhizopus spp (Sigma), was prepared as described previously except that 0.125 g of enzyme preparation was added to 30 ml 0.1M tris-HCl buffer, pH 8.5. After draining the clear enzyme solution into the top of the bed it was followed by 60 ml starting buffer and a salt gradient elution buffer. The latter was dispensed automatically from a gradient apparatus comprising a 500 ml flask containing 0.1M tris-HCl, pH 7.05, and a second containing a similar volume of 0.1M tris-HCl in 0.8M NaCl, pH 7.05. The apparatus is described in ref. 51.

The rate of flow was adjusted to 60 ml/h. The operation was carried out at room temperature (ca 18^{o}).

Monitoring procedure for fractions

The optical density of all fractions was measured at 280 nm. The pH of fractions was checked especially after change of buffer solutions. Chloride concentration was determined.

Preliminary measurement of enzyme activity of fractions

This procedure was carried out as previously described when fractions exhibited a strong absorbance.

Evaluation of purified preparations of amyloglucosidase

After concentrating the enzyme component of fractions exhibiting high absorbance the amylolytic activity of the concentrate was measured by visual means as previously described using wholemeal flour as a substrate. The activity of the enzyme preparation towards dietary fibre was also determined by carrying the semi micro neutral detergent procedure through

to completion.

4.4.2.3 Results

The development pattern shown in Fig. 7 contained two main peaks, the first corresponding to fractions 5-10 in the pre-gradient elution stage, and the second to fractions 15-16 in the gradient elution stage. It was evident from the area covered by the first peak that about half or more of the protein component of the enzyme sample passed through the column without adsorption. Tests with 1% wheat starch at pH 4.6 showed that fractions 5-10 did not possess amylolytic activity.

The two fractions (15-16) of the second peak were found to possess strong amylolytic activity. It appeared that the active amyloglucosidase was eluted by a low salt concentration in the range 0.02-0.05M. Two minor peaks which followed were found to possess weak amylolytic activity.

Fractions 15-16 were combined, cooled to 0^{o}, and treated with cold 95% ethanol to effect concentration of the amyloglucosidase as previously described. The protein residue recovered after centrifugation was dissolved in 2 ml acetate buffer, pH 5.5.

Evaluation of purified enzyme

Small aliquots of the concentrated enzyme solution from fractions 15-16 were used to hydrolyse starch in the first stage of the determination of NDF in wholemeal flour by the semi micro method. An assessment of enzyme activity was obtained by visual means as described previously; the results are shown in Table 30. The determination of NDF was completed and the results were used to calculate the percentage loss of hemicellulose fraction from the cereal fibre caused by the action of amyloglucosidase from Rhizopus spp.

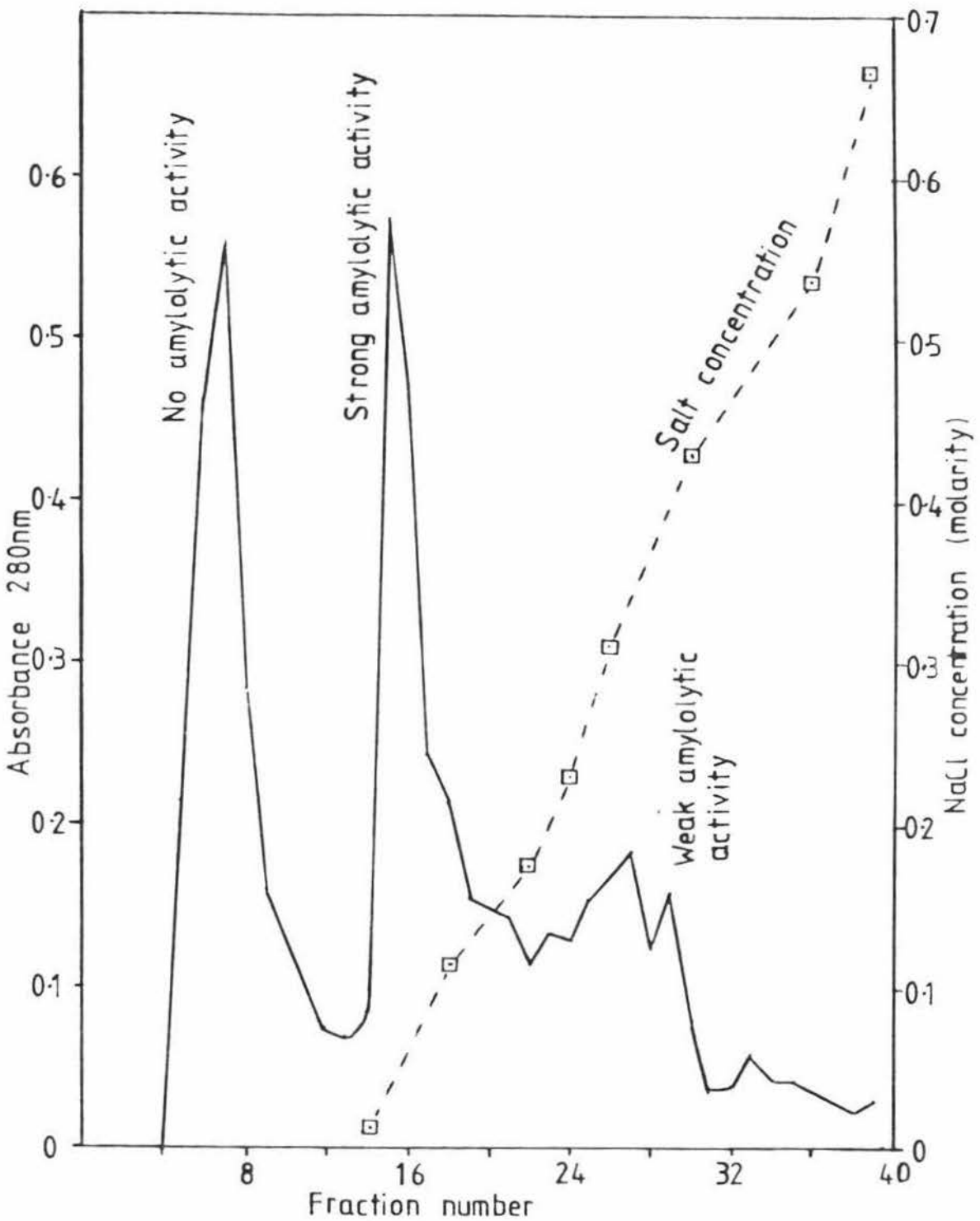

FIG. 7 ELUTION DIAGRAM OF CRUDE AMYLOGLUCOSIDASE (SIGMA) ON DEAE CELLULOSE

Starting buffer : 0.1M tris-HCl, pH8.5
Elution buffer : 0.1M tris-HCl, pH7.05, with a linear salt gradient to 0.8M NaCl

TABLE 30. Measurement of enzyme activity of fraction concentrate using wholemeal flour

Fraction numbers	Vol. of concentrate	Wt of wholemeal flour	Residue heights in tubes after (h)					
			0.5	1.25	2.25	2.75	4.5	24
	ml	g	cm					
f.15-16	0.2	0.08	1.2	0.85	0.75	0.65	0.7	0.65
	0.5		0.8	0.7	0.6	0.6	0.55	0.55

TABLE 31. Evaluation of fraction concentrate containing purified amyloglucosidase from Rhizopus spp

Fraction numbers	Vol. of concentrate	Estimated amount of enzyme added[1]	NDF residue	Calculated loss of hemicellulose fraction[2]
	ml	mg	g/100g dry matter	%
f.15-16	0.2	2	8.4	44
	0.5	5	8.3	46
Controls:				
Crude amyloglucosidase (Sigma)	10 mg (powder)	~ 2	8.5	43
Amyloglucosidase (Boehringer)	0.2 ml	2	11.8	nil

[1] Estimated from graphs in Figs. 5 and 6

[2] Hemicellulose fraction represents 65% of total dietary fibre

The results for hemicellulose fraction losses quoted in Table 31 show that the purified preparation of amyloglucosidase from Rhizopus spp degraded the dietary fibre of wholemeal flour to the same extent as

the crude unpurified enzyme, the concentrations being similar.

4.4.3 Ion exchange chromatography of glucoamylase using DEAE cellulose, tris-HCl buffers and salt gradient elution II.

4.4.3.1 Materials

Enzymes, ion exchange material and glass tubes as for previous experiment.

Tris-HCl buffers:

1. 0.05M, pH 8.6: 6.05 g tris dissolved in 1 litre water containing 15.6 ml 1N HCl
2. 0.075M; 0.05M NaCl pH 7.5: (a) 4.54 g tris dissolved in 500 ml containing 25.3 ml 1N HCl

   (b) 200 ml of above solution + 0.585 g NaCl + 0.5 ml 1N HCl = pH 7.5 at 17°
3. 0.075M; 0.8M NaCl, pH 7.5: 200 ml 0.075M tris-HCl + 9.36 g NaCl + 0.7 ml 1N HCl = pH 7.5 at 17°

Reagents: AnalaR grade.

4.4.3.2 Methods

Column chromatography of crude glucoamylase

The DEAE cellulose used in the previous experiment was regenerated by a washing process with 1M NaCl followed by pure water. The column was re-equilibrated with 0.05M tris-HCl buffer, pH 8.6.

A clear solution of amyloglucosidase, grade II from Rhizopus spp (Sigma) was prepared from 0.12 g powder and 18 ml 0.05M starting buffer.

After draining the enzyme solution into the top of the bed, 80 ml starting buffer was added, followed by salt gradient elution buffer from the automatic dispensing apparatus. Equal quantities of buffers

(2) and (3) were put into the gradient apparatus. The rate of flow was adjusted to 30 ml/h. Room temperature was 17-18°.

The absorbance of fractions was measured at 280 nm. The pH and chloride concentration were monitored in appropriate fractions.

The fractions were evaluated in terms of amylolytic activity and effect on dietary fibre of wholemeal flour as previously described.

4.4.3.3 Results

The development pattern is shown in Fig. 8 and as in the previous experiment produced two peaks, one containing a non-amylolytically active protein component and the other a strongly amylolytically active component. Other minor peaks possessing weak amylolytic activity followed the second main peak.

The active peak corresponding to fractions 21-22 was induced by a salt concentration in the range 0.02-0.04M.

Characteristically, a sharp pH rise was detected in the fractions which immediately preceded the second peak.

The fractions forming the active peak were combined and treated with ethanol in the usual way in order to concentrate the active enzyme component. The concentrate was then evaluated in terms of amylolytic activity and its effect on the dietary fibre component of wholemeal flour using the semi micro neutral detergent method. The results are presented in Tables 32 and 33.

