PRODUCTION OF AN ENZYMIC CASEIN HYDROLYSATE USING A CONTINUOUS MEMBRANE BIOREACTOR

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JULIE MAREE O'SULLIVAN

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To Derek, Ryan and Lauren

ABSTRACT

Milk protein hydrolysates suitable for inclusion in hypoallergenic infant formulae should, in general, contain no material greater than 5000 daltons molecular weight and have less than 10% free amino acids. The aim of this study was to investigate the suitability of a continuous stirred tank membrane bioreactor for the production of such a hydrolysate from casein.

Thirteen commercial protease preparations were evaluated for their effect on casein. The most suitable enzymes for production of the target hydrolysate were selected on the basis of molecular weight profiles obtained by the use of high performance size exclusion chromatography. Novo Alcalase 2.4L and Amano Protease A were selected for the bioreactor experiments.

Hollow fibre polysulphone and spiral wound cellulose acetate membranes, each with nominal molecular weight cut off values of 10,000 and 30,000 daltons, were evaluated for their potential effectiveness in the bioreactor system. The spiral wound membrane with a nominal molecular weight cut off of 30,000 daltons was selected for the bioreactor experiments on the basis of molecular weight profiles of permeates obtained from the ultrafiltration of a casein hydrolysate. This membrane had a high flux, gave the highest recovery of peptides, was not susceptible to particulate fouling and gave the most desirable permeate molecular weight profile relative to the target product.

The effect of hydraulic residence time on the molecular weight profile of products produced using the bioreactor and on the productivity of the bioreactor was investigated. Using Amano Protease A and a short hydraulic residence time it was possible to produce a casein hydrolysate with a low proportion of free amino acids and a large proportion of material in the molecular weight range 3000-5000 daltons. Although the hydrolysate produced contained a small amount of material (approximately 2%) greater than 5000 daltons molecular weight, in all other respects the molecular weight profile of this hydrolysate met the criteria defined above.

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LIST OF ABBREVIATIONS OF UNITS

°C	degrees Celsius
g	gram
h	hour
kPa	kiloPascal
L	litre
m	metre
μm	micrometre
mg	milligram
min	minute
ml	millilitre
mm	milimetre
mol%	percent of peptides by number
nm	nanometre
psi	pounds per square inch
v/w	volume/weight

w/w	weight/weight
w/v	weight/volume

LIST OF OTHER ABBREVIATIONS

CPP	casein phosphopeptide
CSTMR	continuous stirred tank membrane reactor
CSTR	continuous stirred tank reactor
DH	degree of hydrolysis
E:S	enzyme to substrate ratio
HPSEC	high performance size exclusion chromatography
HPLC	high performance liquid chromatography
HRT	hydraulic residence time
MW	molecular weight
MWCO	molecular weight cut off
MWP	molecular weight profile
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis

CHAPTER 1

INTRODUCTION

Broadly speaking, protein hydrolysis is the process of breaking down proteins into peptides and amino acids. Industrially, three hydrolysis methods are available: alkaline hydrolysis, acid hydrolysis and enzymic hydrolysis.

Alkaline hydrolysis is low in cost but results in products with unpleasant flavours. Tryptophan remains intact but arginine and cystine are destroyed. Lysinoalanine, a compound reported to have detrimental nutritional effects, is also formed.

Acid hydrolysis is relatively low in cost, is rapid and is used to produce meaty and brothy flavourings. Nutritionally, acid hydrolysis has the disadvantage of destroying tryptophan.

Enzymic hydrolysis utilizes proteolytic enzymes to break down the protein to peptides and amino acids. The proteolytic reaction, as it occurs between pH 5 and pH 7, can be described by the following equation:

Proteolytic enzymes or proteases are classified according to their source (animal, plant or microbial), their catalytic action (endopeptidases or exopeptidases) and the nature of the cleavage and active sites. Endopeptidases cleave the protein at any point along the protein's amino acid chain whereas exopeptidases cleave one amino acid at a time from either or both ends of the polypeptide chain.

Proteolytic enzymes catalyse the hydrolysis of protein molecules. Trypsin, a digestive protease, is a well-known example of a proteolytic

enzyme; it catalyses the cleavage of peptide bonds on the C-terminal side of lysine and arginine. The rate of hydrolysis is not the same for all of the lysine and arginine residues. Some parts of the protein are more accessible to the enzyme than others and, as hydrolysis proceeds, other sites become available. Hence the bonds of the protein that are cleaved by the enzyme are generally cleaved in a certain order.

In enzyme technology, the word "enzyme" usually refers to a commercial enzyme preparation which may contain several individual enzyme proteins plus compounds for standardizing and stabilizing the preparation. For example, a commercial preparation of pancreatin may contain the enzymes trypsin, chymotrypsin and elastase and, hence, the overall specificity of pancreatin will be a result of the individual specificities of the component enzymes of the preparation.

As hydrolysis of a protein proceeds, the functional properties of the protein change. A small amount of hydrolysis (low degree of hydrolysis (DH)) can improve the functionality of the protein without any detrimental effect on the flavour. For example, hydrolysis can result in a peptide mixture with substantially increased solubility compared with the original protein. A hydrolysate may even be completely soluble at the isoelectric point of the original protein (Mahmoud, 1994). As hydrolysis proceeds, however, bitterness usually develops along with other brothy and meaty flavours. These flavour defects are a major limitation to the use of protein hydrolysates in food products.

Proteolysis has been used as a method for preserving and improving the taste of food for centuries. The Japanese took advantage of proteolysis to create soya sauces. The French used the reaction to produce cheese. In more recent times, hydrolysates based on milk protein have been used as a source of highly digestible protein in nutritional products for patients with Crohn's disease, ulcerative colitis, short bowel syndrome, fistulae, pancreatitis, severe trauma and food allergies and for protein supplementation diets for the elderly, sports nutrition and weight control

diets. These uses for protein hydrolysates have recently been reviewed by Schmidl *et al.* (1994) and Frøkjaer (1994).

There are other advantages to be gained from hydrolysing milk proteins. Some of the peptides generated by hydrolysis of milk proteins, casein in particular, have physiological and immunological effects. One of the most well-known of these peptides is casein phosphopeptide (CPP). CPP actually refers to a group of phosphopeptides, derived by tryptic hydrolysis of α_{s1}-, α_{s2}- and β-caseins, that enhance the absorption of calcium and iron. The range of physiologically and immunologically functional peptides, their origin and function have been reviewed by Meisel & Schlimme (1990), Mills *et al.* (1992) and Fox & Flynn (1992).

A major use of milk protein hydrolysates is in hypoallergenic infant formulae which are specially designed to be fed to infants that are, or have the potential to become, allergic or intolerant to cows' milk protein. The term allergy can be defined as an immunologically mediated hypersensitivity involving IgE, IgG, IgA (types of immunogens) and/or cell-mediated mechanisms. The term intolerance usually refers to an undesirable reaction, such as asthma, eczema and rhinitis, to a certain food or ingredient. "At risk" infants are those who have either one or both parents who are allergic or intolerant to milk protein. The incidence of allergy to milk protein is variously estimated at between 0.3% and 7.5% of live births. Infants who are intolerant or allergic to cows' milk protein are usually not able to be fed soy or other animal milk protein as a substitute for cows' milk protein. However, peptides produced by the hydrolysis of milk protein can be fed to these infants because peptides are not usually recognized by the body as allergens or immunogens. The production of a protein hydrolysate suitable for hypoallergenic infant formulae involves some challenges, however. The resulting hydrolysate must be completely non-allergenic and have an acceptable taste. The hydrolysate must also be low in free amino acids in order to avoid problems such as osmotic diarrhoea in the infants that consume it and the possible loss of nutritional quality as a result of

chemical reactions of the free amino acids. Furthermore, it has been reported that di- and tri-peptides are better absorbed in the digestive tract than the equivalent mixture of free amino acids (Mathews & Payne, 1975; Adibi *et al.*, 1987).

A wide range of milk protein hydrolysate products is possible. The final product depends on the selection of substrate and enzyme and the hydrolysis conditions. The following parameters define a hydrolytic process: the substrate and enzyme; concentration of substrate; enzyme to substrate ratio; pH; temperature; DH or hydrolysis time; enzyme inactivation conditions; post-hydrolysis treatments such as filtration, carbon treatment, precipitation, neutralization, evaporation and drying. The general manufacturing procedure for a milk protein hydrolysate is outlined in Figure 1.1.

Ultrafiltration is often used after hydrolysis to remove residual intact protein and large peptides. A variation on this process is to carry out ultrafiltration and hydrolysis simultaneously in an "enzyme bioreactor". The enzyme bioreactor technique was originally invented as a method to save on enzyme costs and to achieve complete conversion of the substrate to peptides and amino acids. In the enzyme bioreactor system, the retentate from the ultrafiltration part of the process is recycled back to the hydrolysis reaction vessel. The enzyme remains in this retentate stream and is, therefore, effectively recycled back to the reaction vessel to hydrolyse more protein and peptides. Peptide material can pass through the ultrafiltration membrane only if it is small enough. Protein and peptide material that is too large to pass through the membrane is recycled back to the hydrolysis reaction vessel for further hydrolysis. Therefore, the efficiency of the hydrolysis reaction is further increased because products that may inhibit the reaction are continuously removed from the reaction vessel and because fresh substrate, and, hence, the most susceptible bonds for hydrolysis, is always available to the enzyme. That peptide material is removed as soon as it is small enough to pass through the ultrafiltration membrane, may also

prevent the enzyme from hydrolysing the protein to very small peptides and free amino acids, but this will depend entirely on the specificity of the enzyme and the order in which peptide bonds are cleaved.

The purpose of this work was to evaluate enzyme bioreactor technology for the manufacture of a casein hydrolysate, with a defined molecular weight distribution, suitable for use in hypoallergenic infant formulae.

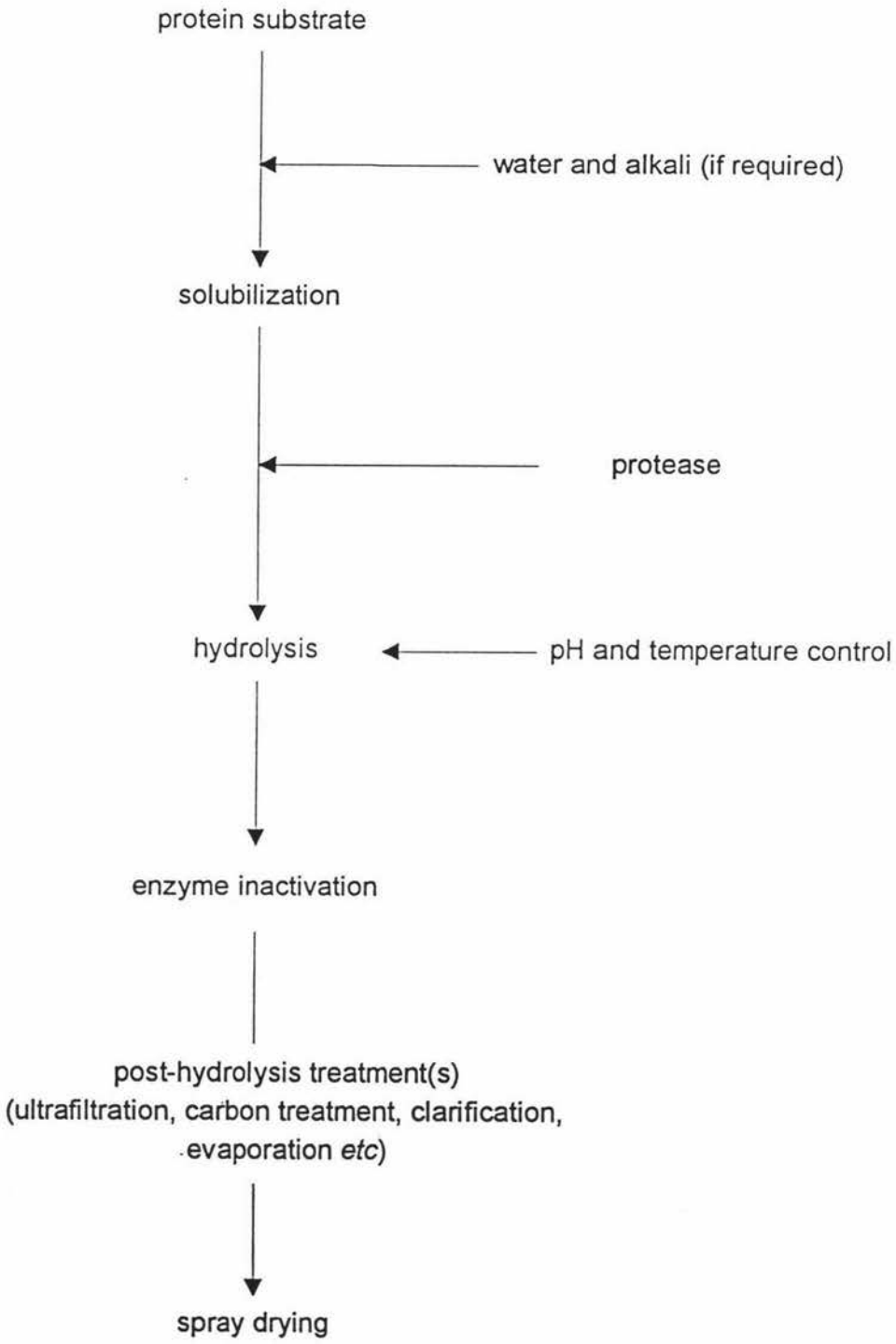


Figure 1.1 A general scheme for the manufacture of a milk protein hydrolysate.

CHAPTER 2

LITERATURE REVIEW

2.1 Protein Hydrolysates in Infant Formulae

2.1.1 Milk-protein-based infant formulae

Making cows' milk more like human milk has been the focus for the development of infant formulae for many years. One of the major differences between cows' milk and human milk is the ratio of casein to whey protein. Cows' milk has a casein to whey ratio of 80:20 whereas human milk protein has a ratio of 30:70. In addition, the types and ratios of the individual proteins vary; for example, both α_{s1}-casein and β-lactoglobulin are present in cows' milk but not in human milk. Matsuda & Nakamura (1993) cite β-lactoglobulin as the most allergenic component of cows' milk and casein (α_{s1}-casein in particular) is also reported to be allergenic. Cows' milk is also harder for the infant to digest. It forms dense and elastic curds in the infant's stomach whereas human milk forms soft, loose curds. The formation of dense curds can delay gastric emptying, leading to problems such as spitting-up (Nakai & Li-Chan, 1987).

In order to adjust the casein to whey protein ratio of cows' milk closer to that of human milk, demineralized whey powder has been added to skim milk to form the protein component of modern commercial infant formulae. Although this makes the composition of the protein fraction of the cows'-milk-based formula more like that of human milk, it does not reduce its allergenicity or the properties of the curds formed from it during digestion.