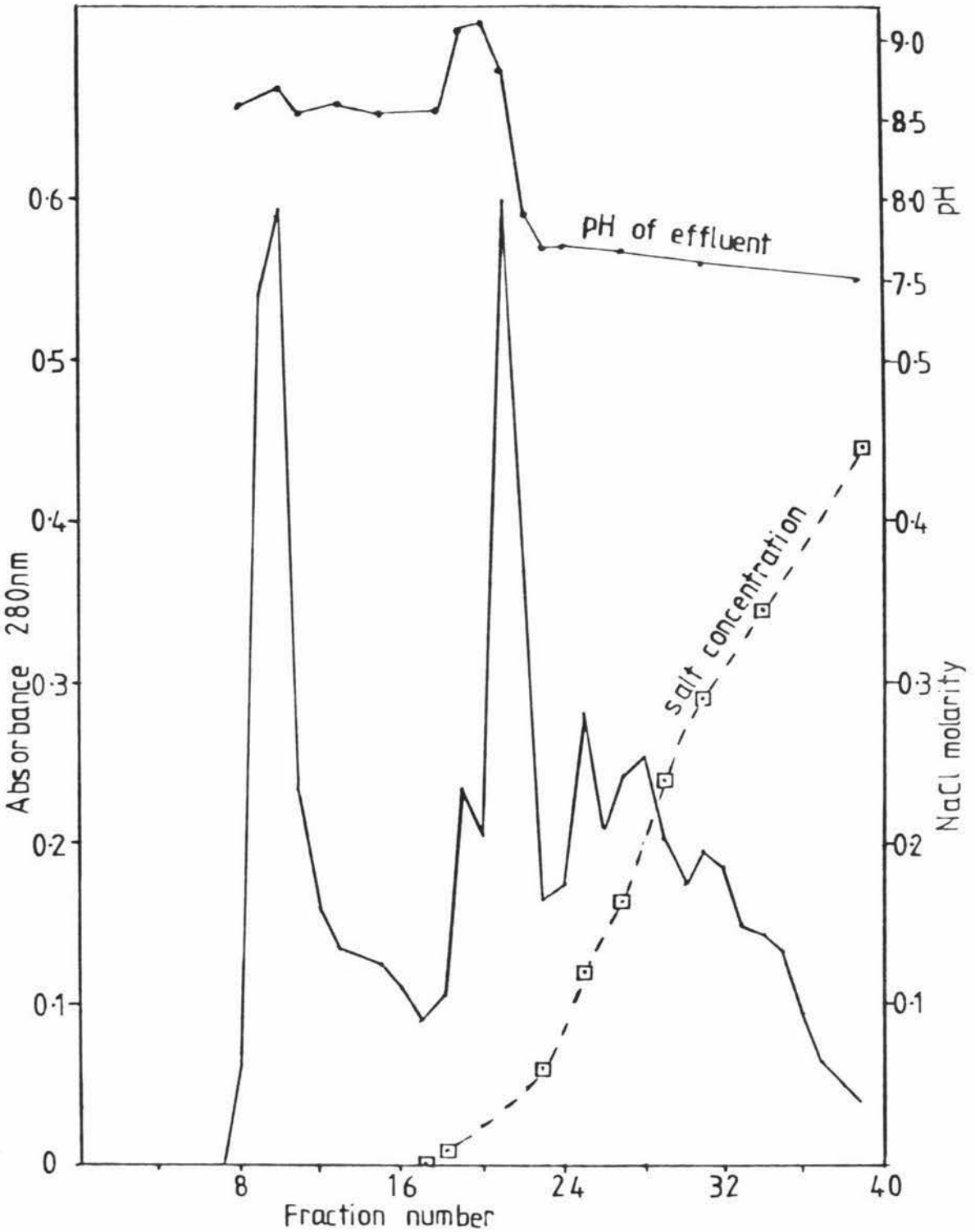

FIG. 8 ELUTION DIAGRAM OF CRUDE AMYLOGLUCOSIDASE (SIGMA) ON DEAE CELLULOSE

Starting buffer : 0.05M tris-HCl, pH8.6
Elution buffer (1) : 0.075M tris-HCl, pH7.5, 0.05M NaCl
Elution buffer (2) : 0.075M tris-HCl, pH7.5, 0.8M NaCl

TABLE 32. Measurement of enzyme activity of fraction concentrate using wholemeal flour (0.2 g)

Fraction numbers	Vol. of concentrate	Wt of wholemeal flour	Residue heights in tubes after (h)		
			3.5	5	24
	ml	g	cm		
f21-22	0.075	0.2	2.0	1.5	1.35
	0.2		1.6	1.5	1.35

TABLE 33. Evaluation of fraction concentrate containing purified amyloglucosidase from Rhizopus spp

Fraction numbers	Vol. of concentrate	Estimated amount of enzyme added[1]	NDF residue	Calculated loss of hemicellulose fraction[2]
	ml	mg	g/100 g dry matter	%
f21-22	0.075	0.5	10.7	14
	0.2	1	9.1	34

1 Estimated from graphs in Fig. 5 and 6.

2 2 mg (est.) crude enzyme causes 43% loss

Reference to the last column of data in Table 33 shows that the purified enzyme, even at comparatively low concentrations (0.5 to 1 mg per determination) strongly attacks the dietary fibre component of wholemeal flour.

4.4.4 Ion exchange chromatography of glucoamylase using DEAE cellulose, tris-HCl buffers and salt gradient elution III.

4.4.4.1 Results

A development pattern prepared from absorbance values (at 280 nm) of fractions collected during the course of a third ion exchange chromatographic experiment with crude amyloglucosidase from Rhizopus spp. using DEAE cellulose and tris-HCl buffers is shown in Fig. 9.

The buffers were of weaker strength (0.02M-0.05M) than used in the previous experiments and the pH of the elution buffer was slightly lower (6.75). A stepwise salt gradient was superimposed on the elution buffer during the final stage.

The peaks of the pattern were found to be less sharp and somewhat broader at the base than those obtained in previous experiments which indicated a less satisfactory purification procedure. However, the experiment served to demonstrate that a change in buffer strength from 0.02M to 0.05M and a fall in pH from 8.5 to 6.75 was insufficient to release the enzyme from the column. The peak containing amylolytic activity did not appear until a salt gradient was introduced.

Fractions of eluate possessing amylolytic activity were not tested by the semi micro NDF method on this occasion.

4.5 Discussion and conclusions

The development patterns obtained from the ion exchange chromatography of crude amyloglucosidase of Rhizopus spp. on DEAE cellulose using two species of buffer were found to have one or two important features in common:

1. A substantial proportion of the protein component of the crude enzyme passed through the column without adsorption and appeared

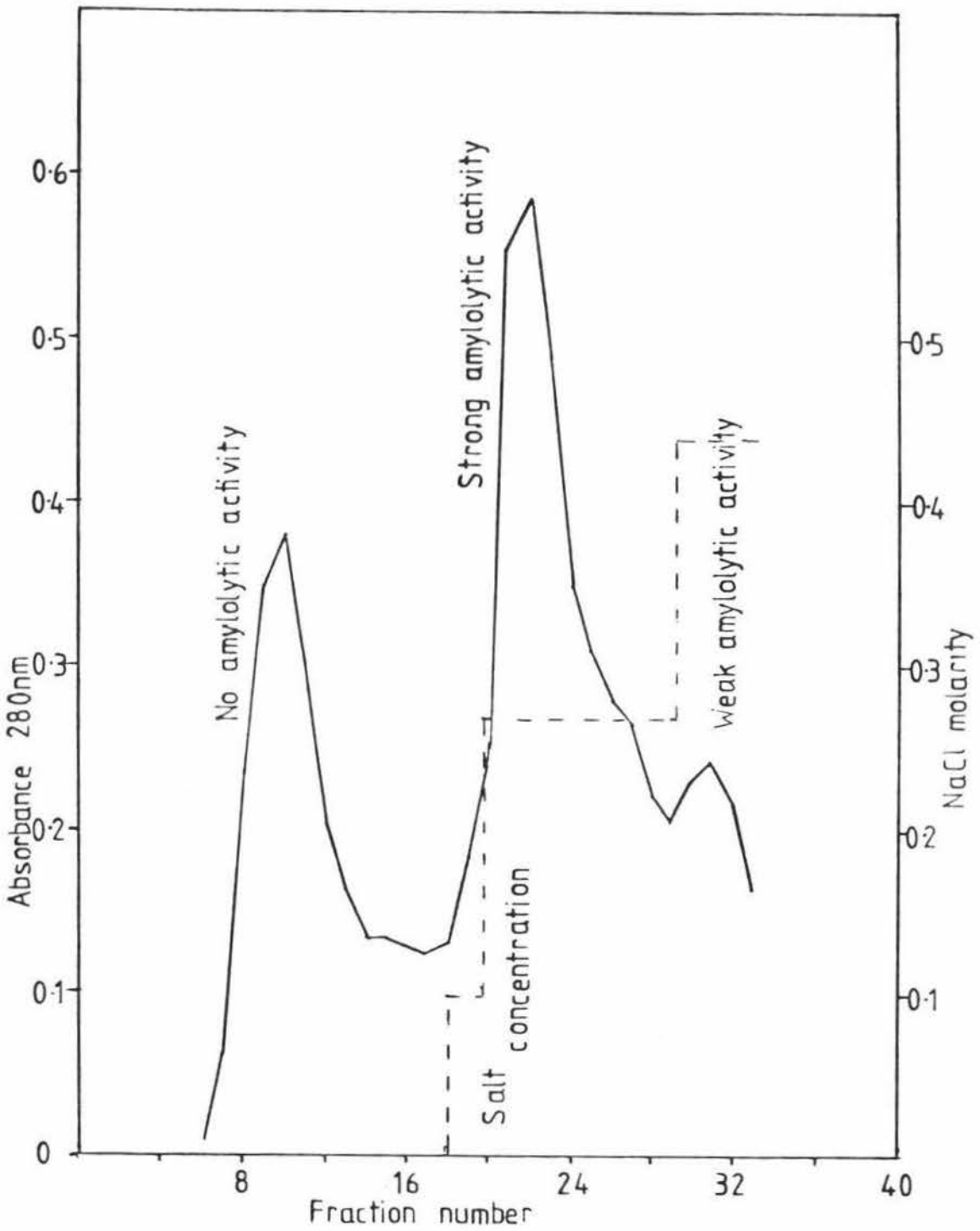

FIG. 9 ELUTION DIAGRAM OF CRUDE AMYLOGLUCOSIDASE (SIGMA) ON DEAE CELLULOSE

Starting buffer : 0.02M tris-HCl, pH8.5
Elution buffer (1) : 0.05M tris-HCl, pH6.75
Elution buffer (2) : 0.05M tris-HCl, pH6.75 with stepwise salt gradient
Weight of enzyme in 30ml starting buffer : 120mg

as a large pre-step or gradient elution peak.

2. The single large peak formed after elution of the enzyme was sharp (especially with citrate-phosphate buffer) and included a characteristic trailing edge. These features are characteristic of the pattern produced by highly purified protein (see diagrams by Lineback et al in Fig. 4).

Further support for the contention that the chromatography described in the present work succeeded in producing fractions containing highly purified amyloglucosidase comes from the observation that the absorbance ratios (280 nm/260 nm) for a group of fractions forming a single peak were found to be constant (Table 27).

During the course of the present studies many samples of wholemeal flour were analysed by the semi micro neutral detergent method using the enzyme prepared by ion exchange chromatography of crude amyloglucosidase from Rhizopus spp. Without exception the results demonstrated that the purified enzyme was capable of destroying an appreciable proportion of the dietary fibre component of wholemeal flour (and Weetbix) when used for the purpose of hydrolysing the starch component of the cereal product. This behaviour was in complete contrast with the action of amyloglucosidase from A. niger (Boehringer) which was found to be practically inactive towards cereal dietary fibre.

The comparative effects of the two types of amyloglucosidase on wholemeal flour when used in the semi micro method of measuring neutral detergent fibre are finally illustrated by the curves in Fig. 10. The treatment with purified amyloglucosidase from Rhizopus spp reduces the NDF value of wholemeal flour to a constant low level of 8.3-8.4%. The accepted normal value is 11.8% as determined with the aid of purified amyloglucosidase from A. niger.

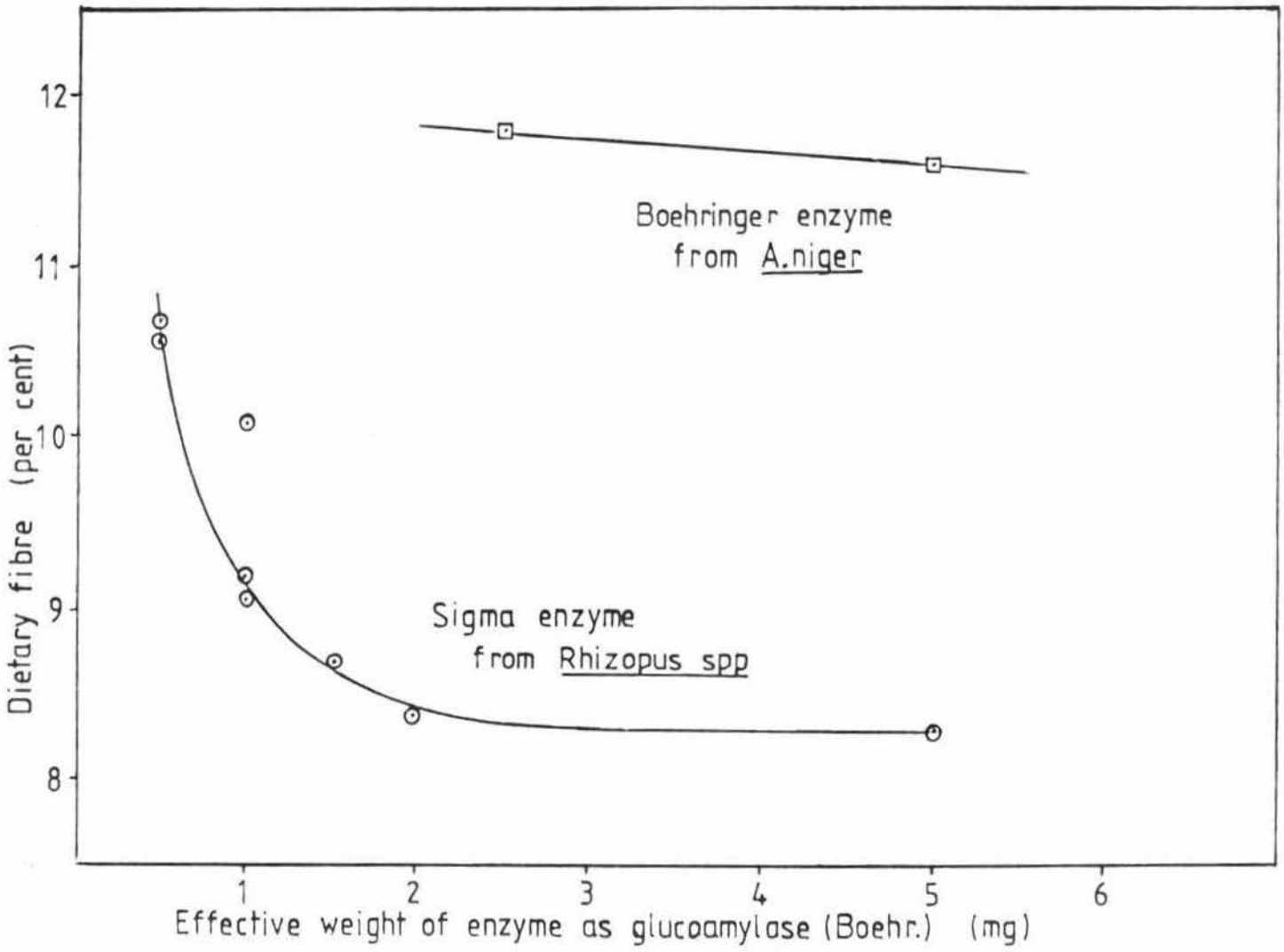

FIG. 10 EFFECT OF PURIFIED GLUCOAMYLASE ON DIETARY FIBRE EVALUATION OF WHOLEMEAL FLOUR

Note: The data used to construct the curves in Fig. 10 were drawn from Tables 26, 29, 31 and 33.

Substrate specificity of amyloglucosidase

The chemical composition of the water insoluble dietary fibre components of wheat flour and bran has been studied by Neukom et al (35). Their analytical results are reproduced in Table 34:

TABLE 34. Content and composition of indigestible polysaccharides (NSP) of flour and bran

Flour type (ash)	NSP content g/100 g flour	β-glucan %	Galactan %	Mannan %	Xylan %	Araban %	Rhamnan %
Wheat (0.44)	2.19	21.5	6.8	2.7	38.8	29.7	0.5
Wheat (0.70)	3.07	23.8	5.2	5.5	37.1	27.4	1.0
Wheat (1.03)	4.21	24.2	4.7	4.0	37.5	28.5	1.0
Wheat (1.80)	10.32	7.7	3.6	1.1	52.1	34.4	1.1
Rye (0.85)	9.27	22.9	3.1	2.8	40.6	30.5	0.1
Wheat bran	-	28.3	1.4	1.5	40.5	27.9	-

Taken from: Neukom et al: ref. 35

The principal constituents of the insoluble indigestible polysaccharides (NSP) of wholemeal flour (ash = 1.8%) comprised 86.5% pentosans and 13.5% hexosans.

Southgate (56) reported 28% hexoses in the dietary fibre of wholemeal flour but this figure almost certainly includes hexoses from water soluble indigestible polysaccharides.

As mentioned earlier in this discussion, NDF values for wholemeal flour

obtained with the aid of amyloglucosidase from Rhizopus spp were 8.3-8.4% or 30% below the true value for the NDF (11.8%). It therefore appears that this enzyme affects the pentosan structure of the non-cellulosic polysaccharides in some way as there is insufficient hexosan present to account for the proportionately large loss of fibre.

Cole (8) studied the physicochemical properties of wheat flour hemicellulose and found that 2% of glucose was present in the purified hemicellulose fraction. He concluded that this glucose could be present in the hemicellulose molecule as a glucose polymer or as part of the hemicellulose molecule.

Pazur and Ando (41) found that the carbohydrate composition of the amyloglucosidases isolated from A. niger and R. delemar differed in important respects and that the electrophoretic properties of the two enzymes were markedly different.

During the course of the present studies on enzyme purification techniques it was found that the conditions relating to buffer ionic strength and pH which were reported by Pazur and Ando (39) and Lineback et al (29) to effect strong adsorption of amyloglucosidase from A. niger onto DEAE cellulose did not succeed with amyloglucosidase from Rhizopus spp (crude preparation). In fact in order to achieve satisfactory binding of the enzyme to the ion exchanger it was found necessary to use a starting buffer with a molar strength only 5% of that recommended by the above authorities. The pH too had to be changed to a higher value.

In the light of the evidence presented above it seems reasonable to conclude that the two amyloglucosidases, while both being excellent starch hydrolysing enzymes, differ markedly in their behaviour towards

the non-cellulosic polysaccharides of cereals, which makes one of them (from Rhizopus species) entirely unsuitable for use in dietary fibre methodology.

In the absence of precise knowledge of the molecular structure of the polysaccharides comprising the dietary fibre component of cereals it is not possible to speculate further on the nature of the enzymatic hydrolysis of hemicellulose caused by the amyloglucosidase from Rhizopus spp. However, the less desirable characteristic of this enzyme may perhaps be exploited to advantage by offering the proposed semi micro neutral detergent method as a means of identifying or confirming the fungal origin of an amyloglucosidase preparation.

APPENDIX 1

REVIEW OF LITERATURE ON THE PHYSICAL AND CHEMICAL INTERPRETATION OF DIETARY FIBRE

Introduction

The term dietary fibre has many connotations and has been extensively discussed in the literature since 1972 when Trowell (69) defined dietary fibre as the indigestible matter in the diet derived from the plant cell wall.

Dietary fibre presents many problems to the analyst because it is not a single chemical entity but comprises a complex mixture of structural and non-structural polymers whose chemistry is not yet fully understood. Some components of dietary fibre are truly fibrous and insoluble whereas others are not and may even be water soluble; certain components may not be totally indigestible.

The dietary fibre content of foods may be determined by a variety of analytical procedures, the results of which may not always be in agreement, due partly to the method chosen, and also to the source of food material.

The concept of dietary fibre

Historically the classical crude fibre method, also known as the Weende method, of measuring fibre in animal feeds, had its beginnings in the early nineteenth century. It became a highly empirical procedure and did not measure any specific component or precise group of components. The procedure consisted of a sequential extraction of the plant material with solvent, hot dilute acid and hot dilute alkali. Van Soest and McQueen (75) have estimated that this procedure

causes the loss of 80% of the hemicellulose or pentosans, 50-90% of the lignin and 20-50% of the cellulose. In the view of Van Soest and Robertson (76), in so far as human foods are concerned, the use of the method and its results should be discontinued.

Southgate (54) believes that the crude fibre method has little or no place in the studies for man and suggests that its continued use can only lead to confusion.

In 1935 Williams and Olmsted (83) used the term non-digestible residue which they defined as those vegetable materials not attacked by digestive enzymes in the mammalian gut, and consisting of lignin, cellulose and non-water soluble hemicelluloses. Unfortunately these authors confused the issue by linking the term with crude fibre.

After the crude fibre era came rapid advances in the methodology of fibre analysis developed by the pioneering efforts of Van Soest and associates with detergent extractants, and by Southgate with an analytical scheme based on the determination of sugars following the acid hydrolysis of the fibre. This situation created a need for a new term to express the concept of the newly discovered form of fibre. The terms that were introduced were, from an analyst's point of view, rather disappointing as they were based on physiological rather than chemical considerations. For example, Van Soest and Robertson (76) favoured cell wall constituents, and also non-nutritive residues which were defined as those substances resistant to animal enzymes. Similarly, Southgate chose unavailable carbohydrates and lignin. The expression non-nutritive residues implies inertness but as later admitted by Van Soest this is not true considering the properties of ion exchange, hydration and bulk volume, and in turn their effects on mineral excretion, transit and stool volume (76). This material may

be digested by intestinal bacteria producing certain metabolites, e.g. volatile fatty acids, which may be absorbed into the systemic circulation or lymph (65).