Soy-protein-based formulae are often prescribed for children who are allergic to milk protein but soy protein can be as allergenic as cows' milk protein (Chandra *et al.*, 1989). In addition to this, Lönnerdal & Glazier (1991) have shown that trace elements are not well absorbed from soy-

protein-based formulae. It is also known that soy protein contains antinutritional factors, which are not destroyed by processing, such as trypsin inhibitors, lectins, plant phyto-estrogens and phytate (Liener, 1994).

Whey protein allergenicity can be reduced, and its curd structure improved, by heat treatment. In general, denaturation of whey proteins by heat causes changes in the tertiary protein structure and hence can destroy the antigens. However, heat treatment can make the protein insoluble. In an attempt to reduce this undesirable effect, manufacturers have decreased the amount of heat treatment used, with the consequence that low allergenicity cannot be guaranteed because denaturation is incomplete (Jost *et al.*, 1987). Casein is extremely heat stable and, whereas heat treatment can be used to decrease the allergenicity of whey proteins, the only way to reduce the allergenicity of casein is by hydrolysis.

2.1.2 Protein-hydrolysate-based formulae

The role of protein hydrolysates in infant nutrition has been reviewed by Levy *et al.* (1991) under a Federal Drug Administration (USA) contract. These authors listed the medical problems of infants requiring hydrolysates, and included allergy, sensitivity to whole protein, abnormalities in digestion, inborn errors of metabolism and infantile colic. Hydrolysates also have the additional advantage that di- and tri-peptides are better absorbed than free amino acids (Mathews & Payne, 1975; Adibi *et al.*, 1987). Enzymic hydrolysis of proteins can involve the use of single enzymes or mixtures of enzymes to simulate digestion. Hydrolysates are often filtered and/or charcoal treated to remove protein-like residuals. Overcoming allergies to milk protein requires more severe hydrolysis of the protein than does overcoming gastrointestinal and metabolic disorders. Casein has been traditionally used for hydrolysis because it is a readily available protein of high nutritional quality. Whey protein has been used less because it is not so readily available and because it contains the allergen β-lactoglobulin.

Peptide (also called semi-elemental) formulae based on extensive

casein hydrolysates have been available for more than 50 years. The first of these formulae was Nutramigen which was released by Mead-Johnson in 1941. These semi-elemental diets have proved to be well tolerated by infants with allergies. However, the prolonged use of these formulae is complicated by low palatability and high costs. Brady *et al.* (1986) stated the disadvantages of Nutramigen to be the high cost (three times that of standard infant formulae), difficulty mixing the product and unpalatability for older infants (over 6 months). In addition to these disadvantages, the use of semi-elemental diets is not fail-safe. There have been reports of severe, and even life-threatening, allergic reactions to formulae such as Nutramigen and Pregestimil (Mead-Johnson), which are generally considered to be the casein-hydrolysate-based products of choice for allergic infants (Saylor & Bahna, 1991).

Cows' milk allergy is an immunopathy which results when cows' milk proteins are absorbed intact from the intestinal mucosa and antibodies are produced against them (Otani, 1992). Infants are thought to be more susceptible to cows' milk allergy during the first few months of life, when the digestive tract is not completely formed and cows' milk protein can easily pass through.

Knights (1985) stated that, based on molecular weight alone, hypoallergenic infant formulae should not contain any peptides greater than 1200 daltons molecular weight. This statement is supported by Jost *et al.* (1987), who found that hydrolysis on its own may be insufficient to deal with the antigens present in whey and claimed that complete transformation of the protein into peptides was essential to preventing allergic reactions. Mahmoud *et al.* (1992) found that hydrolysis of casein by pancreatin gave allergenicity reduction that followed three-phased first order kinetics. Furthermore, Mahmoud *et al.* (1992) found that there was extensive loss of allergenicity during the first 10% of hydrolysis time. Siemensma *et al.* (1993), however, claimed that the relationship between DH and allergenicity is unknown.

The manufacture of protein hydrolysate formulae depends on selective enzymic hydrolysis, and, in the case of whey protein, heat treatment, to reduce the allergenicity of the protein. The main challenges are to choose enzymes that will reduce the allergenicity and to control the hydrolysis reaction to minimize bitterness. Protein-specific epitopes are a function of the tertiary structure of the protein. The epitopes could be broken down at an early stage and at a low DH. However, sequential epitopes, which are a result of the amino acid sequence of the protein, require much more extensive hydrolysis. A high DH is, in general, achieved at the expense of functionality (particularly emulsification of fat) and flavour. Traditional protein-hydrolysate-based formulae contain about 60% free amino acids (Otani, 1992). The brothy, meaty flavour of free amino acids is unpleasant and is often not tolerated by infants over the age of 6 months. In addition to their unpleasant taste, free amino acids are not as well absorbed by the digestive system as di- and tri-peptides.

Lee (1992) described processing effects to alter the allergenic potential of milk-based formulae. Partial hydrolysis of proteins will give large peptides whereas extensive hydrolysis will result in large and small peptides and free amino acids. Hydrolysates are often ultrafiltered to remove these large peptides and unhydrolysed casein or whey protein. Free amino acids often need to be added to the final product to restore the amino acid balance that has been altered as a consequence of ultrafiltration.

2.1.3 The case for prophylactic use of protein-hydrolysate-based infant formulae

There have been numerous reports of allergic reactions to whey-protein-hydrolysate based formulae. Saylor & Bahna (1991) claimed that Nestlé's Good Start contains 0.5-1% intact whey protein, whereas Businco & Cantani (1991) suggested that β-lactoglobulin and α-lactalbumin peptide epitopes and traces of unhydrolysed casein could be the cause. Ellis *et al.* (1991) implicated Nestlé AlfaRé and Good Start as being allergenic. Lorenz

et al. (1988) showed that, although some formulae (including AlfaRé) had hardly any detectable proteins greater than 10,000 daltons molecular weight, casein epitopes were detected by an immunologically-based ELISA test. Sampson *et al.* (1991) considered Alimentum to be as safe as Nutramigen and Pregestimil but the flavour was not acceptable to older children. These workers also found that Good Start contained a significant portion greater than 4000 daltons molecular weight as well as immunologically detectable cows' milk protein, and, therefore, recommended that this formula not be given to allergic infants. Businco *et al.* (1989) reported that AlfaRé can produce allergic reactions. They also reported that the whey protein hydrolysate used in AlfaRé had a DH of 25-30% and contained 20-25% free amino acids. The molecular weight distribution of the peptides contained in the formula is 45-50% less than 500 daltons, 25-30% 500-6000 daltons and 0.1-0.3% greater than 6000 daltons. Intact proteins were not detected; therefore epitopes are probably present in this formula.

Following the backlash against whey protein hydrolysates, there have been a number of studies (some sponsored by infant formula manufacturers) showing the efficacy of whey protein hydrolysates in prophylactic formulae. Prophylactic formulae for non-allergic infants have been defined by Leary (1992) as formulae with sufficient reduction in allergenicity to delay or prevent sensitization that may cause clinical symptoms of allergy. Formulae for allergic infants, on the other hand, should have minimal residual molecular structure required for recognition of antigen by the infant's immune system and, therefore, require extensive hydrolysates. Prophylactic formulae may contain partial hydrolysates not intended for consumption by the allergic infant. Mallet & Henocq (1992) carried out a longterm study and found that early feeding of protein hydrolysate formulae to at risk children had a long term preventative effect on the prevalence of asthma. Schmitz *et al.* (1992) showed that postponing the introduction of cows' milk by 4-6 months may reduce the

subsequent occurrence of allergy in children from allergic families. Guersry *et al.* (1991) stated that breast feeding can prevent allergy but only if it is exclusive for the first 4-6 months of life and the mother avoids allergens in her diet. These authors defined the difference between therapeutic and prophylactic formulae to be that prophylactics may still give a positive skin test in highly sensitized children.

Moran (1992) showed that feeding whey-protein-hydrolysate-predominant formulae to infants resulted in lower levels of some indicators of atopic reactions. The nutritional adequacy of the whey-protein-hydrolysate-based formulae was similar to that of breast milk and a non-hydrolysed-whey-predominant formula.

2.1.4 Defining the DH of protein-hydrolysate-based formulae

There have been calls for standardization of the definition of the term hypoallergenic formula(e). Kleinman (1992) stated that hypoallergenic formulae should be subjected to rigorous pre-clinical and clinical testing, and that as a minimum the formulae should not provoke allergic signs or symptoms in 90% of infants with documented cows' milk allergy when tested in double-blind, placebo-controlled trials. Bindels (1992) made a similar statement when he said that 90% of subjects with a proven allergy to cows' milk protein should tolerate an experimental formulation with 95% confidence. Leary (1992) also called for controlled clinical studies to demonstrate conclusively the biological efficacy of hypoallergenic formulae and placed the responsibility for this with the infant formula manufacturers.

Levy *et al.* (1991) defined complete hydrolysis as 90-99% DH and partial hydrolysis as < 50% DH. However, a more recent classification scheme for protein hydrolysates in infant formulae is that proposed by Siemensma *et al.* (1993). According to these authors, hypoallergenic formulae based on milk proteins can be classified according to the nature of the hydrolysate used. Protein hydrolysates are characterized as first, second or third generation.

First generation hydrolysates are defined as mostly casein-based hydrolysates with high levels of free amino acids (70 mol%). In general, the remaining 30 mol% of the hydrolysate is made up of peptides with less than eight amino acid residues. The two products discussed previously, Nutramigen and Pregestimil, are examples of first generation hydrolysate formulae.

Second generation hydrolysates are usually whey-protein-based hydrolysates with, typically, 40-60 mol% free amino acids. Peptides up to 10-12 amino acid residues long make up the remaining material in these hydrolysates. Most of the whey-protein-hydrolysate-based formulae containing second generation hydrolysates have been on the market for 10-20 years.

Third generation hydrolysates have less than 20 mol% free amino acids and contain detectable peptides with up to 15 amino acid residues. According to Siemensma *et al.* (1993), these third generation hydrolysates are nutritionally superior to previous generations of hydrolysates. Absorption of di- and tri-peptides is more rapid than for the equivalent amount of free amino acids and peptides are less hypertonic than amino acids, so the use of these formulae reduces osmotic problems such as chronic diarrhoea. Hydrolysates also taste better than the equivalent amount of free amino acids. Another advantage of hydrolysates is that the transport of di-peptides in the gut is not affected as much as the transport of free amino acids in the presence of carbohydrates.

Mahmoud (1994) stated that there is no precise, widely accepted specification for extensive and partial hydrolysates. He proposed the following scheme for the classification of hydrolysates (Table 2.1). This scheme is illustrated graphically in Figure 2.1. Mahmoud's classification scheme describes slight and extensive hydrolysates well but is deficient in the description of moderate hydrolysates. Moderate hydrolysates can have a molecular weight distribution that is skewed much more towards the low molecular weight end of the scale than shown in Mahmoud's scheme.

Graph d in Figure 2.1 shows an additional molecular weight distribution that could also be described as moderate. This extension to Mahmoud's classification scheme is required to adequately describe the range of protein hydrolysates used in infant formulae.

Table 2.1 Classification of protein hydrolysates (Mahmoud, 1994)

Molecular Weight Fraction (daltons)	DH		
	a. Slight	b. Moderate	c. Extensive
	% in each fraction		
< 500	3.8	9.8	90.2
500-1000	1.1	13.5	5.5
1000-2000	1.8	13.7	2.9
2000-5000	3.7	16.6	1.4
> 5000	89.7	46.4	0

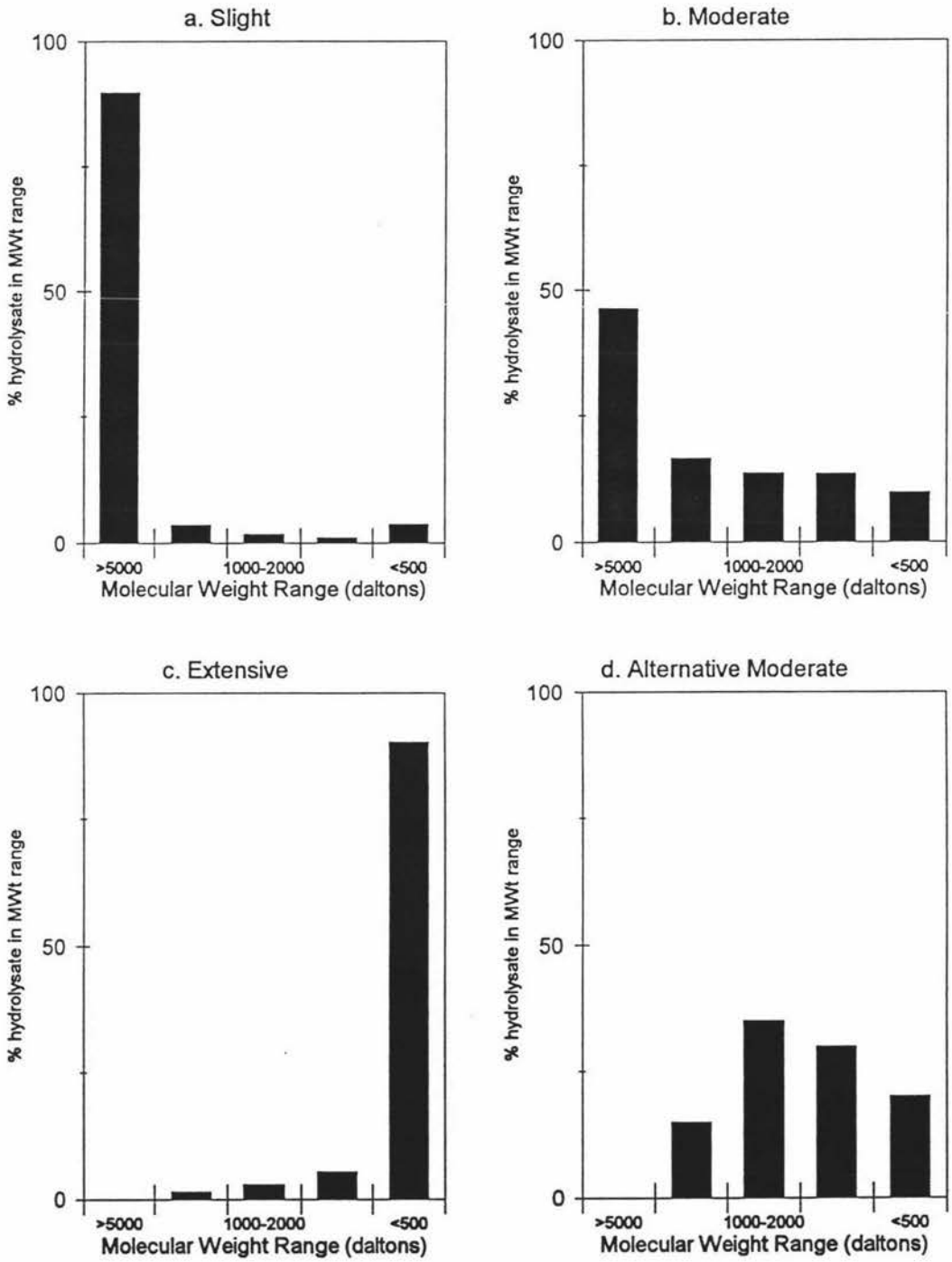


Figure 2.1 Molecular weight distribution graphs drawn from the hydrolysate classification scheme of Mahmoud (1994).