In 1974 Trowell defined dietary fibre as the remnants of the plant cells resistant to hydrolysis by the alimentary enzymes of man. Later (1976) Trowell et al (71) redefined dietary fibre to include undigested storage polysaccharides present within the contents of the cell as well as undigested polysaccharides and lignin present in the cell wall.

In the opinion of Southgate (56) the above definition is a philosophical one. He regards the term as applying to all polysaccharides and lignin that may be imagined to reach the large intestine. Southgate et al (63) have recently defined dietary fibre as the sum of lignin and the polysaccharides that are not digested by the endogenous secretions of the human digestive tract. In their view the inclusion of indigestible protein, lipids, waxes, cutin and inorganic constituents is not justifiable. They believe that these substances should be regarded as dietary constituents which modify the properties of the major components of dietary fibre, that is, the polysaccharides.

Unfortunately both the original definition by Trowell and the later one by Trowell et al are now invested in the term dietary fibre which means that it is at present necessary to qualify the term in order to be precise in meaning.

Spiller et al (65) have suggested the use of the term 'plantix' which would include cellulose, lignin and water insoluble hemicellulose, pectins, gums and mucilages. This would avoid any possible confusion with substances of animal origin that might be undigested by human

gastro intestinal enzymes. However, Trowell (68) objects to the association with matrix on the grounds that it means the middle lamella and consequently is rich in pectic substances.

Furda (18) has proposed that dietary fibre should be fractionated into water soluble polymers and water insoluble polymers. He believes that the term dietary fibre could be replaced with a more appropriate term which would demonstrate its nutritional and chemical character. To fulfil this requirement the term 'partially digestible plant polymers' or 'partially digestible biopolymers' is proposed.

If dietary fibre is regarded as having an imprecise meaning the same criticism cannot be applied to neutral detergent fibre (NDF) and acid detergent fibre (ADF). Water soluble indigestible polymers are not involved with these residues. However, Van Soest and Robertson (76) have pointed out that the lignin component of these residues is not well defined. The apparent lignin in many diets may be composed more of heat damaged artifacts than true lignin. The artifacts arise through heating or cooking of the food. The products of the Maillard reaction whereby carbohydrates are degraded and react with amino acids to form dark coloured polymers are insoluble and indigestible and have the physical properties of lignin.

Southgate (56) has warned food scientists not to seek rapid methods for total dietary fibre as these values will conceal the types of polysaccharide which are present. He explains that at the present time it is not known which components of dietary fibre are important in influencing such physiological properties as faecal bulking, water retention in the stool or binding of bile salts.

Components of dietary fibre

The components of dietary fibre have been conveniently categorized by Southgate (55) (see Table A-1):

TABLE A-1 Components of dietary fibre

Principal sources in the diet	Description	Classical nomenclature
Structural materials of the plant cell wall	Structural polysaccharides	Pectic substances Hemicelluloses Cellulose
	Non carbohydrate constituents	Lignin Minor components
Non structural materials either found naturally or used as food additives	Polysaccharides from a variety of sources	Pectic substances Gums Mucilages Algal polysaccharides Chemically modified polysaccharides

Taken from: Southgate D.A.T. (ref. 55)

1. Hemicelluloses

Eastwood (12) defines hemicellulose as a mixture of linear and highly branched polysaccharides containing various sugar residues, e.g. xylose, arabinose, glucose, mannose, with about 4% of the residues consisting of uronic acid. Hemicelluloses act as plasticisers and are intertwined with lignin and laid down around cellulose fibres. This type of association can hinder the enzymic hydrolysis of polysaccharides as well as their extraction with chemicals (67).

Cummings (10) describes hemicellulose molecules as usually much smaller than those of cellulose with between 150 and 200 sugar units making up the molecule. They are also more amorphous than cellulose molecules, although some xylan molecules exhibit a crystalline structure. Together with pectin the hemicelluloses form the matrix

of the plant cell wall in which are enmeshed the cellulose fibres. The neutral hemicelluloses are typified by those isolated from cereals. These have a backbone of (1 → 4)β-D-xylose with short side chains of arabinose with occasional (about 5%) glucuronic acid residues.

Three properties of hemicelluloses could prove important in human physiology: their water holding capacity; their digestibility and their capacity to bind ions (10).

Bailey (5) prefers the term non-cellulosic polysaccharides to the classical term hemicellulose - still commonly used as a collective term for the non-cellulosic matrix polysaccharides other than pectin.

Neukom et al (35) studied the indigestible non-starchy polysaccharides of wheat flour and concluded that these substances were derived from the insoluble endosperm cell walls. They were surprised to find that the hemicelluloses (pentosans) rather than cellulose formed the bulk of the water insoluble material of wheat flours and bran - for the reason that xylo-arabans are generally considered to be water soluble.

2. Cellulose

Cellulose belongs to the class of fibre polysaccharides as distinct from the associated class of matrix polysaccharides (pectic substances and hemicelluloses) (67).

The cellulose is largely crystalline and exists as micro-fibrils which are considered to be held together by hydrogen bonds in a cement of largely amorphous matrix polysaccharides including lignin and some proteins (67).

Cells are surrounded by a thin primary wall, and the middle lamella or intercellular substance is found in the spaces between these walls. The primary wall contains relatively small amounts of rather unorientated micro-fibrils of cellulose but larger amounts of non-cellulosic compounds, particularly pectic substances. The middle lamella contains no cellulose but much pectic substances. During the cell wall thickening highly orientated cellulose and hemi-celluloses are deposited to form the secondary wall - sometimes, as illustrated in Fig. A-1, in different layers having fibrillar structures. At the end of the thickening phase formation of lignin becomes noticeable, beginning around the primary wall at the cell corners and extending from there into the secondary cell wall.

Cellulose is a linear polymer of high molecular weight built up from β-D-(1 → 4) linked glucose units. The molecule has a degree of polymerisation of up to 10,000 (67).

The disordered non-crystalline regions seen in all cellulose fibrils may represent areas where other sugar or uronic acid residues are incorporated in the molecule. These regions are most accessible to many reagents because the hydroxyl groups are not capable of forming hydrogen bonds as strongly as in the crystalline regions (55).

Within the context of dietary fibre chemistry the most important properties of cellulose concern, first, the susceptibility of the molecule to hydrolysis and, second, its capacity to absorb water (55).

3. Lignin

The lignin molecule is built up by the condensation of phenolic alcohols and is essentially an aromatic complex branched polymer containing a number of functional groups. It is extremely resistant

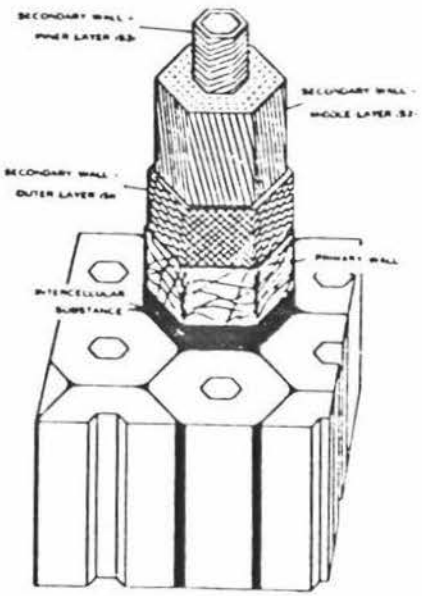

FIG. A-1 SIMPLIFIED STRUCTURE OF THE CELL WALL OF A HARDWOOD FIBRE

Taken from : Theander, O. (ref 67)

to both chemical and enzymatic degradation and is possibly the most resistant substance found in nature (60). It is slowly soluble in organic solvents such as hot butanol but is classically isolated as the residue insoluble in 72% W/W H_2SO_4. Lignin fractions prepared in this way contain cutin and suberin, and for this reason many values for lignin in foodstuffs are too high (60). In addition protein-carbohydrate interactions occur when foodstuffs are heated to give artifacts which analyse as lignin.

The role of lignin in the cell wall is to lend strength and support by permeating other constituents. It is generally considered, but not clearly proven, that lignin is partly linked to polysaccharide cell wall components (67).

Lignin is thought to have bile salt binding properties. It has been claimed that bacterial degradation products of bile acids which are found in the colon and faeces are strongly adsorbed by the lignin component of vegetable material (11).

4. Pectic substances

Pectins are common to all cell walls and are present also in the intercellular layers. As a group they are biochemically less well defined than other polysaccharides but in general are smaller with molecular weights in the region 60,000-90,000. In man ingested pectin may be completely metabolized in the gut, probably in the colon, with less than 5% being recovered (10).

Pectic substances are extracted with a solution of EDTA (ethylene diamine tetra acetic acid as sodium salt) which presumably chelates calcium in the insoluble pectates and releases the pectic substances into solution. The recovery from aqueous solution can be effected

by ethanol precipitation (55).

5. Plant gums and mucilages

The plant gums and mucilages constitute a very heterogeneous group of complex polysaccharides which are not part of the cell wall structure but which are in general indigestible (55).

Biochemically the plant gums present a complex group of highly branched uronic acid-containing polymers with neutral sugars such as xylose, arabinose and mannose (10).

Mucilages structurally resemble hemicelluloses and are usually mixed in with the endosperm or storage polysaccharides of plant seeds. They have been isolated from the endosperm of cereals and are characteristically highly branched with a backbone of (1 → 4)β-D-xylose with L-arabinose side chains (10).

6. Cuticular substances

While the cuticular substances form a small proportion of the total plant lipids they are associated with the plant cell wall and provide an extremely hydrophobic layer on the outer surface of the plant. Cuticular substances may be divided into two broad groups: waxes and cutins. Waxes may be extracted by simple organic solvents but cutins need to be saponified before extraction.

Cuticular substances are extremely resistant to digestion and in turn are thought to impair the digestibility of the other cell wall constituents in a manner similar to lignin (10).

7. Protein

Small amounts of protein are usually associated with isolated cell

walls. It is now clear that a fairly well defined class of plant proteins, sometimes called extensins, form an integral part of the cell wall. These proteins are characteristically rich in hydroxyproline, the hydroxy group of which forms bonds with the hemicellulosic polysaccharides (55).

Systems of partitioning the dry matter of forage

In 1968 Fonnesbeck (16) summarized the then present knowledge of partition methods for the dry matter of forages with the aid of a simple diagram (Fig. A-2) which is still of current interest.

Systems 2-5 represent crude fibre, acid detergent fibre, neutral detergent fibre and dietary fibre, respectively. System 6 combines cellulose and hemicellulose (and presumably pectic substances) into holocellulose which represents the fibrous carbohydrate of forage.

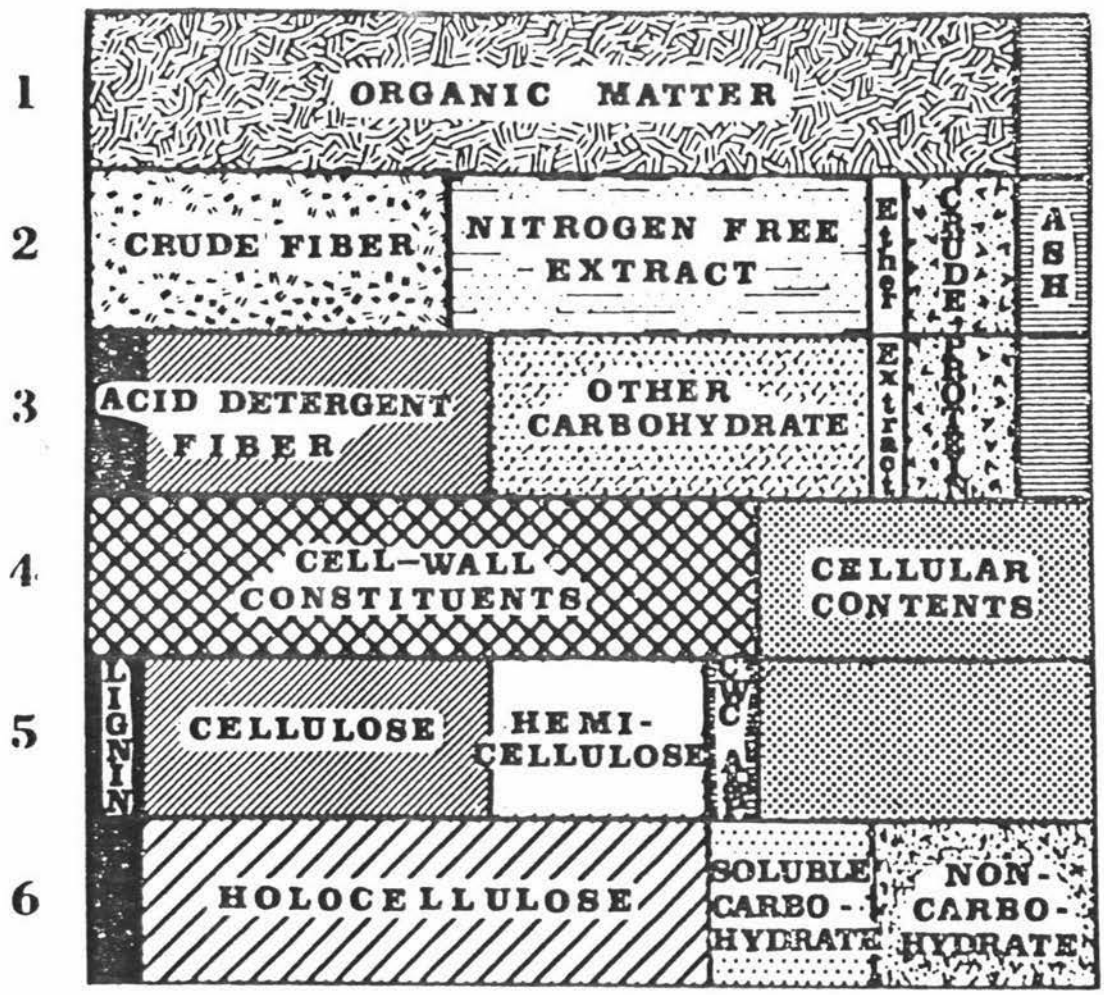

FIG. A-2 DRY MATTER OF FORAGE

APPENDIX 2

REVIEW OF LITERATURE ON METHODOLOGY IN DIETARY FIBRE ANALYSIS

A. Non-enzymatic methods

1. Crude fibre method

The presently accepted official procedure is that of the AOAC (4).

The crude fibre method was favoured for the purity it offered, for the ratio of C to H as in carbohydrate, and for a low ash and nitrogen content (76).

Cereals and other members of the monocotyledon class that contain fibres high in hemicellulose and which are moderately high in lignin are the lowest in recovery of crude fibre (76). Highest recoveries are in the dicotyledonous non-legume vegetables where cell walls contain a high proportion of cellulose.

An example of cell wall constituents being grossly under-estimated by the crude fibre method is provided by the value quoted for whole-meal flour (2%). Total cell wall constituents actually amount to 11-12% (13).

2. Normal acid fibre method (Walker and Hepburn, 1955)

The normal acid fibre procedure proposed by Walker and Hepburn (79) as an alternative to the crude fibre method has not been accepted as widely as the crude fibre method (54). It differs from the latter mainly in the omission of the alkali extraction stage and an increase in the acid strength to 1N. The total nitrogen content is determined, and using the factor 6.25 the crude protein value is deducted from the

ash free residue to give normal acid fibre (79).

3. Acid detergent fibre (Van Soest, 1963)

Van Soest (74) modified the normal acid fibre method by using a quaternary detergent, cetyl trimethyl ammonium bromide (CTAB) in 1N H_2SO_4 as the extractant. This procedure yields a fraction with a low nitrogen content (0.2-0.8%) and also gives an accurate measure of cellulose and lignin in a forage (54).

Van Soest (72) reported that acid detergent fibre will recover lignin, cellulose, pectin and only 15% or so of pentosans from food materials.

A summary of the stages in the acid detergent fibre method is given in Fig. A-3.

4. Buffered acid detergent method (Baker, 1976)

The procedure proposed by Baker (6) is similar to that for determining acid detergent fibre except that a buffer solution of pH 1.5-2.0 is prepared by mixing 260 ml 0.2M HCl and 1000 ml 0.2M KCl. 20 g CTAB is dissolved in 1 litre of buffer solution and this solution is used to extract the food sample. The buffer solution is a much less corrosive reagent than 1N H_2SO_4 and is within the pH range of the human stomach digestive medium.

5. Neutral detergent fibre method (Van Soest, 1963)

This method, developed by Van Soest (73), is considered to give an accurate measure of cell wall constituents in a vegetable foodstuff (54). Van Soest (72) claims that the method will recover lignin, cellulose and hemicellulose but not pectin. The extraction is performed with a buffered solution of sodium lauryl sulphate at pH

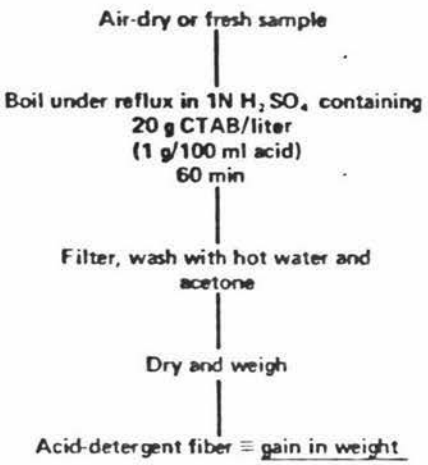

FIG. A-3 ACID DETERGENT FIBRE METHOD

Taken from : Southgate, D.A.T. (ref 54)

7.0. The method is fully described in reference 78.

A summary of the stages in the neutral detergent method is given in Fig. A-4.

B. Methods using enzymes

1. Remy (1931)

The first biological method to be used for the separation of cellulose, hemicellulose and lignin from other plant constituents using an enzymatic digestion process is attributed to Remy (46). He determined the fibre content of various vegetable materials with the aid of 3 enzymes: pepsin-HCl, neutral malt diastase, and pancreatin-sodium carbonate. The results were about twice the crude fibre values and would probably have been larger still but for the protracted (8 day) fermentation period which undoubtedly caused part destruction of the hemicellulose fraction.

2. Williams and Olmsted (1935)

Williams and Olmsted (83) improved on Remy's procedure by substituting pancreatin in neutral solution for malt diastase. The latter was found to degrade a substantial part of the hemicellulose fraction whereas neutral pancreatin removed starch without concurrent loss of hemicellulose. They also found that alkaline conditions during enzyme treatment led to a 40% loss of hemicellulose in some types of vegetable material.

3. McCance, Widdowson and Shackleton (1936)

Southgate (54) draws attention to the work of McCance and Laurence (31) in 1929 when they developed the first simple method of measuring unavailable carbohydrates. McCance et al (32) measured total unavailable carbohydrates in a range of fruits, nuts and vegetables

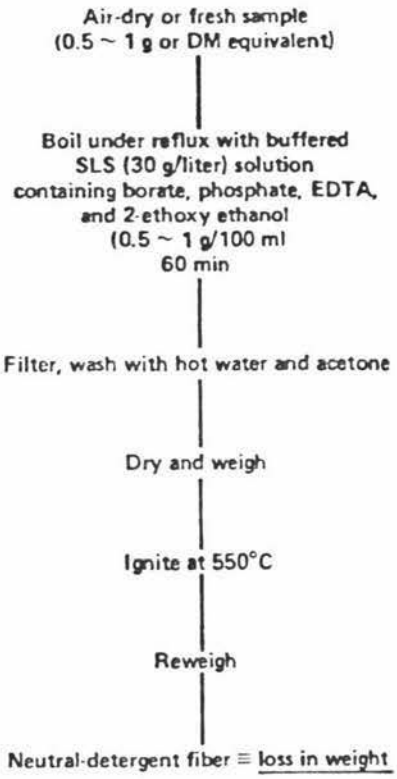

FIG. A-4 NEUTRAL DETERGENT FIBRE METHOD

Taken from : Southgate, D.A.T. (ref 54)

by determining the residue insoluble in 80% V/V ethanol. The starch content of this residue was measured enzymatically by means of a Takadiastase preparation. Corrections were made for both starch and protein in the residue:

Unavailable carbohydrate = residue insoluble in alcohol less (starch + crude protein) (54)

4. Williams, Wicks, Bierman and Olmsted (1940)

Williams et al (84) used pancreatic digestion in buffered bile salt solution at pH 8 to remove starch and protein from the insoluble residues of various fruits and vegetables. The components of the residues were then analysed separately by chemical methods. It was found that ca 70% of the hemicellulose fraction of the residues could be hydrolysed into non-fermentable sugars (mainly pentoses) which could be analysed by chemical means. Cellulose was determined as glucose after acid hydrolysis.

5. Weinstock and Benham (1951)

Weinstock and Benham (80) effected improvements to previous enzymatic procedures by preparing the food sample more adequately and shortening the time of enzyme treatment. The sample was placed in phosphate buffer at pH 4.9 and autoclaved in order to gelatinize the starch following which it was treated with Rhozyme S for 24 h (the enzyme was supplied by Rohm & Haas Co., Philadelphia). Using corn meal as a substrate, fibre values 4.4 times larger than the crude fibre values were realized by this procedure.