2.1.5 Manufacturing procedures for protein hydrolysates for use in hypoallergenic infant formulae

Knights & Manes (1987) have described a general scheme for the manufacture of a casein hydrolysate. This procedure has been used since the 1940s by Mead-Johnson to produce first generation casein hydrolysates. Casein is treated with a combination of pancreatic proteases under conditions of controlled pH and temperature for a specified time. The enzymes are then denatured by heating the hydrolysate. The resultant hydrolysate is then treated with activated charcoal to remove residual antigens. This carbon treatment step also improves the flavour of the hydrolysate, mainly by the removal of tyrosine (which contributes to bitterness). Jost & Pahud (1988) have described the procedures for the production of whey protein hydrolysates for use in the Nestlé products AlfaRé and Beba HA. In the case of AlfaRé, whey protein isolate is hydrolysed with a combination of trypsin, chymotrypsin, elastase and carboxypeptidases to achieve a DH of approximately 30%. This hydrolysate is then heat coagulated and fractionated to produce a lactose-free oligopeptide fraction with 21% free amino acids. The molecular weight distribution of the product is as follows: < 500 daltons, 72.7%; 500-1000 daltons, 26.7%; 1000-1500 daltons, 0.6%; > 1500 daltons, 0%. This could be described as a second generation hydrolysate.

The Beba HA formula is manufactured from partly demineralized whey and ultrafiltered whey. These substrates are hydrolysed by trypsin and chymotrypsin (15:1) with intermediate heating steps to give a DH of about 18%. This hydrolysate contains less than 2% free amino acids and, hence, could be classified as third generation. However, peptides greater than 4000 daltons molecular weight have been suspected of causing allergic reactions to this product and to Nestlé's equivalent products Nan HA and Nidina HA.

2.1.6 The physiological role of milk-protein-based peptides

Otani (1992) stated that the challenge is to develop hydrolysates in which only immunogenic structures or antigenicity have been selectively destroyed so that the physiological function of the protein is maintained. Casein and its digestion products, which arise naturally from the hydrolysis of casein by enzymes in the gut, have been shown to have a physiological role. The physiological and immunological functions of milk-protein-based peptides have been reviewed by Meisel & Schlimme (1990), Mills *et al.* (1992) and Fox & Flynn (1992). The bioactive peptides derived from bovine milk proteins are listed in Table 2.2.

Table 2.2 Bioactive peptides derived from bovine milk proteins (Meisel & Schlimme, 1990)

Bioactive peptide	Protein precursor	Bioactivity
Casomorphins	α- and β-Casein	Opioid[1] agonists (elicits opioid response)
Casoxins	κ-Casein	Opioid antagonists (suppresses opioid response)
α-Lactorphin	α-Lactalbumin	Opioid agonist
β-Lactorphin	β-Lactoglobulin	Opioid agonist
Immunopeptides	α- and β-Casein	Immunostimulants
Casein phosphopeptides	α- and β-Casein	Mineral carriers

[1] **Having pharmacological similarities to opium *e.g.* prolongs gastro-intestinal transit time, has anti-diarrhoeal properties.**

Hachimura *et al.* (1993) have presented evidence that there are immuno-suppressive fragment(s) in tryptic digests of casein and suggested that these may be of use in preventing allergy. In this study mice were fed a tryptic digest of casein that contained peptides smaller than 6000 daltons molecular weight. The mice that were fed the digest responded poorly to subsequent immunization with casein, indicating that the animals had developed oral tolerance to casein.

2.1.7 The "ideal" protein hydrolysate for use in hypoallergenic infant formulae

In summary then, the "ideal" protein hydrolysate for use in hypoallergenic infant formulae can be defined as follows. The hydrolysate should have a low proportion of free amino acids (< 10%) in order to minimize the negative flavour contribution of these free amino acids and to prevent problems such as osmotic diarrhoea in infants consuming a formula containing the hydrolysate. The hydrolysate should not contain any peptides greater than 5000 daltons molecular weight in order to reduce allergenicity and it should contain the greatest proportion possible of peptides in the molecular weight range 1500-3500 daltons to maximize the possibility of retaining the physiological function of the peptides. Traditional batch-type hydrolysis processes alone, such as those described earlier, cannot be used to produce hydrolysates with these properties. Hydrolysates from these processes contain either a high proportion of free amino acids or a large proportion of material with molecular weight greater than 5000 daltons. Additional technologies, such as ultrafiltration, are required to produce hypoallergenic, third generation hydrolysates.

2.2 Protein Hydrolysate Production Using a Continuous Membrane Bioreactor

2.2.1 A comparison of batch and continuous hydrolysis processes

Protein hydrolysates are usually manufactured using batch-type operations, with or without post-hydrolysis processes such as ultrafiltration. Batch hydrolysis reactions have been carried out for many years and are, in general, simple processes. There are some advantages inherent in the familiarity and simplicity of such reactions (Adler-Nissen, 1986).

However, batch hydrolysis reactions have some disadvantages. Batch reactions must be carefully controlled. Small variations in process parameters (such as pH, temperature, hydrolysis time, substrate concentration or enzyme to substrate ratio) could result in batches of product with slightly different molecular weight distributions. These differences would be particularly significant for product destined for use in hypoallergenic infant formulae.

Long times are needed to complete the batch hydrolysis reactions. This is related to substrate depletion, product inhibition and decreasing enzyme concentrations in the hydrolysis vessel. Cheryan & Deeslie (1980) have stated that batch reactions rarely go to completion due to product inhibition; therefore, yields are less than optimum.

Capital costs for equipment can be high due to low productivity (Cheryan, 1986). In addition to this, batch processes are inherently less efficient than continuous processes due to their start-up and shut-down nature.

Extensive post-hydrolysis treatments are usually required before drying, to "make the product useful" (Cheryan & Deeslie, 1980). For example, ultrafiltration is often used to remove large peptides and any unhydrolysed material from the hydrolysate so that it can be used for hypoallergenic applications.

Batch reactions usually have an enzyme to substrate ratio in the

range from 1:100 to 1:300. The enzyme is not reused or recovered, leading to low productivity and high costs per unit of enzyme used. The enzyme is usually inactivated by heating the hydrolysate, which not only adds to the processing costs but also can lead to reduced nutritional quality of the hydrolysate as a result of Maillard reactions and lysinoalanine formation.

The advantages of continuous hydrolysis processes over batch hydrolysis processes have been described by various authors (Iacobucci *et al.,* 1974; Cheryan & Deeslie, 1980; Deeslie & Cheryan 1981a, 1981b, 1988; Cheryan, 1986). There are two approaches to continuous enzymic hydrolysis of protein: hydrolysis using immobilized enzymes and hydrolysis using an enzyme bioreactor. According to Cheryan (1986), the major disadvantages of enzymes immobilized on to solid supports are: (a) loss of enzyme activity (as much as 90%) due to steric hindrance, enzyme-substrate orientation and diffusional restriction; (b) immobilization is expensive; and (c) there can be problems with gas hold-up and high pressure drops. Cheryan (1986) described hollow fibre bioreactors as an alternative form of "immobilization". Takahata *et al.* (1991) described a process that utilizes both bioreactor and immobilization technologies simultaneously. One of the advantages of bioreactor-type continuous hydrolysis processes is the inherent efficiency of a continuous process. Batch processes are usually energy and labour intensive and the equipment may require more floor space than for the equivalent continuous-mode equipment (Deeslie & Cheryan, 1981b). However, in some industries, such as the dairy industry, existing equipment may be utilized for batch hydrolysis processes.

In a bioreactor process, the enzyme is theoretically retained in the system and is re-used. The ultrafiltration membrane is chosen so that only permeation of the product occurs, and the enzyme is retained on the retentate side of the membrane. Re-using the enzyme allows higher enzyme to substrate ratios to be used without adding to the cost of the

process.

Furthermore, inhibitory end-products are continuously removed provided they are small enough to permeate the membrane. This prevents the enzyme from losing activity due to product inhibition as hydrolysis proceeds.

Because an ultrafiltration membrane is used, the product stream is free of cells and particulate matter; hence, downstream costs are reduced. Control of the molecular weight of the product is possible by choosing the appropriate membrane size, *i.e.* the upper molecular weight of the product can be controlled by the selection of the membrane. At constant ultrafiltration membrane flux, steady state is reached quickly and quantitative conversions of protein to peptides can be sustained for extended periods of time at constant rates of production. In addition to this, high throughputs can be used in a bioreactor system so that productivity is higher than for an equivalent batch system.

Continuous hydrolysis in a bioreactor has the further advantage that the control of process parameters such as pH and temperature is easier. This is because there is only one vessel in which the hydrolysis reaction takes place rather than a series of batch vessels or a series of batches. This feature of continuous hydrolysis processes reduces batch-to-batch variation in the product.

Continuous hydrolysis processes also have disadvantages. The process is complex and may, therefore, require specially trained operators. The choice of enzyme for use in the bioreactor must be made carefully. The enzyme must maintain activity and stability over several hours at relatively high temperatures (Deeslie & Cheryan, 1981a; Boudrant *et al.*, 1976). If the protein substrate is difficult to hydrolyse then un-hydrolysed material can build up in the membrane reactor (Deeslie & Cheryan, 1981b). If this material is subsequently bled from the reactor then the resultant enzyme losses can result in a level of enzyme use approaching that of batch processes (Iacobucci *et al.*, 1974). Finally, control of concentration

polarization is essential to the avoidance of membrane fouling and subsequent failure of the reactor (Cheryan, 1986).

2.2.2 The continuous stirred tank membrane reactor (CSTMR)

Three different configurations for continuous membrane hydrolysis have been described in the literature. Cheryan & Deeslie (1980) have described "dead-end cell" and CSTMR processes and Turgeon & Gauthier (1990) have described a two-step continuous hydrolysis and ultrafiltration process based on the CSTMR design.

The dead-end cell was first described by Blatt *et al.* (1968) who used the technique to hydrolyse whey protein with chymotrypsin. The configuration of the dead-end cell is shown schematically in Figure 2.5. An ultrafiltration "cell", maintained at the reaction temperature, is charged with substrate and enzyme and the cell is then pressurized. As hydrolysis proceeds, the substrate is broken down into peptides small enough to permeate the ultrafiltration membrane. This permeate is the reactor product. Fresh substrate is added to the cell as the permeate is collected in order to maintain a constant reaction volume in the cell. The main problem with these types of continuous reactors is that the flux decreases over time due to concentration polarization and membrane fouling (Cheryan & Deeslie, 1980).

The development of hollow fibre membranes made a second type of continuous hydrolysis system achievable. Continuous tangential flow across a membrane helps to reduce the phenomena associated with decreasing flux. This system, called a continuous stirred tank membrane reactor or CSTMR, is shown schematically in Figure 2.6. A continuous stirred tank reactor (CSTR) is connected to a membrane module. The substrate is continuously fed into the CSTR which is charged with the enzyme. The reaction mixture, which consists of unhydrolysed protein, peptides of various sizes and enzyme, is pumped through the membrane module under pressure. The permeate from the membrane module is

collected and forms the product. The retentate from the membrane module is recycled back to the CSTR for further reaction. The CSTR is fed substrate continuously at a rate equivalent to the permeate flowrate in order to maintain constant volume in the CSTR. The CSTR is maintained at a constant temperature and there is usually provision for base or acid to be added to maintain a constant pH.

Turgeon & Gauthier (1990) have described a further variation of the CSTMR system. The configuration used in their studies is shown in Figure 2.7. In this case, the CSTMR was connected to a further membrane module in order to separate the resulting hydrolysate further by removing free amino acids and small peptides.

The various studies that have been carried out to investigate the continuous hydrolysis of proteins are summarized in Table 2.3.

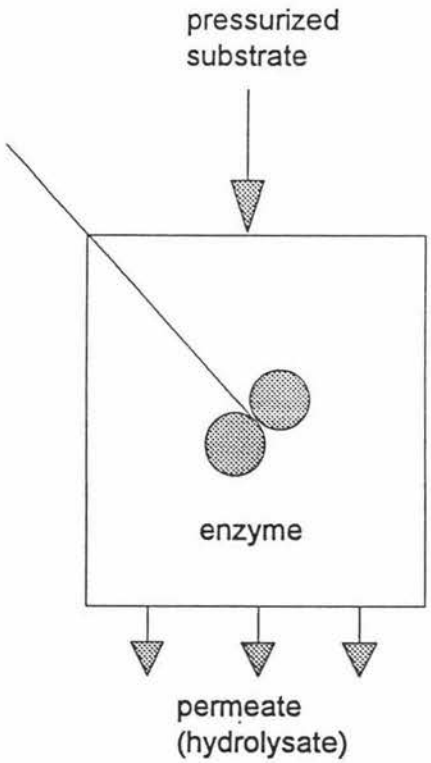


Figure 2.5 Dead-end cell membrane reactor (Cheryan & Deeslie, 1980).

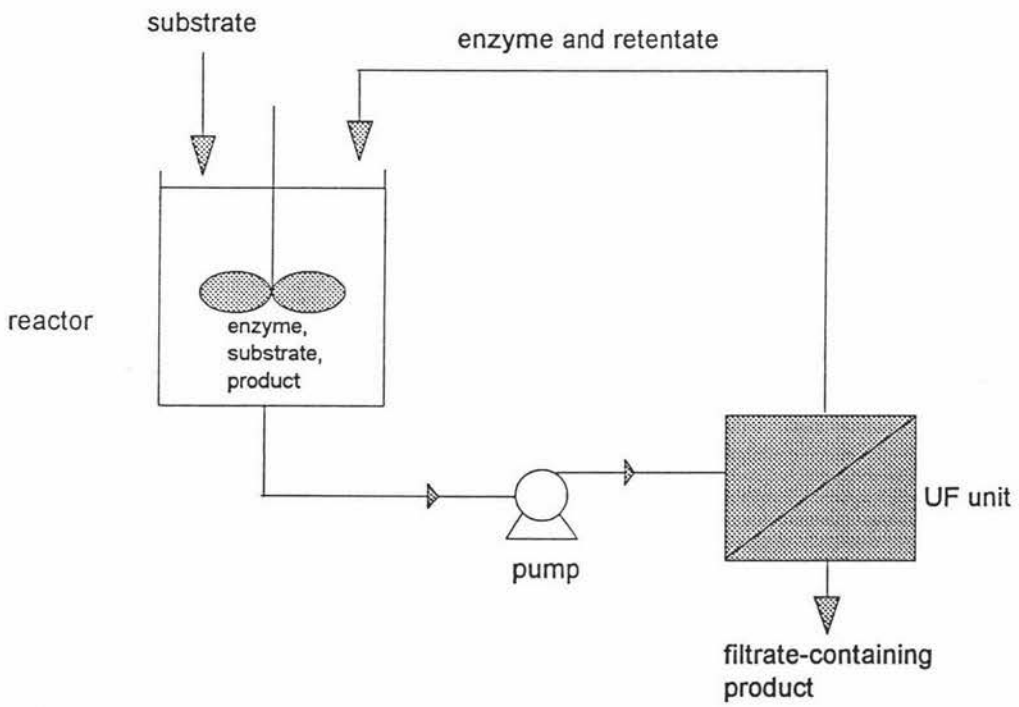


Figure 2.6 The continuous stirred tank membrane reactor.