6. Fraser, Brandon-Bravo and Holmes (1956)

Fraser et al (17) combined the procedure of Weinstock and Benham with chemical analysis of the residues. Takadiastase and trypsin were

used in place of Rhozyme as this was not available. Pentosans were determined as furfural (after acid distillation) by spectrophotometric estimation of colour using orcinol-iron reagent. The results obtained with wholemeal flour showed serious discrepancies in the pentosan/cellulose ratios (4.0/4.2 compared with 6.25/2.46 by Southgate's method - ref. 61).

7. Southgate (1969)

Southgate pioneered a new technique of measuring available and unavailable carbohydrates in foods after becoming aware of the need for more precise methods during an investigation by Southgate and Durnin (62) concerning the use of energy conversion factors in the calculation of the energy value of mixed diets. Details of the analytical procedure were published in 1969 (57) followed by modifications to the procedure in 1976 (58). A schematic outline of the method is reproduced in Fig. A-5.

Southgate originally used Takadiastase supplied by Parke Davis for the purpose of hydrolysing the starch component of a food sample. As this enzyme is no longer available Southgate (54) has recommended α-(1 → 4) amyloglucosidase from Aspergillus niger supplied by Boehringer or Sigma as an alternative, but advises potential users that the enzyme appears to have slight activity towards galactomannans in guar gum and that chemically modified starches may not be hydrolysed.

8. Hellendoorn, Nordhoff and Slagman (1975)

A simple method which depends on the use of pepsin and pancreatin is described by Hellendoorn et al (20) for the determination of the indigestible residue (dietary fibre) content of food. An 18 h predigestion treatment of the sample with pepsin in dilute HCl at

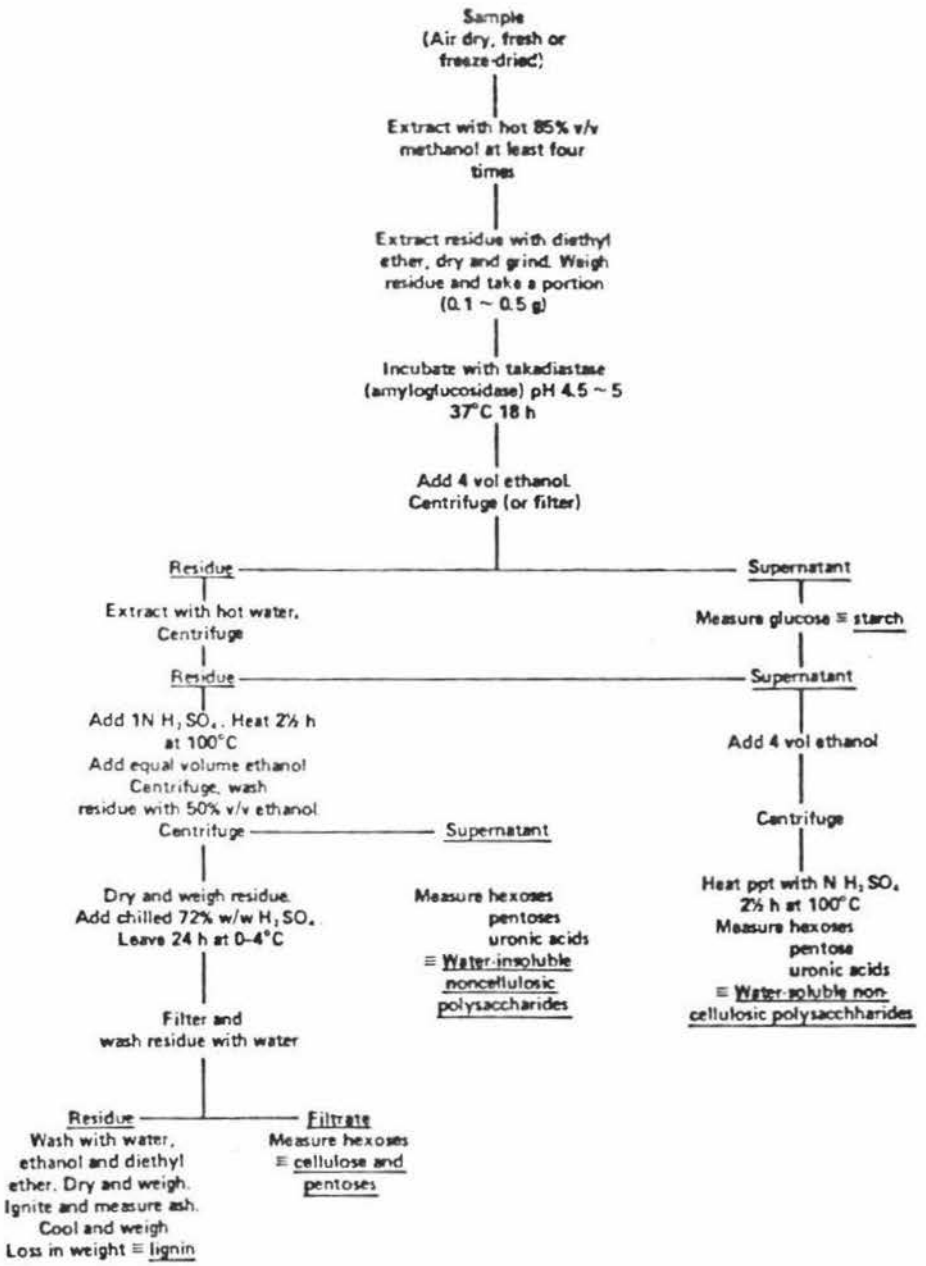

FIG. A-5 SCHEMATIC OUTLINE OF METHOD FOR MEASURING THE COMPONENTS OF THE UNAVAILABLE CARBOHYDRATES OF DIETARY FIBRE

Taken from : Southgate, D.A.T. (ref 54)

40° is followed by a 1 h neutral pancreatin digestion also at 40°. The residue is washed by a centrifugation procedure using water and successive amounts of acetone followed by filtration, drying and weighing. The results obtained with cereal products agree reasonably well with Southgate's figures.

9. Elchazly and Thomas (1976)

Elchazly and Thomas (14) have described a new and simple enzymatic method for the determination of total water insoluble unavailable carbohydrates (dietary fibre) and their components - hemicellulose, cellulose and lignin. The food sample is placed in buffer solution at pH 4.7 and autoclaved to gelatinize starch. It is then treated successively with amyloglucosidase for 3 h at 37° (or Takadiastase for 18 h at 37°) and with trypsin or pancreatin for 18 h at 37°. The residue is washed by a centrifugation procedure, filtered, dried and weighed.

The components of the residue are determined by Southgate's method or by Van Soest's acid detergent method.

A table of statistical data relating to the results of analyses of wheat and rye samples is reproduced below in Table A-2.

Comment: Having regard to the findings described in Chapter 2 (2.3.3.4) relating to the adverse effect of using unspecified sources of amyloglucosidase on cereal products in general and even of amyloglucosidase (Boehringer) for the determination of NDF in corn-flakes and rolled oats (see Table 6), the method of Elchazly and Thomas cannot be expected to give valid results with these and similar products.

TABLE A-2. Reproducibility of dietary fibre values

Statistical constant (n=10)	Dietary fibre content in g/100 g dry matter			
	Wheat	Wheat flour 60% extr.	Rye	Rye meal flour 60% extr.
Mean value	8.6	1.9	7.95	3.90
S.D.	0.455	0.443	0.398	0.431
C	5.29	23.26	4.99	11.05

Taken from: Elchazly, M. and Thomas, B. (ref. 14).

10. Southgate, Hudson and Englyst (1978)

New ideas on fractionation procedures involving the measurement of water soluble and water insoluble non-cellulosic derived hexoses and pentoses using gas liquid chromatography are described by the authors (63).

Englyst's modifications of the Southgate method are described as providing more precise and informative results.

11. McQueen and Nicholson (1979)

The method devised by these authors (33) is based on the use of bacterial α-amylase at 40° for 12-18 h in pH 7 buffer before determination of NDF. The enzyme, α-amylase from Bacillus subtilis, is dissolved in 0.067M phosphate buffer, pH 7, at 0.1% concentration, filtered and 30 ml added to 0.4-0.6 g air dried sample to be analysed. After overnight incubation the NDF is determined as directed by Van Soest and Wine (78).

The bacterial α-amylase was found to contain some proteolytic activity but the hydrolysis of starch was considered to be more important. The enzyme was found to be most effective in reducing

both filtering time and the estimated NDF content (compared with the conventional procedure).

The NDF value for rolled oats using the α-amylase pretreatment procedure is given as 3.8 $\pm$ 0.3 and compares with 9.4 $\pm$ 1.8 by the conventional NDF procedure (results expressed as % of dry matter $\pm$ SD).

APPENDIX 3

EFFECT OF GEL FILTRATION ON CRUDE AMYLOGLUCOSIDASE (SIGMA) IN CITRATE-PHOSPHATE BUFFER

Materials and Methods

A solution of 1 g amyloglucosidase from Rhizopus spp (Sigma) in 30 ml 0.025M citrate-phosphate buffer at pH 8.0 was submitted to gel filtration using a column prepared from 10 g dry Sephadex G50 grade gel, particle size 20-80 µm, previously equilibrated with 0.025M citrate-phosphate buffer.

Fractions, each 10-12 ml, were collected and tested for amylolytic activity with 1% wheat starch solution buffered to pH 4.5.

Results

A development profile of absorbance measurements taken at 280 nm is shown in Fig. A-6. Most of the starch hydrolysing activity was concentrated in fractions 6-9 but weak activity was present in fractions well outside this range.

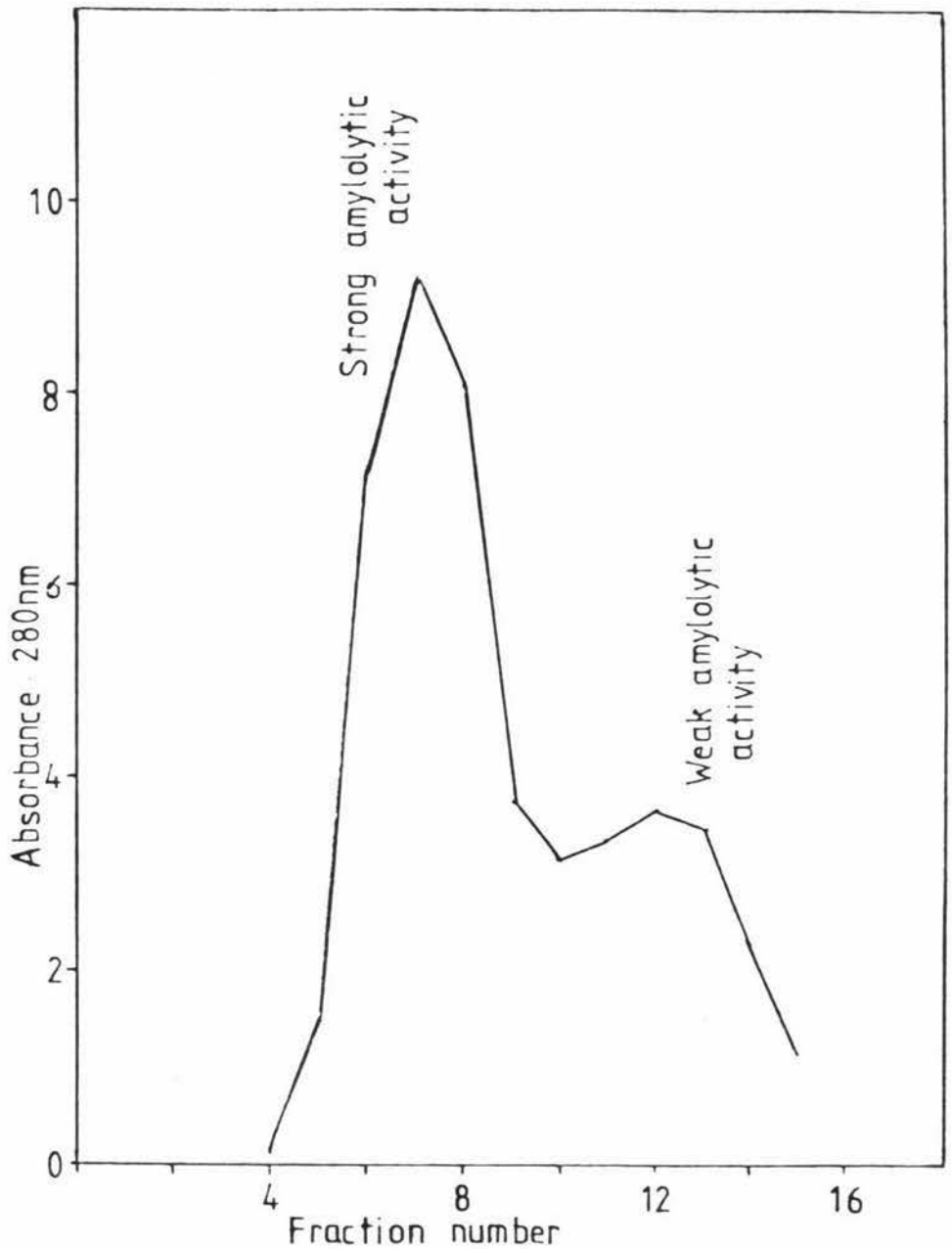

FIG. A-6 GEL FILTRATION PROFILE OF CRUDE AMYLOGLUCOSIDASE FROM *RHIZOPUS spp* USING SEPHADEX G50

Buffer : 0.025M citrate-phosphate, pH8
Sample weight : 1g

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METHODOLOGY IN DIETARY FIBRE ANALYSIS

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Most food composition tables include data relating to fibre content but unless the information is of very recent origin the values quoted will almost certainly be derived from crude fibre determinations. Today, in the context of food analysis, one speaks of 'dietary fibre' - a concept which has little in common with crude fibre but which is certainly much more meaningful.

Crude fibre is determined by a sequential extraction procedure depending on the use of boiling mineral acid and alkali. Dietary fibre, on the other hand, represents the residue from a comparatively mild form of extraction based usually on hot neutral detergents. It may be preceded by enzymatic hydrolysis.

Dietary fibre was defined by Trowell[1] in 1972 'as the remnants of the plant cell wall that are not hydrolysed by the alimentary enzymes of man'. Four years later, after further advances had been made in this area, Trowell, Southgate and others[2] proposed a new definition to include indigestible matter derived from the plant cell as well as from the cell wall. This would involve gums, mucilages, algal polysaccharides and the like. Redefined then, dietary fibre may be regarded as consisting simply of plant polysaccharides and lignin which are resistent to hydrolysis by the digestive enzymes of man. And there the matters rests for the time being.

The polysaccharide fraction of dietary fibre comprises cellulose, hemicellulose, pectin, gums and - in the view of some authorities - water soluble mucilages. In addition to polysaccharides and lignin small amounts of extensin protein and mineral matter are irretrievably bound to the fibre complex. With such heterogeneous material it is not surprising that food analysts have difficulty in producing consistent dietary fibre values for complex food products - and cereal products in particular.

To begin with the analyst has the choice of a number of official and unofficial methods. For example, the fibre may be extracted, dried and weighed, either with or without the aid of amylolytic or proteolytic enzymes. Or the determination may be approached in an entirely different manner if the analytical procedure pioneered by Southgate[3] is adopted. In this method the fibre is first separated into its

main components of hemicellulose, cellulose, pectin and lignin. The polysaccharide fractions are hydrolysed by means of mineral acid into simple sugars and uronic acids which are measured colorimetrically. The lignin is weighed. From this information it is possible to calculate the original unavailable carbohydrate and lignin present as dietary fibre.

Despite the variety of methods and modifications applicable to the analysis of dietary fibre the results from them appear to show a reasonable measure of agreement for many types of food material such as fruits and vegetables. However, where much starch or lipid is present discrepancies occur, and it was this feature of the analysis that attracted our attention at Massey and which provides the main topic of discussion for this paper.

The simplest and certainly the most convenient method of determining dietary fibre in foods is the one based on extraction with boiling neutral detergent devised by van Soest and his associates.[4] But, as Southgate has pointed out, the presence of starch can be a source of difficulty by causing frothing problems during the boiling stage with neutral detergent and by creating filtration problems in the final stage. These difficulties can be overcome by an initial hydrolysis of the starch using amyloglucosidase. What the analyst may not realise, however, is that only a pure enzyme is suitable for this operation. The relatively cheap crude fungal preparations such as one can obtain from Sigma may be excellent starch degraders, but they also attack hemicelluloses and possibly other components of dietary fibre. This unwanted interference can be demonstrated by plotting enzyme concentration against yield of fibre residue from food products such as Weetbix and rolled oats:

Figures 1 and 2

It will be seen that with increasing concentration of crude enzyme the fibre residue weight may fall off sharply at first and then more slowly as the saturation point is reached.

If, on the other hand, a relatively pure form of the enzyme is employed a substantially higher fibre residue weight will be recorded which is not significantly influenced by varying the concentration of enzyme. Such an enzyme may be obtained from Boehringer or Sigma - at a price! 100 mg costs about $30 at present day prices, which is about 1000 times more expensive than the crude preparations. Alternatively, as we have done, the crude enzyme can be purified in the laboratory by means of anion exchange chromatography using DEAE cellulose and a Tris-HCl buffer at pH 8.5

Fig 1 EFFECTS OF ENZYME PURITY AND CONCENTRATION ON RESIDUE WEIGHTS OF NEUTRAL DETERGENT FIBRE

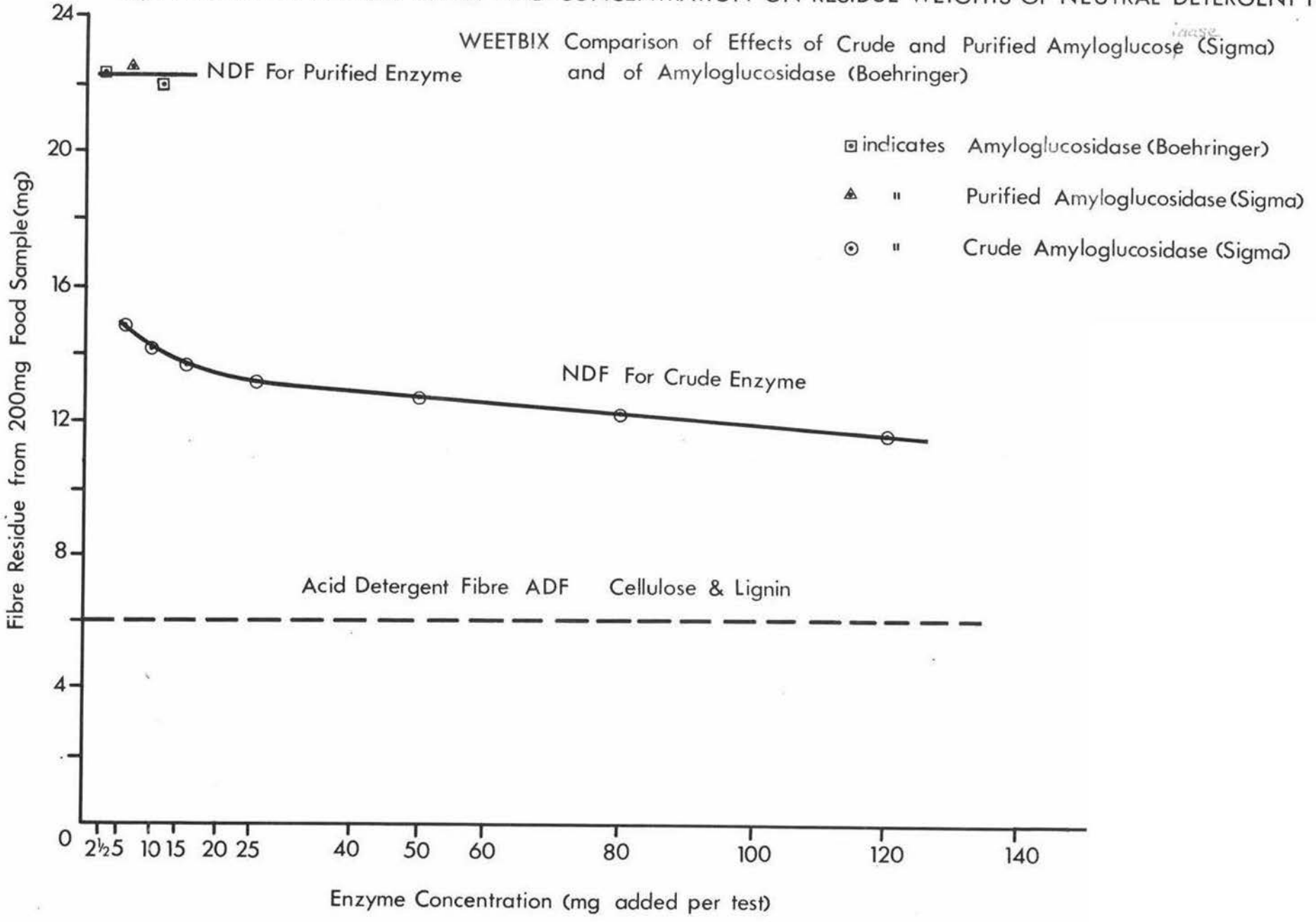
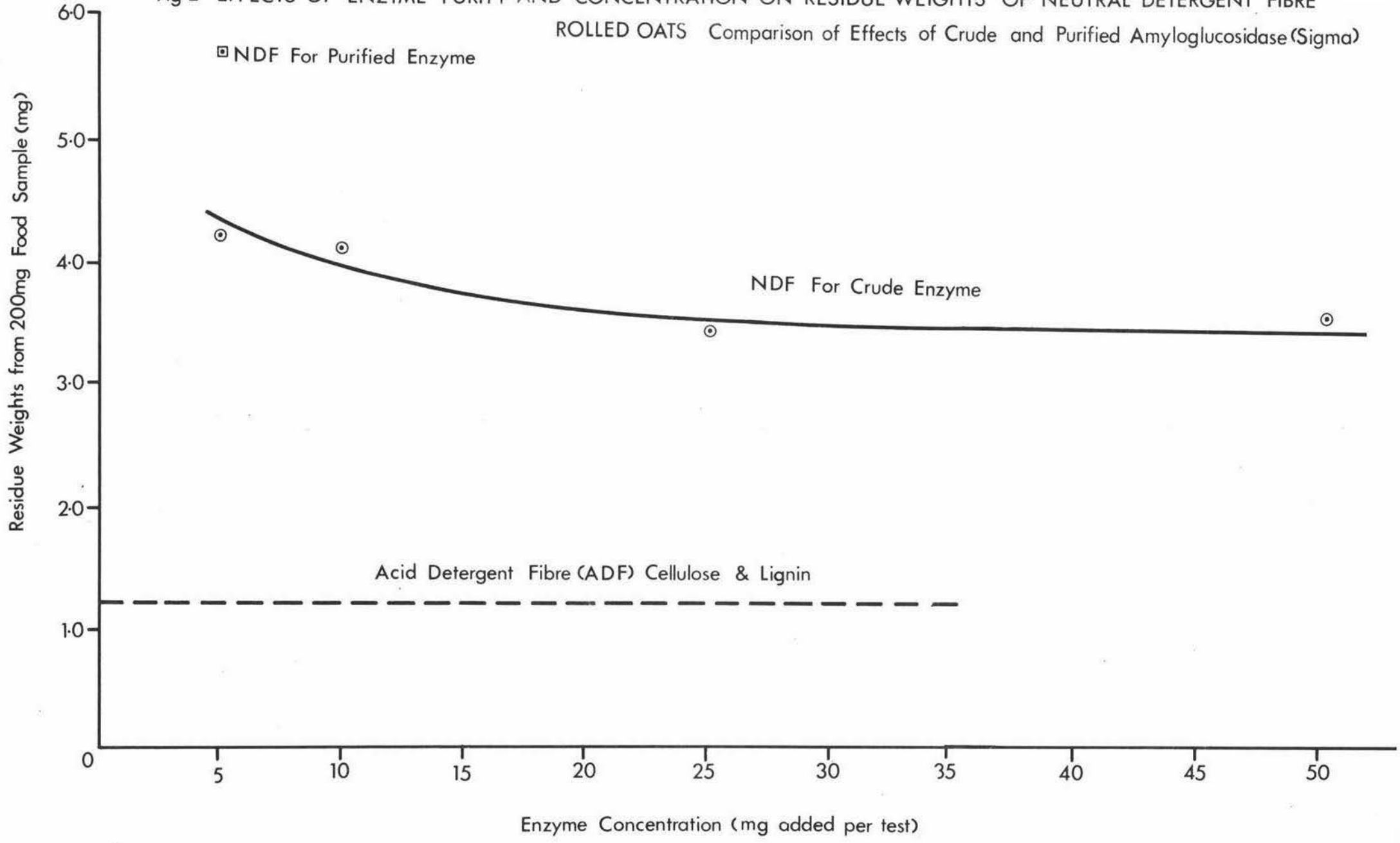