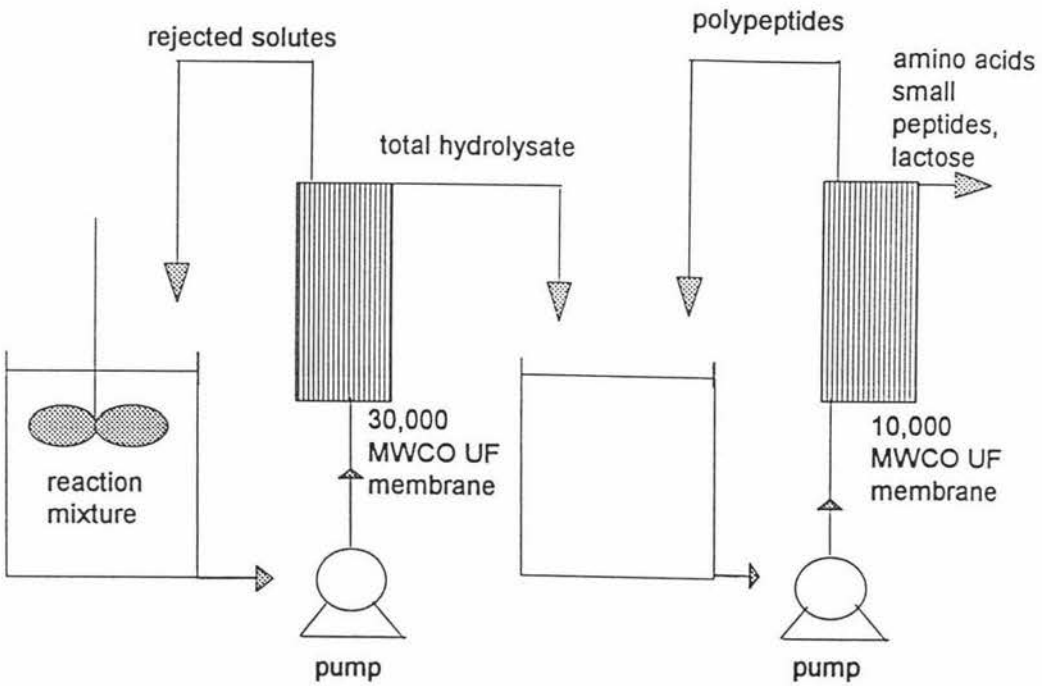


Figure 2.7 Two-step hydrolysis and ultrafiltration process (Turgeon & Gauthier, 1990).

Table 2.3 Summary of continuous proteolysis studies

Authors	Reactor Type	Substrate(s)	Enzyme(s)	Purpose
Blatt *et al.* (1968)	Dead-end cell	Whey from milk	α-Chymotrypsin	First demonstration of continuous hydrolysis process
Cheftel (1972)	Dead-end cell	Fish protein concentrate	Pronase	Studies with enzyme recycle added to dead-end cell process
Roozen & Pilnik (1973)	Dead-end cell	Soybean protein isolate	Pankreaproteinase A (Rohm GmbH, Germany) Bakterienproteinase N (Rohm GmbH, Germany) FAAN type A (Schweiz Ferment, Germany) Saure Protease SPR (Schweiz Ferment, Germany) HT Proteolytic 200 (Takamine, Miles Labs Inc, USA) Rhozyme P53 (Rohm and Haas, USA)	To examine the effect of bioreactor system on bitterness
Iacobucci *et al.* (1974)	CSTMR	Soybean protein isolate	Acid-stable protease from *Penicillium duponti*	Optimized process and developed kinetic and mechanistic descriptions of the process
Boudrant *et al.* (1976)	-	-	Alcalase	Stabilization of Alcalase so that it did not permeate the membrane

Table 2.3 (continued)

Authors	Reactor Type	Substrate(s)	Enzyme(s)	Purpose
Boudrant & Cheftel (1976)	CSTMR	Casein	Alcalase	Selection of appropriate enzyme and continuous hydrolysis experiments
Bhumiratana *et al.* (1977)	Dead-end cell	Fish protein concentrate	Trypsin	Comparison of batch, semi-batch and continuous membrane reactors
Cunningham *et al.* (1978)	Dead-end cell	Cottonseed storage protein	Pepsin	Continuous modification and recovery of cottonseed storage protein
Payne *et al.* (1978)	Dead-end cell	Leaf protein concentrate	Trypsin	Comparison of batch, semi-batch and continuous membrane reactors
Cheryan & Deeslie (1980)	CSTMR	-	-	Review

Table 2.3 (continued)

Authors	Reactor Type	Substrate(s)	Enzyme(s)	Purpose
Deeslie & Cheryan (1981a)	CSTMR	Soy protein isolate	Pronase	Kinetic model development
Deeslie & Cheryan (1981b)	CSTMR	Soy protein isolate	Trypsin Chymotrypsin Pronase Prolidase Alcalase	Studies of the effect of operational factors on productivity
Olsen & Adler-Nissen (1981)	CSTMR	Soy protein isolate	Alcalase	Development of kinetic models
Deeslie & Cheryan (1982)	CSTMR	Soy protein isolate	Pronase	Study of factors affecting long term operational stability

Table 2.3 (continued)

Authors	Reactor Type	Substrate(s)	Enzyme(s)	Purpose
Cheryan & Deeslie (1983)	CSTMR	Soy protein isolate	Pronase	Development of CSTMR system and demonstration of advantages over batch production
Cheryan (1986)	CSTMR	-	-	Review
Deeslie & Cheryan (1988)	CSTMR	Soy protein isolate	Pronase Alcalase	Investigation of the functional properties of CSTMR products
Visser *et al.* (1989)	CSTMR	β-Casein	Plasmin	A model system to study continuous production of peptide fractions
Mannheim & Cheryan (1990)	CSTMR	Casein	Alcalase	Optimized process for hydrolysis of casein

Table 2.3 (continued)

Authors	Reactor Type	Substrate(s)	Enzyme(s)	Purpose
Turgeon & Gauthier (1990)	CSTMR with two ultrafiltration membranes	Whey protein	Trypsin Chymotrypsin	Production of hydrolysates with specific functional and nutritional properties
Takahata *et al.* (1991)	CSTMR	Whey protein	Trypsin immobilized on Sepharose	Demonstrated that α-lactalbumin and β-lactoglobulin were not present in product
Bouhallab *et al.* (1992)	CSTMR	Casein glycomacropeptide	Trypsin	Model of continuous production of bioactive peptides
Bouhallab *et al.* (1993)	CSTMR	β-Casein	Chymosin	Continuous and selective extraction of β-casomorphin
Pouliot *et al.* (1993)	CSTMR with two ultrafiltration membranes	Casein	Trypsin Chymotrypsin	Characterization of the fractionation of hydrolysates by polysulphone membranes

2.2.3 Selection of enzymes for use in continuous hydrolysis processes

As discussed earlier, the selection of an enzyme is a very important consideration when developing a continuous hydrolysis process. Different authors who have published in the area of continuous hydrolysis processing have based their selection of enzymes on different criteria. Flavour of the resulting hydrolysate is often used as the criterion for enzyme selection. For example, Roozen & Pilnik (1973) studied several different enzymes with the intention of producing a "bland" soy protein hydrolysate. No specific enzyme selection technique was used prior to continuous hydrolysis experiments. Blandness was assessed only in the final products. Bhumiratana *et al.* (1977) chose trypsin for their experiments on the solubilization of fish protein because trypsin was effective on this substrate at a neutral pH. In this instance, an enzyme active at alkaline pH was preferred to one active at acid pH so that the essential amino acid tryptophan was not destroyed. Cunningham *et al.* (1978) used pepsin and Molsin (a commercial protease) to hydrolyse cottonseed protein. It is unclear why these enzymes were selected. Payne *et al.* (1978) selected trypsin, on the basis of previous work studying the various enzymes, for the hydrolysis of leaf protein concentrate. Iacobucci *et al.* (1974) combined several criteria for enzyme selection. They stated that the enzyme selected is important in terms of availability, cost, rate and specificity. They selected a microbial protease (for its availability and cost) that produced a non-bitter hydrolysate under conditions (pH and temperature) that reduced the chances of microbial spoilage. In all of these studies enzymes were chosen for reasons other than their activity and the molecular weight distribution of the final product.

More recent studies have aimed to produce certain peptides using continuous hydrolysis. The studies published by Turgeon & Gauthier (1990) and Takahata *et al.* (1991) both used trypsin for the hydrolysis of whey protein. It is not clear why these authors chose trypsin for their work, even though Turgeon & Gauthier (1990) mentioned the fact that peptides that are greater than 20 amino acids long will retain some

emulsification capacity and that small peptides are absorbed in the gut better than free amino acids. Both studies targeted products suitable for infant formulae so it could be that trypsin was selected in order to mimic natural digestion in the gut. Trypsin is also less likely to produce large amounts of small peptides and free amino acids from casein due to its specificity. Visser *et al.* (1989) used plasmin to study the hydrolysis of bovine β-casein in a membrane reactor, as a precursor to the production of specific biologically active peptides. Bouhallab *et al.* (1992, 1993) used trypsin and chymosin respectively in order to produce bioactive peptides from casein-based substrates. In all of these cases, the selection of the enzyme was an integral part of the process, or the desired product, being studied because if a different enzyme had been used, then the products appropriate to the study would not have been produced. However, reactors of this type may lack long term stability if the enzyme's activity is not high enough to prevent the build-up of unhydrolysed material in the bioreactor.

Another group of authors who have published in the area of continuous hydrolysis processes have based their selection of enzymes on maximum activity. In their review, Cheryan & Deeslie (1980) reported that the enzyme selected for continuous hydrolysis should have as high an activity as possible to prevent the build-up of unhydrolysable material in the CSTMR system. A build-up of unhydrolysed material would hinder the long term stability of the process by decreasing membrane flux. They also stated that in order to achieve this a mixture of exo- and endopeptidases may be required. However, the inclusion of an exopeptidase in the enzyme system would lead to the production of a hydrolysate containing a reasonable proportion of free amino acids. Throughout all of their work (Deeslie & Cheryan, 1981a; 1981b; 1982; Cheryan & Deeslie, 1983) and in the work of Mannheim & Cheryan (1990), enzymes for the hydrolysis of various substrates were selected only on the basis of enzyme activity. A further limitation of their enzyme selection methods is that the measurement of enzyme activity was, in all cases, based on batch experiments in spite of the statement made by Cheryan & Deeslie (1980) that "preliminary batch

hydrolysis experiments with the native, soluble enzyme are necessary to determine kinetic parameters such as optimum enzyme-to-substrate ratio, pH *etc.,* but are a poor indication of long-term stability". They also stated that "enzyme manufacturers' and published data on the effects of temperature, activators, substrate inhibition effects, *etc.* are based on short-term batch hydrolysis experiments, frequently with low molecular weight substrates which may not be directly applicable in real systems". Cheryan & Deeslie (1980) based these statements on their own work and on their review of the work carried out by Cheftel (1972).

A more rigorous study used both activity and some functional properties in order to select an enzyme. Boudrant & Cheftel (1976) selected Alcalase, a commercial enzyme preparation from *Bacillus licheniformis*, for their continuous hydrolysis experiments. They selected this enzyme on the basis of its activity, operating pH and the physical characteristics and flavour of the hydrolysis product produced in batch mode.

To summarize, many authors have often put one property ahead of others to select an enzyme and have thereby compromised either the efficiency of the reactor or the properties of the final product. Another group of authors have used only maximum activity as the basis for enzyme selection.

A number of authors (Blatt *et al.,* 1968; Roozen & Pilnik, 1973; Boudrant & Cheftel, 1976; Cunningham *et al.,* 1978; Visser *et al.,* 1989; Turgeon & Gauthier, 1990) have mentioned that continuous membrane hydrolysis processes would be useful for separating the intermediate products of hydrolysis and Turgeon & Gauthier (1990) have alluded to the fact that hydrolysates low in free amino acids could be produced. This focus on the intermediate products of hydrolysis reactions arises from the understanding that these products should be less bitter and retain some functionality compared with smaller peptides. However, these authors did not develop this idea because they did not use the appropriate analytical methods to examine enzyme specificity and bond hierarchy.

The work reported in this thesis is the first published work to use the molecular weight distribution of the products produced by hydrolysis to select enzymes that will operate in a CSTMR and achieve a defined molecular weight distribution.

2.2.4 The effect of membrane choice on the continuous hydrolysis process

Early studies in the field of the continuous hydrolysis of proteins (Blatt *et al.,* 1968; Cheftel, 1972; Roozen & Pilnik, 1973) were based on the dead-end cell configuration shown in Figure 2.5. All authors reported that the use of this membrane configuration resulted in an unacceptable decrease in membrane flux and, hence, reactor productivity. Roozen & Pilnik (1973) suggested that the use of a flow-through ultrafiltration cell could help to overcome flux problems. Bhumiratana *et al.* (1977) and Cunningham *et al.* (1978) also used the dead-end cell configuration but made no statement about membrane flux and its effect on productivity. Subsequent work in the area of CSTMR hydrolysis of proteins has not included any form of membrane evaluation. Membranes appear to have been selected primarily on the basis of availability, with the use of Amicon (USA) hollow fibre membranes prevailing.