Fig 2 EFFECTS OF ENZYME PURITY AND CONCENTRATION ON RESIDUE WEIGHTS OF NEUTRAL DETERGENT FIBRE ROLLED OATS Comparison of Effects of Crude and Purified Amyloglucosidase (Sigma)



Whichever form of purified enzyme is used the results with Weetbix are very similar and strongly indicate that the residue weights obtained by this treatment are a true representation of dietary fibre content.

One way of minimising the cost of using purified amyloglucosidase is to employ the van Soest neutral detergent method on a semi micro scale. Unfortunately, this contrivance creates fresh difficulties although these are not serious. Firstly, there is the problem of preparing a subsample of food material in a sufficiently finely divided state that it will truly represent the material to be analysed. Southgate's preliminary procedure with a methanol and ether extraction is probably the best answer as after extraction of lipids and sugars the material is usually easily ground to a fine powder. The second problem is how to prevent fine particles of fibrous residue, produced by the grinding operation just mentioned, from passing through the sintered glass crucible during the filtration stage. It has been reported by Heller et al[6] that wheat samples ground through a 60 mesh sieve showed a loss of 20% in weight after neutral detergent extraction and filtration using a sintered glass crucible of coarse porosity. Fortunately, there is a simple answer to this problem. Instead of filtering the fibrous residue in an aqueous suspension and risking the loss of not only fine particles of fibre but of gelatinous hemicellulose-like material as well, the residue is washed by centrifugal means and dehydrated with acetone before transferring it to the sintered glass filter. In the dehydrated state no significant loss of fibrous particles is observed. The residue is then dried and accurately weighed.

By modifying the van Soest neutral detergent method as just outlined the new semi micro procedure requires only 200mg of air dried food material for each analysis. In our experience the results are both reliable and reproduceable. The cost factor arising from the use of purified enzyme preparations is kept well within acceptable limits as the amount of enzyme needed can be quite small. For example, 2½ to 5mg of amyloglucosidase Boehringer appears to be adequate for the hydrolysis of starch present in 200mg of most starchy cereal products, including rolled oats.

The quality of results achieved by the proposed semi micro technique depends to a considerable extent on the skill of the analyst, particularly in regard to the transfer of minute amounts of fibrous residue from one piece of glassware to another and also to the weighing of this small amount of material.

Some comparative results of the application of the new semi-micro technique to the analysis of breakfast cereals and bread are given in Tables 1 and 2, together with results obtained by more conventional means by other workers in this field.

Tables 1 and 2

The high value (10.4) quoted for the neutral detergent fibre content of rolled oats by the unmodified method (table 1) is probably accounted for by the contamination of the residue with starch. On the other hand, the lower value (9.1) quoted for the much less starchy Weetbix product, also by the unmodified method, is probably caused by the loss of gelatinous xylan polymers during the filtration stage.

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Table 1 - DIETARY FIBRE CONTENTS OF BREAKFAST CEREALS

(Expressed as percentage of dry weight)

Cereal Product	Starch Content	Neutral Detergent Fibre (N.D.F.) - van Soest's Method						
		Unmodified method			Modified method (Semi micro scale)		Unmodified Method N.D.F. Values quoted from other sources	
	%	range	mean	SD	range	mean	Spiller & Amen[1]	Holloway [2]
All-Bran Kelloggs	6	31.4-32.4 (2)	31.9		32.1-32.4 (2)	32.3	34.0	29.7
Weetbix	8	7.9-10.2 (6)	9.1	0.94	12.4-12.8 (2)	12.6		12.9
Cornflakes Kelloggs	27	4.0-11.0 (8)	6.9	2.22	4.9-5.1 (2)	5.0	7.9	
Rolled Oats	46	9.5-11.7 (4)	10.4		3.1-3.2 (2)	3.1	10.4	

NOTE: Numbers in parentheses indicate number of replicates

1. Crit. Rev. Fd. Sci & Nutr. 1975, 7, 44

2. Private communication 8th February 1977.

Table 2 - DIETARY FIBRE CONTENT OF BREAD

(expressed as percentage of dry weight)

Description of Bread	Neutral detergent fibre (N.D.F.) - van Soest's Method					
	Unmodified method	Modified Method (Semi micro method)	Unmodified method N.D.F. values quoted from other sources van Soest[1]	Southgate[1]	Spiller and Amen[2]	Holloway[3]
White	9.9(2)	2.7(2) 3.6(2)	2.86	(3.17)	3.3	4.2
Brown or wholemeal		6.7(2) 3.9(1) 5.0(2)	5.61	(5.62)	14.9	4.8
Fibre enriched		8.4(1) 13.2(2) 10.6(2)				

NOTE: Numbers in parentheses indicate number of replicates

1. Nutrition Reviews 1977, 35, 31-37
2. Crit. Rev. Fd. Sci & Nutr. 1975, 7, 44.
3. Private Communication 8th February 1977.

METHODOLOGY IN DIETARY FIBRE ANALYSIS

Determination of neutral detergent fibre by means of a semi-micro scale modification of Van Soest's method

REAGENTS

Amyloglucosidase, Boehringer Mannheim
Acetate buffer 0.5M pH 4.5
Neutral detergent. For method of preparation see van Soest and Wine [1]
Sodium sulphite, acetone.

APPARATUS

Refluxing apparatus: Berzelius beakers, 6 x 500 ml, and condensers made from 250 ml r-b flasks
Centrifuge tubes, 6 x 15ml cap. and 6 x 50 ml cap.
Sintered glass crucibles, coarse porosity, 6 x 20 ml cap.

DETERMINATION

Grind air dried food material to pass 18 or 20 mesh screen. If food material contains lipids and/or hard granular fragments extract 4-5g with successive 25 ml portions of boiling 85% V/V methanol followed by diethyl ether as described by Southgate[2]. Air dry residue and weigh to determine conversion factor for dry matter in original food material. Grind to small particle size.

Weigh 200mg finely ground food sample into 15ml centrifuge tubes. Add 5ml water or 7.5ml if much starch is present. To reduce evaporation loss insert small filter funnels into necks of tubes and place in boiling water bath for 20 minutes to gelatinize starch. Stir once with micro spatula or glass rod with rubber policeman to disperse solid material. Cool, add 1ml 0.5M acetate buffer pH 4.5 to each tube followed by 2½ to 5mg amyloglucosidase - the amount depends on the skill of the operator and on how much starch is present. Add 1 drop toluene and incubate tubes overnight at 37^{0}.

After enzyme action is complete centrifuge tubes and carefully discard supernatants. Transfer residues to 500ml tall beakers with 50 ml quantities of neutral detergent solution. Add 1 glass bead and 0.2g sodium sulphite to each beaker. Boil gently under reflux for 1 hour exactly. Allow to cool and transfer bulk of fibre residue suspensions to 50ml centrifuge tubes. Centrifuge and carefully remove supernatants. Wash remainder of residue into each centrifuge tube with 45ml hot water and with aid of glass rod and rubber policeman. Centrifuge and reject supernatants as before.

Add 20ml acetone slowly to each tube with swirling and carefully transfer dehydrated residues to previously weighed sintered glass crucibles, using a further 10ml acetone and aided by the rod and rubber policeman. Apply suction to remove surplus acetone, dry at 100^0 for 1 hour and cool in dessicator. Crucibles should be weighed to ± 0.1mg, always in the same order with a limit of 2 to 3 per desiccator and after a cooling time of precisely 10 min.

To determine ash contents ignite residues at 550^0 for 2-3hours and reweigh as directed above.

Calculate NDF values for original food material on dry matter basis.

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