Some of the advantages of CSTMR processes for hydrolysate production are based on the assumption that the enzyme is retained on the retentate side of the ultrafiltration membrane. The molecular weight of the enzyme being used then, should have a bearing on the molecular weight cut-off (MWCO) of the membrane chosen. Boudrant & Cheftel (1976) studied the retention of glutaraldehyde-stabilized Alcalase with 30,000 MWCO membranes. They found that the enzyme did leak through the membrane. The molecular weight of Alcalase is 27,287 daltons (Adler-Nissen, 1986). However, membrane MWCO are not absolute; there is a distribution of pore sizes of which 10-15% may be larger than the stated MWCO (Cheryan, 1986); therefore, some leakage of intact enzyme is to be expected. Boudrant & Cheftel (1976) attributed enzyme leakage through the membrane to self-digestion of the enzyme to produce small fragments

that retained some proteolytic activity. Interestingly, Deeslie & Cheryan (1982) and Mannheim & Cheryan (1990) reported enzyme losses through the membrane but did not report any treatment of the permeate from the CSTMR in any way to prevent further hydrolysis occurring during storage prior to analysis. Deeslie & Cheryan (1982) studied the effect of the membrane on the enzyme and attributed the loss of enzyme activity in their CSTMR system to "membrane poisoning" of the enzyme, *i.e.* irreversible binding of the enzyme to the membrane. It appears that many authors were unaware of enzyme leakage or were oblivious to its consequences. Several authors (Iacobucci *et al.,* 1974; Boudrant *et al.,* 1976; Bhumiratana *et al.,* 1977; Visser *et al.,* 1989) attributed an apparent decrease in enzyme activity either partially or wholly to thermal inactivation of the enzyme over the course of the continuous process.

Olsen & Adler-Nissen (1981) reviewed the area of membrane processing of enzymatically modified proteins. This review concentrated on the properties of the resulting fractionated hydrolysates rather than the selection and availability of different membranes. Similarly, Nakamura *et al.* (1992) studied the effect of different molecular weight fractions, obtained by hydrolysis and subsequent ultrafiltration, on the allergenicity of whey proteins.

In spite of the availability of an increasingly wide range of ultrafiltration membranes, there have been few systematic studies reported on the effect of membrane type on the characteristics of hydrolysate products. Audinos & Branger (1992) investigated the difference between the use of inorganic and organic membranes to fractionate casein hydrolysates. Deeslie & Cheryan (1992) examined the effect of membrane pore size on the molecular weight distribution and selected functional properties of a soy protein hydrolysate. These authors concluded that it was possible to produce peptides of differing molecular weight distributions using membranes with different MWCOs but that the currently available membranes had broad pore size distributions which resulted in a broad distribution of peptide sizes in the permeates. They also found that small

variations in the molecular weight distributions of peptide mixtures could have a significant effect on the functionality of the product. Their final conclusion was that "The ultrafiltration reactor, especially with better ultrafiltration membranes, could be useful in producing new fractions of proteins with unique functional properties." Pouliot *et al.* (1993) examined the fractionation of casein hydrolysates using polysulphone ultrafiltration hollow fibre membranes (Amicon, USA, and Romicon, USA). They used amino acid analysis to characterize the fractionation profile of casein hydrolysates using the two-step ultrafiltration process developed by Turgeon & Gauthier (1990). These authors related their results to the specificity of the enzymes used. As such studies are carried out, the importance of membrane selection on the CSTMR process will be able to be better assessed.

An evaluation of different membrane types was carried out as part of the work reported in this thesis.

2.2.5 Analysis of the molecular weight distribution of hydrolysis products

In any study of the hydrolysis of proteins, it is important to be able to measure the molecular weight distribution of the resulting hydrolysate. Various methods have been used to characterize the products of CSTMR reactions in terms of their molecular weight distributions. The methods used to characterise the molecular weight distribution of continuous hydrolysis products and the authors who have used them have been summarized in Table 2.4.

These methods could all be improved upon to obtain more detailed information on the molecular weight distribution of the products. For example, a better choice for the detection wavelength would be 205 nm which is closer to the wavelength of maximum absorbance for the peptide bond (190 nm) (Motion, 1991). Phosphate buffer, although transparent to ultra-violet light at wavelengths less than 210 nm, has been shown to give a poorer separation of the lower molecular weight species (Motion, 1991). Gel electrophoresis of hydrolysates does not retain all of the smaller

peptides and amino acids on the gel and hence some valuable information is lost.

Table 2.4 Summary of methods used to characterize continuous hydrolysis products by their molecular weight distribution

Authors	Method
Roozen & Pilnik (1973)	Gel filtration Column: Sephadex G-25 Eluent: ammonium bicarbonate-acetate Detection wavelength: 280 nm
Boudrant & Cheftel (1976)	Gel filtration Column: Biogel P 10 Eluent: sodium phosphate buffer Detection wavelength: 280 nm
Bhumiratana *et al.* (1977)	Gel filtration Column: Sephadex G50 Eluent: phosphate buffer Detection wavelength: 280 nm
Cunningham *et al.* (1978)	Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE)
Deeslie & Cheryan (1981b, 1988)	Gel filtration Column: Sephadex G-15 and G-50 Eluent: phosphate buffer Detection wavelength: 215 nm
Visser *et al.* (1989)	Reversed-phase high performance liquid chromatography (HPLC) and SDS- and urea-PAGE
Turgeon & Gauthier (1990)	Gel filtration Column: TSK-2000SW (Toso-Haas) Eluent: not reported Detection wavelength: not reported

Table 2.4 (continued)

Authors	Method
Takahata *et al.* (1991)	Gel filtration Column: not reported (supplied by Toso Co.) Eluent: sodium sulphate Detection wavelength: 280 nm
Bouhallab *et al.* (1992, 1993)	Reversed-phase HPLC Eluent: acetonitrile and sodium phosphate Detection wavelength: 214 nm
Pouliot *et al* (1993)	High performance size exclusion chromatography (HPSEC) Column: TSK-2000SW Eluent: not reported Detection wavelength: not reported Amino acid analysis using a Beckmann High Performance Amino Acid Analyzer System

2.2.6 The effect of residence time on the molecular weight distribution of CSTMR products

Proteolytic enzymes cleave proteins in a very specific fashion. This specificity relates not only to which peptide bonds are cleaved but also to the order in which the bonds are cleaved. It follows then, that peptides produced in a CSTMR would pass through the ultrafiltration membrane before they can be hydrolysed further by the enzyme, which is predominantly trapped in the CSTR. The CSTMR parameter residence time could be controlled in order to produce hydrolysates with a particular MWP. Of all the published work, only Boudrant & Cheftel (1976) examined the effect of residence time on the molecular weight distribution of the product. Their work was flawed, however, by their choice of an inappropriate method for the determination of the molecular weight distribution of the product.

2.2.7 The measurement of bioreactor productivity

Mathematical models describing the behaviour of membrane bioreactors have been described by Olsen & Adler-Nissen (1981), Deeslie & Cheryan (1981a, 1981b, 1982) and Iacobucci *et al.* (1974). The differences between the various models have been reviewed by Cheryan (1986). Although the derivation of the mathematical models differs, the expressions arrived at to describe reactor yield and productivity are the same. The conversion percentage or reactor yield, is a function of the feed concentration, the enzyme concentration, the reactor volume, the flux and the proteolytic reaction rate constant, *k*. It is usually expressed as the ratio between the nitrogen concentration in the permeate and the feed. The productivity of the reactor is expressed as the ratio of hydrolysate produced to enzyme used.

2.2.8 Patented CSTMR processes for milk protein hydrolysis

The Institut National de la Recherche Agronomique (France) has patented a CSTMR process for the production of a whey protein hydrolysate, using pancreatin, that contains "at least 50% of the peptides with 2-5 amino acids, 70-90% of the nitrogen present as peptides and less than 10% free amino acids". They did not claim the use of any substrate other than whey protein. However, these authors used the characteristics of CSTMR production of hydrolysates to assure themselves of a product with a lower proportion of free amino acids than could be obtained from batch hydrolysis methods achieving the same degree of protein solubilization. This is the only patent related to CSTMR production of milk protein hydrolysates.

CHAPTER 3

ENZYME SCREENING

3.1 Introduction

A wide range of commercially available food grade enzymes was screened with casein as the substrate. The hydrolysis conditions were based on optima described in the manufacturers' information bulletins. The resulting hydrolysates were assessed on the basis of their MWPs, as analyzed by HPSEC, to determine the suitability of the enzymes for bioreactor trials. This approach was different from those used by previous authors.

Deeslie & Cheryan (1981a, 1981b) and Mannheim & Cheryan (1990) used a systematic evaluation of enzyme activity to select enzymes for use in CSTMR processes. Mannheim & Cheryan (1990) described a "pH-drop method" for enzyme selection which they adapted from Hsu *et al.* (1977). By using this technique, these authors selected enzymes on the basis of enzyme activity alone, without regard for enzyme specificity, in order to obtain maximum productivity from the bioreactor. The productivity of the CSTMR was their primary consideration so no attempt was made to use the properties of the enzyme as a tool to develop hydrolysates with particular molecular weight distributions. The method of Hsu *et al.* (1977), as described by Mannheim & Cheryan (1990), was also used in the present study to compare the two methods of enzyme selection.

3.2 Materials and Methods

3.2.1 Materials

3.2.1.1 Enzymes

The following enzymes were evaluated for their effect on casein: Rohm Corolase N (Rohm Enzyme, Rohm GmbH, Darmstadt, Germany); Biocon Fungal Protease Powder, Batch 5910819 (Biocon, Australia); Novo Neutrase 0.5L, Batch PWN0186 93-8 (Novo Enzymes, Novo Industri

A/S, Enzymes Division, Denmark);
Novo Alcalase 2.4L, Batch PMN 5036 93-2 (Novo Enzymes, Novo Industri A/S, Enzymes Division, Denmark);
Rohm Corolase 7089 (Rohm Enzyme, Rohm GmbH, Darmstadt, Germany);
Amano Protease A, Batch PRP10503A (Amano Pharmaceuticals Ltd, Japan);
Amano Protease B, Batch PRP12524 (Amano Pharmaceuticals Ltd, Japan);
Amano Papain W40, Batch R02503 (Amano Pharmaceuticals Ltd, Japan);
Rohm Corolase S50 (Rohm Enzyme, Rohm GmbH, Darmstadt, Germany);
Rohm Bromelain (Rohm Enzyme, Rohm GmbH, Darmstadt, Germany);
Amano Bromelain (Amano Pharmaceuticals Ltd, Japan);
Rhozyme P64, Batch 15.89299.01 (Genencor Inc., South San Francisco, California, USA);
Rhozyme P41, Batch 14.89299.01 (Genencor Inc., South San Francisco, California, USA).

3.2.1.2 Substrate

The substrate used for all experiments was Alacid Acid Casein (30 mesh, lactic acid casein from Factory 1032, Batch J4176) supplied by the New Zealand Dairy Board. The substrate was stored in its original packaging, a multiwall paper bag with a plastic liner, at ambient temperature (approximately 20°C), away from direct sunlight. A sub-sample of the substrate was submitted to the Analytical Chemistry Section at the NZDRI for analysis of total nitrogen (TN), non-protein nitrogen (NPN), moisture, fat, lactose, ash, sodium and potassium (Appendix II). The methods used have been described by Crofts & Gray (1991).

3.2.1.3 Chemicals

"Analar" grade sodium hydroxide was obtained from BDH Laboratory Supplies (Poole, England). Sodium hydroxide solutions were prepared using demineralized water.

Acetonitrile (HPLC, "Far uv" Grade) was obtained from BDH Laboratory Supplies (Poole, England). Trifluoroacetic acid (HPLC-Spectrograde) was obtained from Pierce Chemical Company (Rockford, Illinois, USA). HPLC eluent was prepared using Milli-Q water (Millipore Associates, Bedford, Massachusetts, USA).

3.2.2 Methods

3.2.2.1 Selection of enzymes for screening

Enzymes for screening were selected on the basis of the supplier's information. Those enzymes with exopeptidase activity, according to the manufacturer's information, were not tested because these enzymes would be considered to produce a higher proportion of free amino acids than would endopeptidases.

3.2.2.2 Hydrolysis experiments at constant pH

The hydrolyses were carried out using the temperature and pH conditions listed in Table 3.1. The conditions and enzyme concentrations for the enzymes screened were selected on the basis of manufacturers' information. A general laboratory-scale hydrolysis method has been described by Armstrong (1991). A caseinate solution (10% total solids) was prepared by adding casein to demineralized water, adjusting the pH of the slurry to approximately pH 7 with 4 N sodium hydroxide, heating the slurry to 60°C and holding for approximately 1 h. The resulting solution was weighed (1 kg) into a beaker for each hydrolysis experiment. The beaker was then placed in a water bath and allowed to reach the hydrolysis temperature shown in Table 3.1. The pH of the solution was checked and adjusted with 4 N sodium hydroxide, if necessary, to the pH shown in Table 3.1 before enzyme was added to give an enzyme to substrate ratio of 1:100 (w/w). The pH was maintained during hydrolysis by the addition of 4 N sodium hydroxide, using an autotitrator system consisting of a Radiometer type PHM 82 pH meter, a Radiometer type TTT 80 titrator and a Radiometer type ABU 80 autoburette (Radiometer Pacific, Auckland, New

Zealand). The amount of sodium hydroxide used to maintain the pH throughout the reaction was recorded manually. A "base consumption" curve was prepared for each enzyme by plotting the amount of 4 N sodium hydroxide used against hydrolysis time.

3.2.2.3 Storage and sampling

Samples of hydrolysed material were collected for molecular weight profiling at 0 (before enzyme addition), 5, 10, 15, 30, 45, 60, 90, 120, 150 and 180 min. In some cases a 150 min sample was not taken and in other cases samples were taken half-hourly or hourly for up to 5 h. In the case of hydrolysis using Amano Protease B, samples were taken at various intervals for up to 21 h. The total hydrolysis time for each experiment is shown in Table 3.1. The length of each hydrolysis experiment was varied according to the rate of alkali consumption. The hydrolysis was allowed to continue for longer if the rate of alkali consumption had not slowed to almost zero, indicating that the hydrolysis reaction had not approached completion. In most cases, the hydrolysis reaction was approaching completion after 180 min. Hydrolysate (1 ml or 0.25 ml) was added to 9 ml or 4.75 ml of HPLC eluent (36% acetonitrile, 0.1% trifluoroacetic acid, in water) for MWP analysis using HPSEC (Motion, 1991). All samples were taken in duplicate and stored under refrigeration (approximately 4°C) prior to analysis.

Table 3.1 Summary of enzymes assessed, conditions used and analyses performed

Enzyme	Enzyme Source	pH	Temperature (°C)	Total Hydrolysis Time (min)
Rohm Corolase N	Bacterial	7.0	50	240
Biocon Fungal Protease	*Aspergillus niger*	7.0	50	330
Novo Neutrase 0.5L	*Aspergillus oryzae*	7.0	45	300
Novo Alcalase 2.4L	*Bacillus licheniformis*	8.0	60	420
Rohm Corolase 7089	*Bacillus subtilis*	7.0	50	180
Amano Protease A	*Aspergillus oryzae*	7.0	50	180
Amano Protease B	*Penicillium sp.*	6.0	50	1260 (21 h)
Amano Papain W40	Papaya	6.0	50	180
Rohm Corolase S50	Papaya	7.0	60	180
Rohm Bromelain	Pineapple	7.0	50	180
Amano Bromelain	Pineapple	7.0	50	180
Genencor Rhozyme P64	*Bacillus licheniformis*	7.0	60	180
Genencor Rhozyme P41	*Aspergillus oryzae*	7.0	50	180

3.2.2.4 Analytical methods

DH was calculated from alkali addition. This method allows the proportion of peptide bonds cleaved to be calculated from the amount of alkali required to maintain the pH of the solution undergoing hydrolysis at a constant value. This method has been described by Adler-Nissen (1986). A DH curve was prepared for each hydrolysis experiment by plotting DH against time.

HPSEC analysis was performed by the Food Science Protein Analytical Laboratory at the NZDRI according to the method described by Motion (1991). This method separates peptides based on their molecular weight using size exclusion chromatography. A TSK-2000SW column (Toya Soda Company, Japan) was used with water:acetonitrile:trifluoroacetic acid (64:36:0.1) eluent, at a flowrate of 0.5 ml/min and a detection wavelength of 205 nm. The HPSEC column was calibrated using the protein standards shown in Table 3.2. The absorbance data was collected and analyzed using Maxima GPC (Gel Permeation Chromatography) software (Waters, Millipore Corporation, Milford, Massachusetts, USA). The individual MWPs were imported into the software package Quattro Pro (Borland International Inc., Scotts Valley, California, USA). The molecular weight scale was calculated form the time data by applying the relevant calibration equation. The absorbance and molecular weight data were then used to prepare a time series MWP for each enzyme.

Table 3.2 Molecular weight standards used for MWP analysis

Protein Standard	Molecular Weight (daltons)
Glyceraldehyde-3-phosphate dehydrogenase	36,000
Carbonic anhydride	29,000
Soybean trypsin inhibitor	20,100
Egg-white lysozyme	14,300
Aprotinin	6,500
Insulin	5,730
Insulin B-chain	3,400
Bacitracin	1,420
Phenylalanine	165

3.2.2.5 pH-drop experiments

The experiment has been described by Mannheim & Cheryan (1990). A 1% (w/v) casein slurry in demineralized water was prepared and adjusted to pH 8 with 0.1 N sodium hydroxide. A 50 ml aliquot of the resulting solution was heated to 50°C, while stirring, in a water bath. The enzyme (0.0705 g) being tested was mixed with 5 ml of demineralized water and the pH of the solution was adjusted to pH 8 using 0.1 N sodium hydroxide. The enzyme solution was added to the casein solution while stirring in a 50°C water bath. The pH of the solution was measured using a Radiometer type PHM 82 pH meter (Radiometer Pacific, Auckland, New Zealand) and recorded manually over a 10 min period.

The pH of the solution was plotted against time for each enzyme. The total pH-drop over 10 min and the initial slope of the pH-drop curve were calculated.

3.3 Results

3.3.1 Notes on the interpretation of MWPs

The MWP method has been described in detail by Motion (1991, 1993). Care is required in the interpretation of the lower molecular weight part of the MWP. Amino acids do not have a peptide bond and only the aromatic amino acids (tryptophan, tyrosine, phenylalanine and methionine) absorb strongly at 205 nm. Therefore, although most amino acids run together in the same peak on a MWP, the MWP should not be used to quantify levels of free amino acids in a hydrolysate (Motion, 1991). The free amino acid peak can, however, be used as a qualitative indicator for the levels of free amino acids in a hydrolysate. The method is only semi-quantitative for very short peptides. These species have a lower absorbance than their mass would suggest because the ratio of peptide bonds to amino acids in very short peptides is lower than that for larger peptides (Motion, 1991). A peak often appears to the right of the free amino acid peak. This peak can be positive or negative and is related to factors such as the salt concentration of the sample (Motion, 1991, 1993). The characteristics of the lower end of the molecular weight scale have been summarised in Table 3.3. It should also be noted that peptides containing basic amino acids (arginine, lysine and histidine) are eluted a little earlier than would be expected on the basis of their molecular weight (Motion, 1991).

Table 3.3 Characteristics of the low molecular weight range of MWPs

Elution position (log Molecular Weight)	Species
3.0	Tetra- and larger oligo-peptides, lysine-lysine
2.6	Tri-peptides and basic di-peptides
2.4	Di-peptides and basic amino acids
2.2	Free amino acids

3.3.2 Time series MWPs and DH curves

The time series MWPs and the DH and base consumption curves for each enzyme are presented in Figures 3.1 to 3.26. The raw data for the base consumption curves and calculated DH values are given in Appendix II. The following observations were made after examination of Figures 3.1 to 3.26.

3.3.2.1 Rohm Corolase N

(Figures 3.1 and 3.2)

Hydrolysis occurred slowly. An amino acid peak was present after 5 min and remained constant throughout the hydrolysis. Some starting material was still apparent after 180 min of hydrolysis.

3.3.2.2 Biocon Fungal Protease

(Figures 3.3 and 3.4)

Hydrolysis occurred at a moderate rate. A moderate free amino acid peak was present after 5 min and increased during the hydrolysis. Some starting material was still present after 180 min of hydrolysis.

3.3.2.3 Novo Neutrase 0.5L

(Figures 3.5 and 3.6)

Hydrolysis occurred at a moderate rate. A small free amino acid peak was present after 5 min of hydrolysis and this did not increase during the hydrolysis. Some high molecular weight material was present after 180 min of hydrolysis.

3.3.2.4 Novo Alcalase 2.4L

(Figures 3.7 and 3.8)

Hydrolysis proceeded at a rapid rate with complete breakdown of the starting material after 120 min. A free amino acid peak was not formed during the 420 min of hydrolysis.

3.3.2.5 Rohm Corolase 7089

(Figures 3.9 and 3.10)

Hydrolysis occurred slowly. A small free amino acid peak was present after 5 min of hydrolysis and did not increase during hydrolysis. Some starting material was present after 180 min of hydrolysis.

3.3.2.6 Amano Protease A

(Figures 3.11 and 3.12)

Hydrolysis proceeded at a rapid rate. A small free amino acid peak appeared after 10 min of hydrolysis and this increased throughout the hydrolysis until, at 180 min, a significant free amino acid peak was present. The starting material was broken down completely after 180 min of hydrolysis.

3.3.2.7 Amano Protease B

(Figures 3.13 and 3.14)

Hydrolysis occurred very slowly. Free amino acids were present after 5 min of hydrolysis and increased throughout hydrolysis. There was little difference between MWPs after 120 min of hydrolysis. A large proportion

of the starting material was not hydrolysed.

3.3.2.8 Amano Papain

(Figures 3.15 and 3.16)

Hydrolysis was very rapid. Free amino acids were present after 5 min of hydrolysis and increased slightly during hydrolysis. All of the starting material was broken down after 10 min of hydrolysis.

3.3.2.9 Rohm Corolase S50

(Figures 3.17 and 3.18)

Hydrolysis was very rapid. Free amino acids were present after 5 min of hydrolysis and increased throughout the reaction to form a significant proportion of the 180 min MWP. All of the starting material had been hydrolysed after 5 min.

3.3.2.10 Rohm Bromelain

(Figures 3.19 and 3.20)

Hydrolysis was very rapid. Free amino acids were present after 5 min of hydrolysis and increased throughout the reaction to form a moderate proportion of the 180 min MWP. All of the starting material had been hydrolysed after 5 min.

3.3.2.11 Amano Bromelain

(Figures 3.21 and 3.22)

Hydrolysis was rapid. Free amino acids were present after 5 min of hydrolysis and increased throughout the reaction to form a significant proportion of the 180 min MWP. All of the starting material had been hydrolysed after 180 min.

3.3.2.12 Rhozyme P64

(Figures 3.23 and 3.24)

Hydrolysis occurred slowly. Free amino acids were present after 5

min of hydrolysis and did not increase significantly during the hydrolysis reaction. A moderate free amino acid peak was present after 180 min of hydrolysis. A significant proportion of the starting material remained unhydrolysed after 180 min.

3.3.2.13 Rhozyme P41

(Figures 3.25 and 3.26)

Hydrolysis occurred slowly. A small free amino acid peak was present after 5 min of hydrolysis and this increased throughout the hydrolysis. A significant proportion of high molecular weight material was present after 180 min of hydrolysis.

3.3.3 pH-drop curves

The graph derived from the results of the pH-drop experiments is shown in Figure 3.29. The difference between the pH at 0 min and after 10 min of hydrolysis was plotted against the slope of the initial straight-line portion of the pH-drop curve (Figures 3.27 and 3.28). Hence, an enzyme that is represented by a circle at the top, right-hand-side of the diagram has high activity for casein as its substrate. Based on the results obtained, Amano Protease A, Novo Alcalase 2.4L and Rohm Bromelain are examples of enzymes with high activity for casein.

The raw data for the pH-drop curves are given in Appendix III (Table A3).

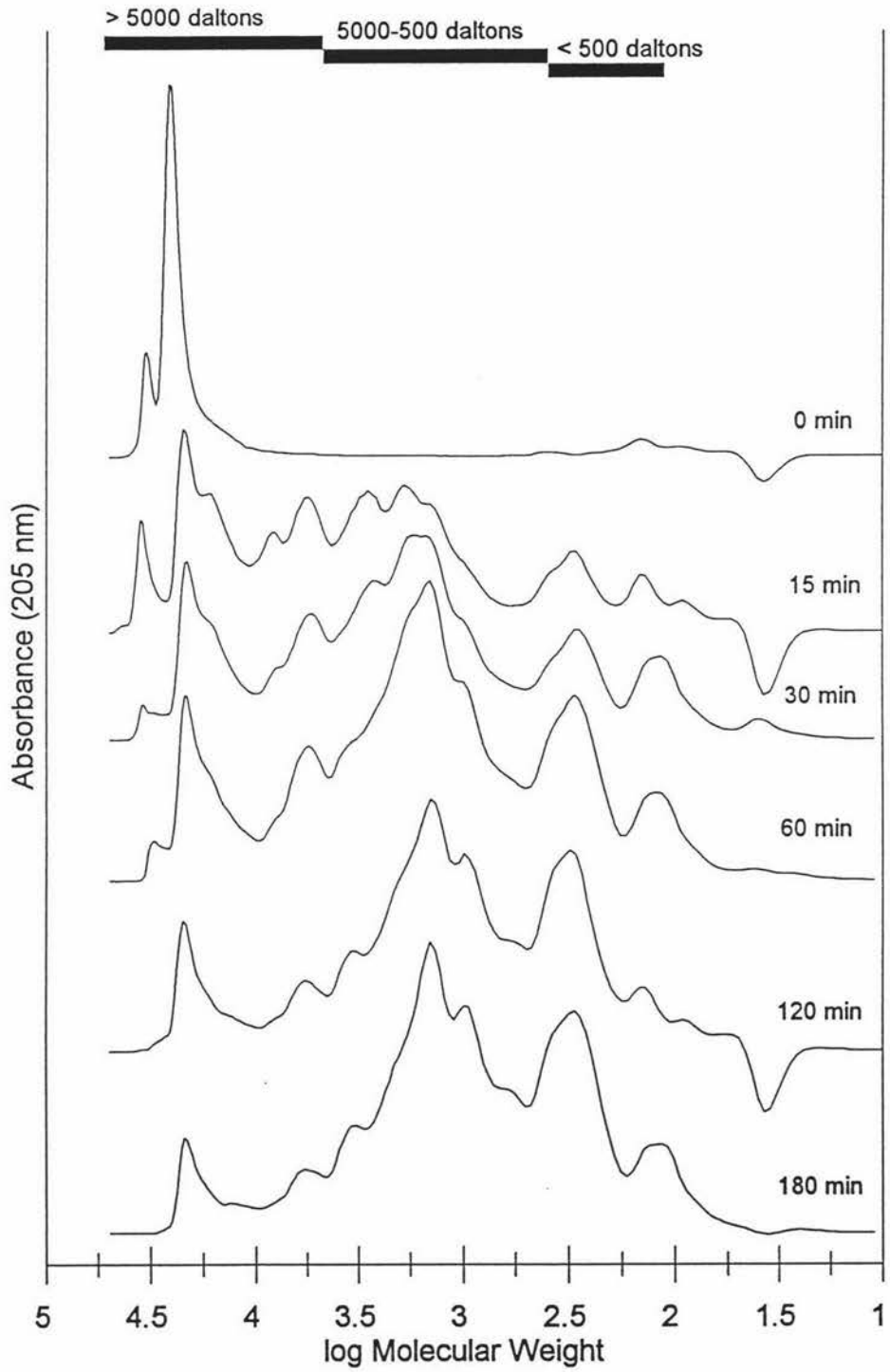


Figure 3.1 Time series MWP for Rohm Corolase N.

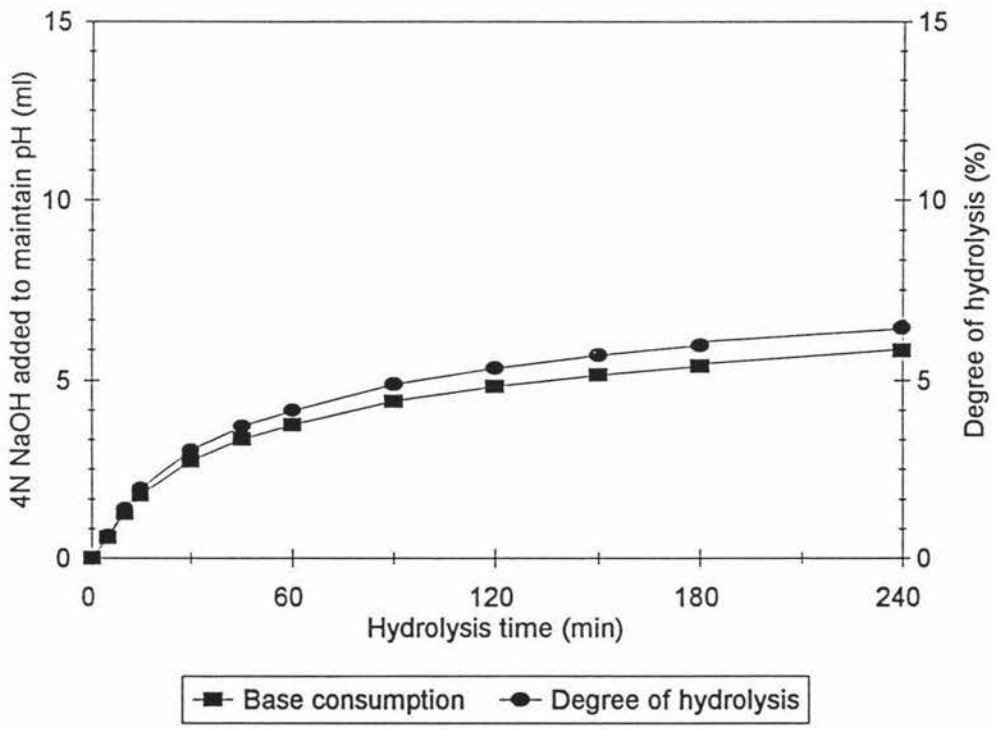


Figure 3.2 Base consumption and DH curves for Rohm Corolase N.

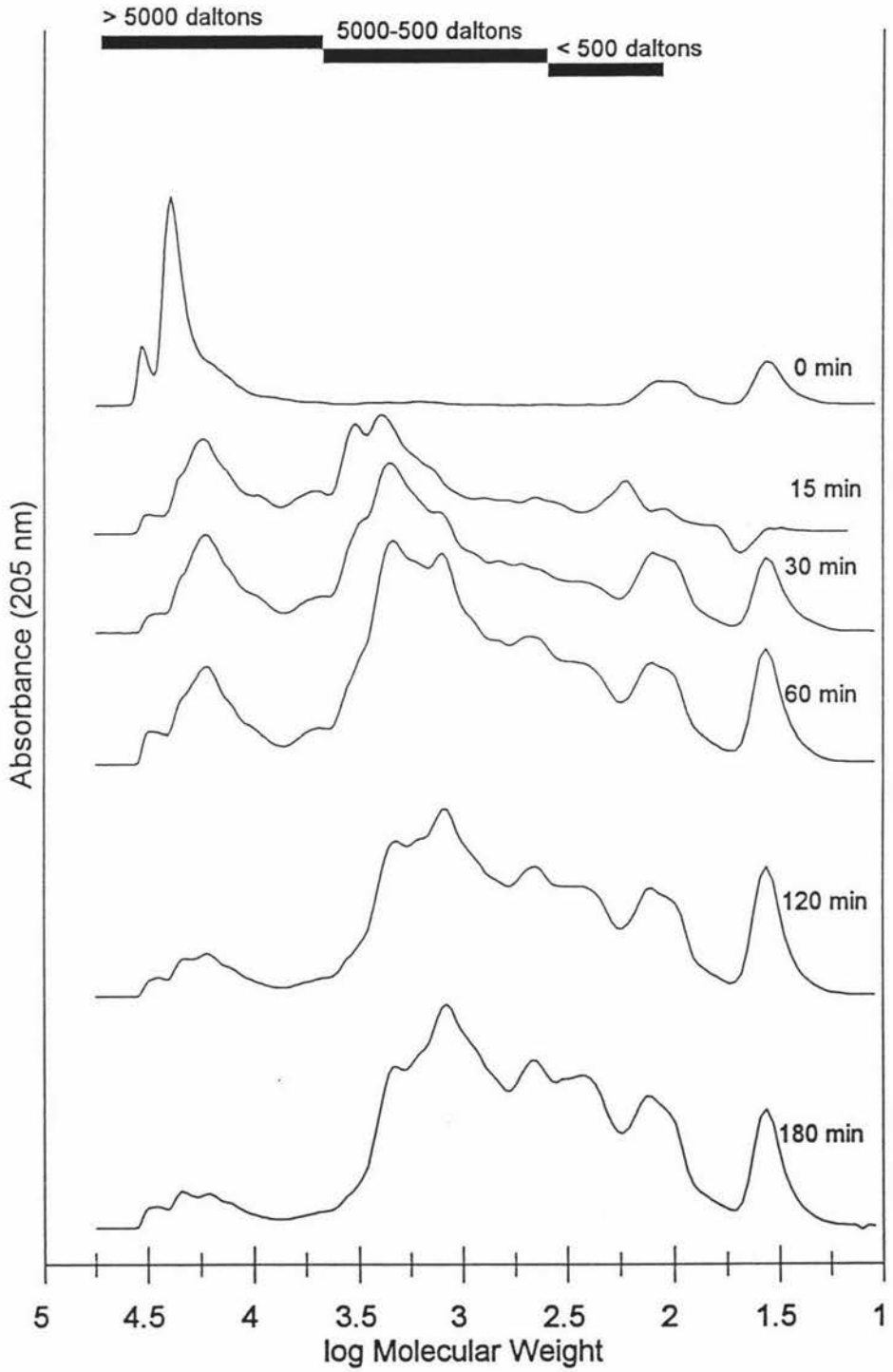


Figure 3.3 Time series MWP for Biocon Fungal Protease.

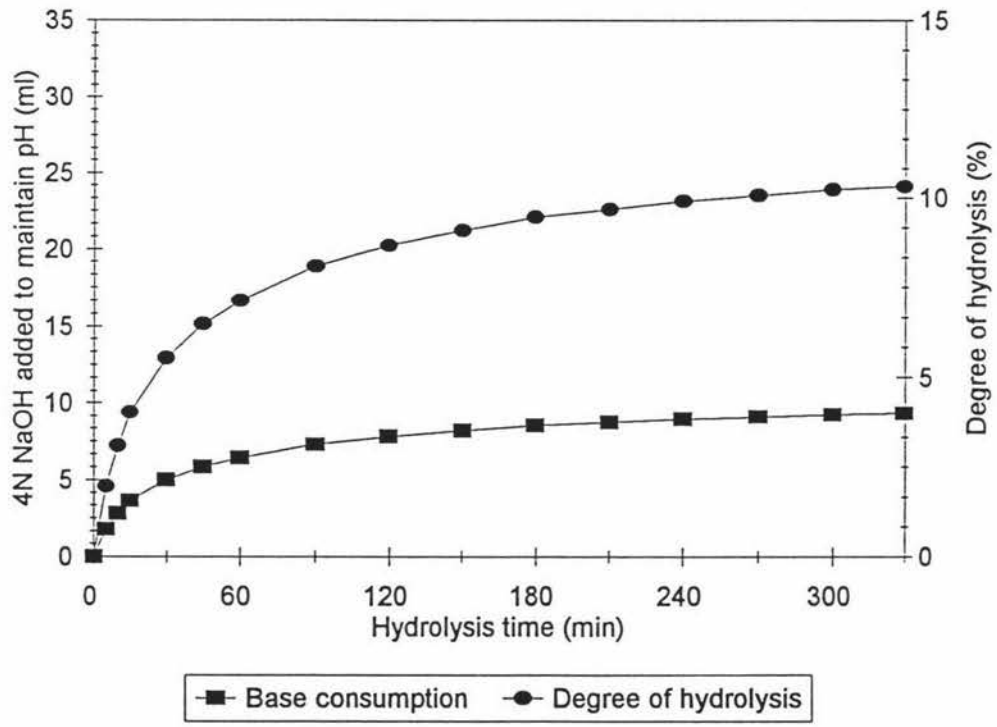


Figure 3.4 Base consumption and DH curves for Biocon Fungal Protease.

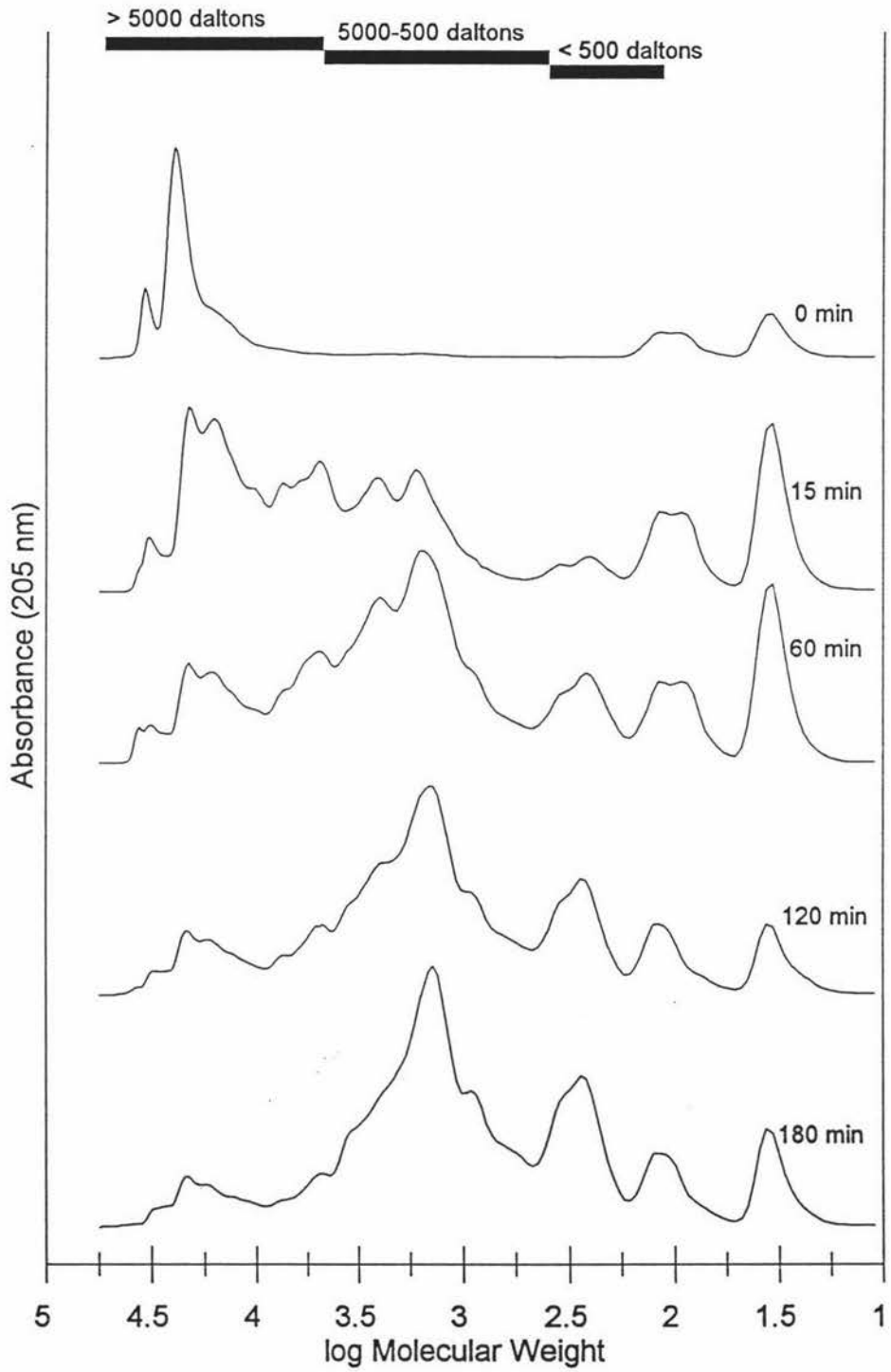


Figure 3.5 Time series MWP for Novo Neutrase 0.5L.

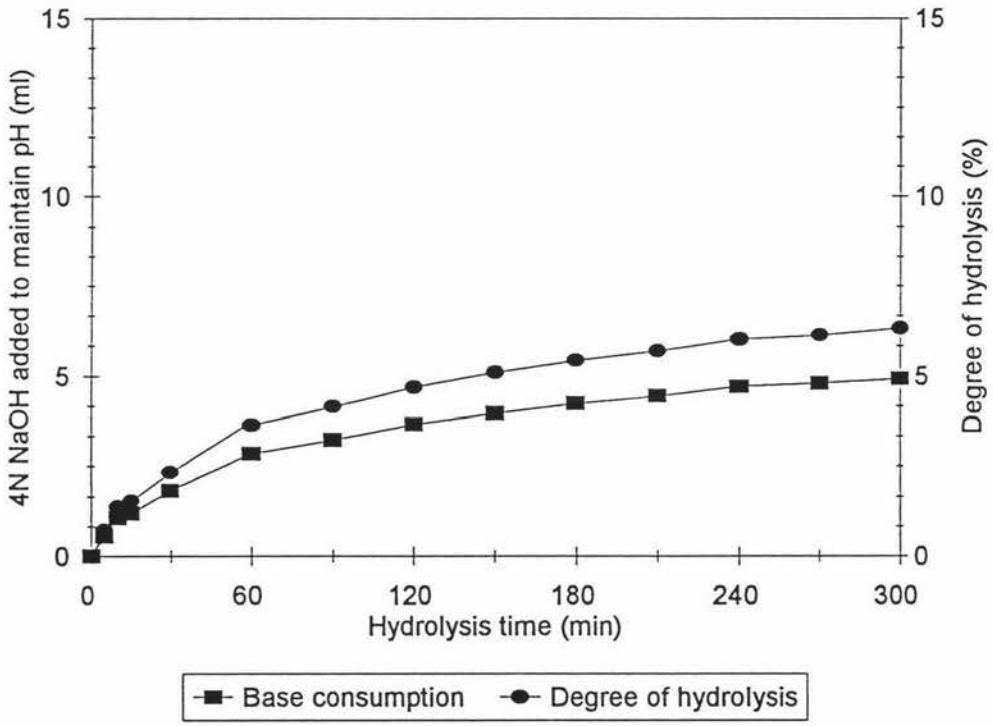


Figure 3.6 Base consumption and DH curves for Novo Neutrase 0.5L.

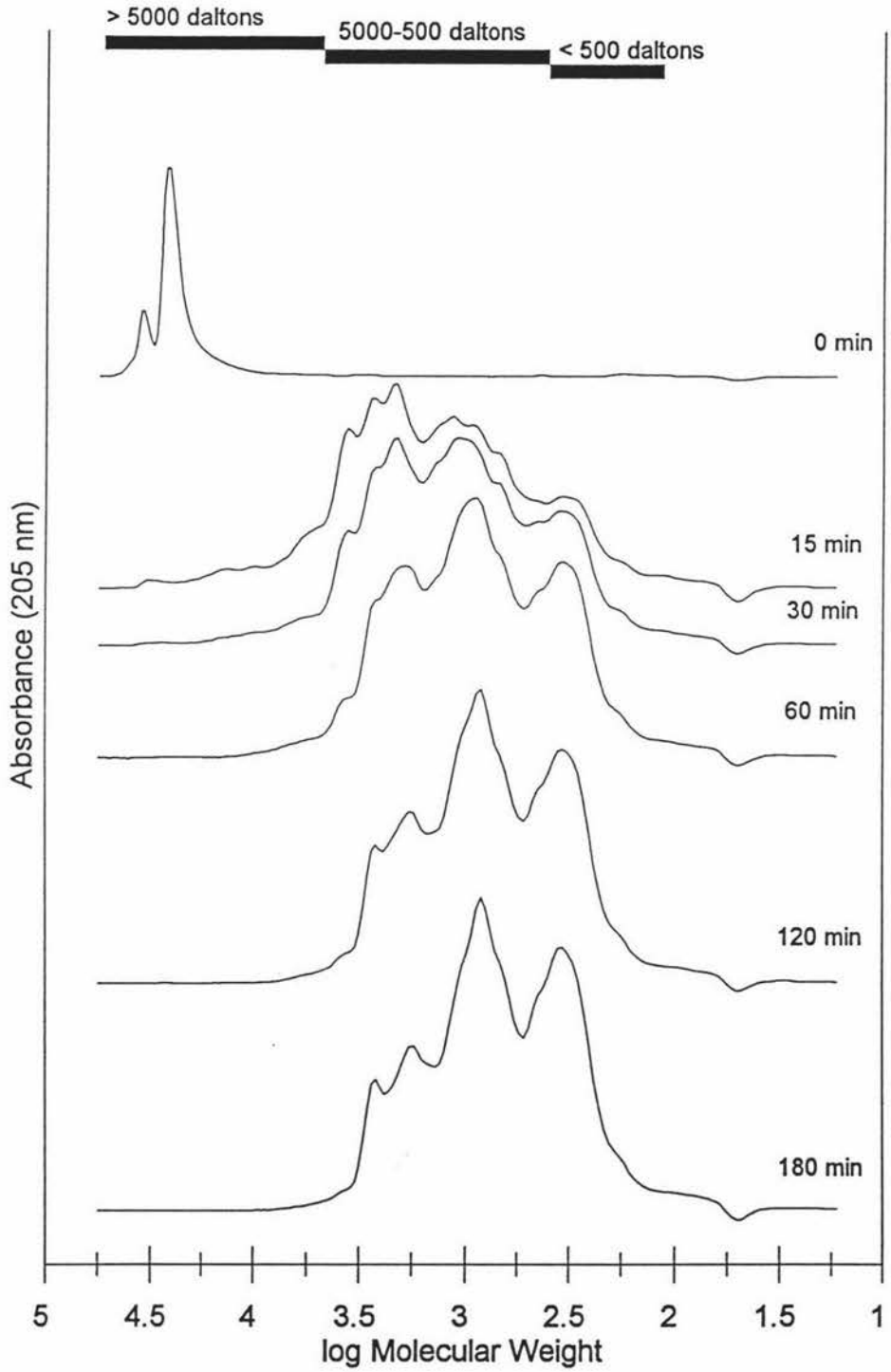


Figure 3.7 Time series MWP for Novo Alcalase 2.4L.

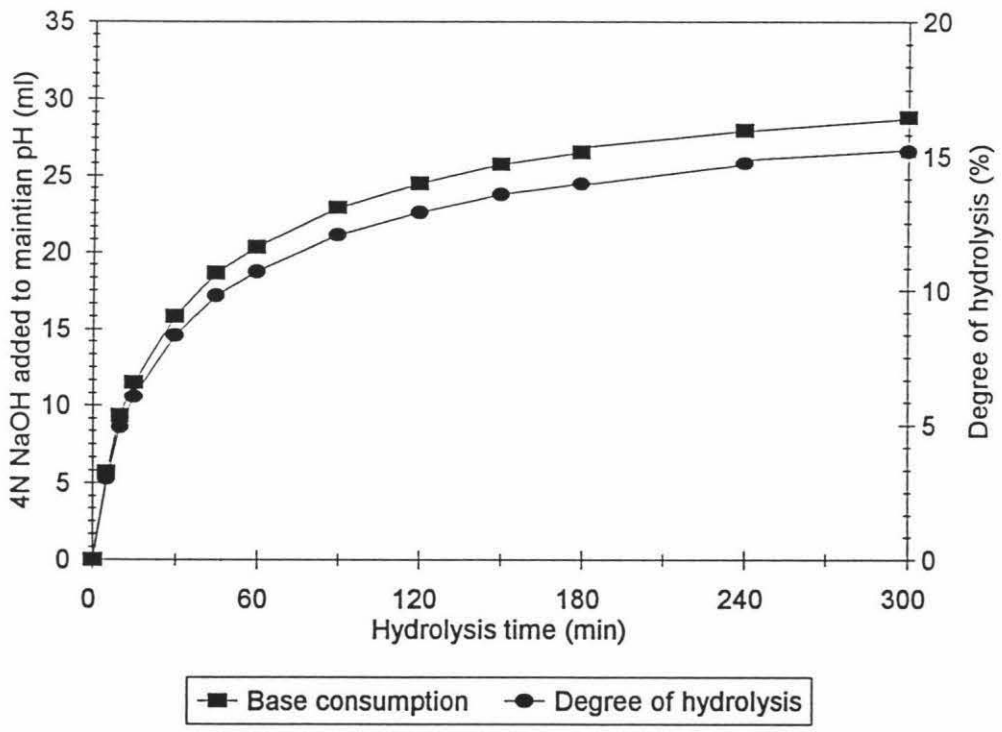


Figure 3.8 Base consumption and DH curves for Novo Alcalase 2.4L.

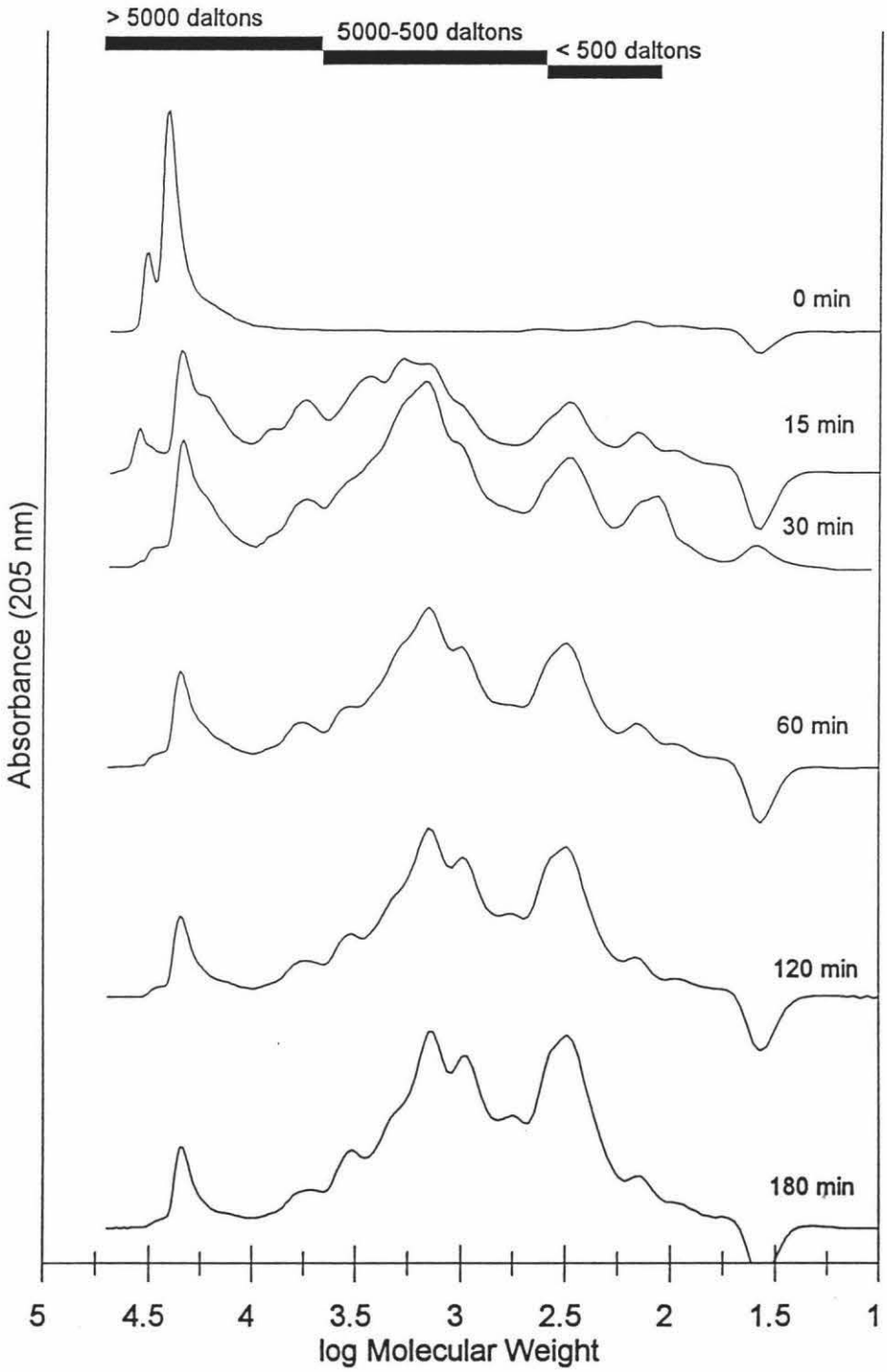


Figure 3.9 Time series MWP for Rohm Corolase 7089.

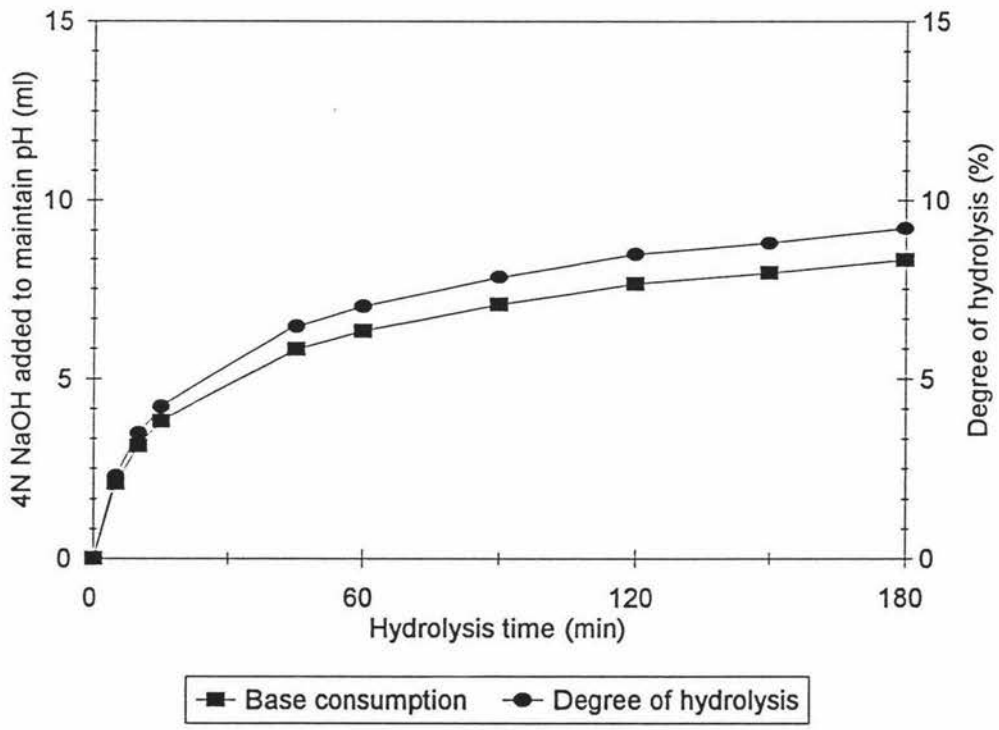


Figure 3.10 Base consumption and DH curves for Rohm Corolase 7089.

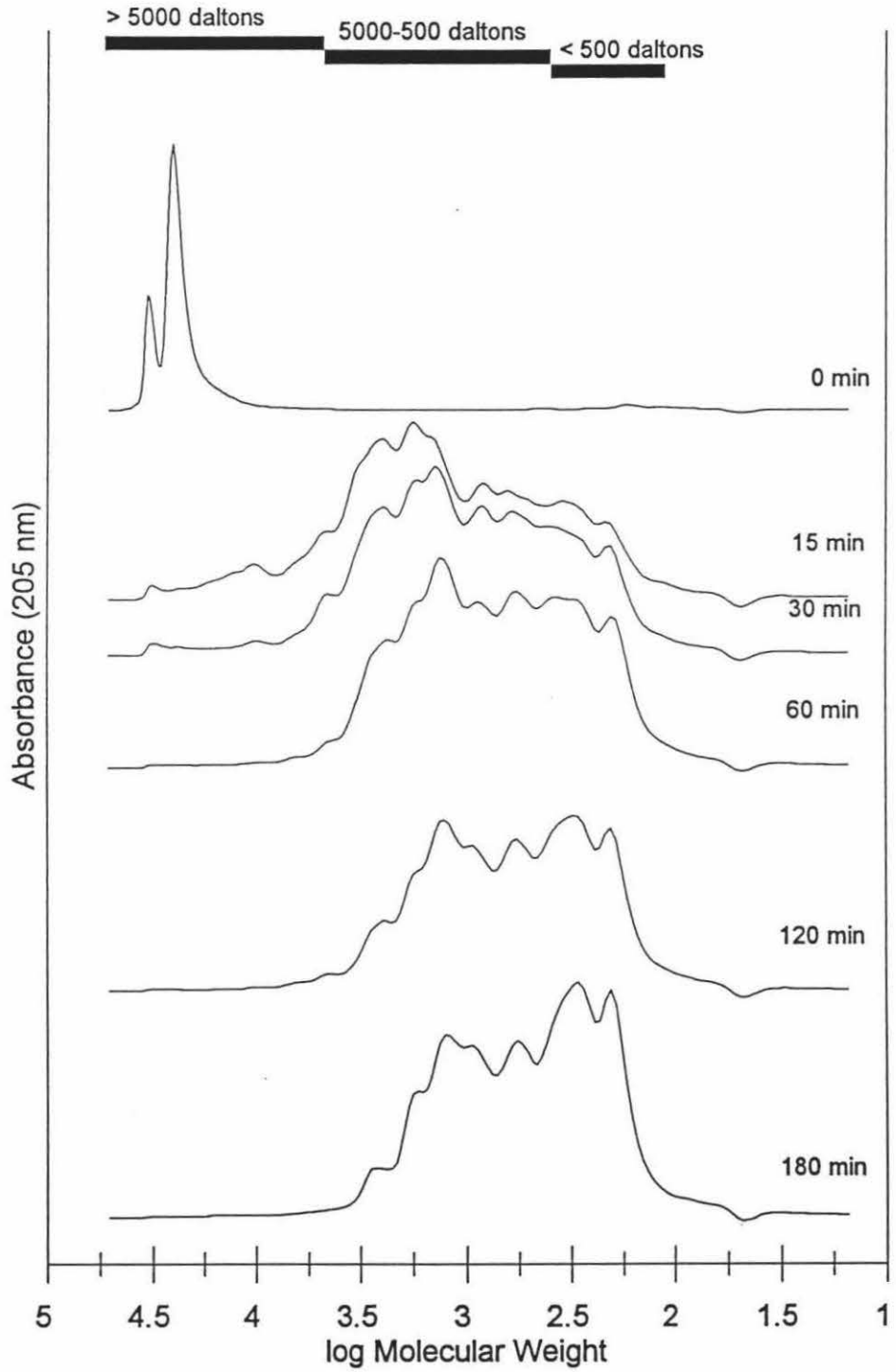


Figure 3.11 Time series MWP for Amano Protease A.

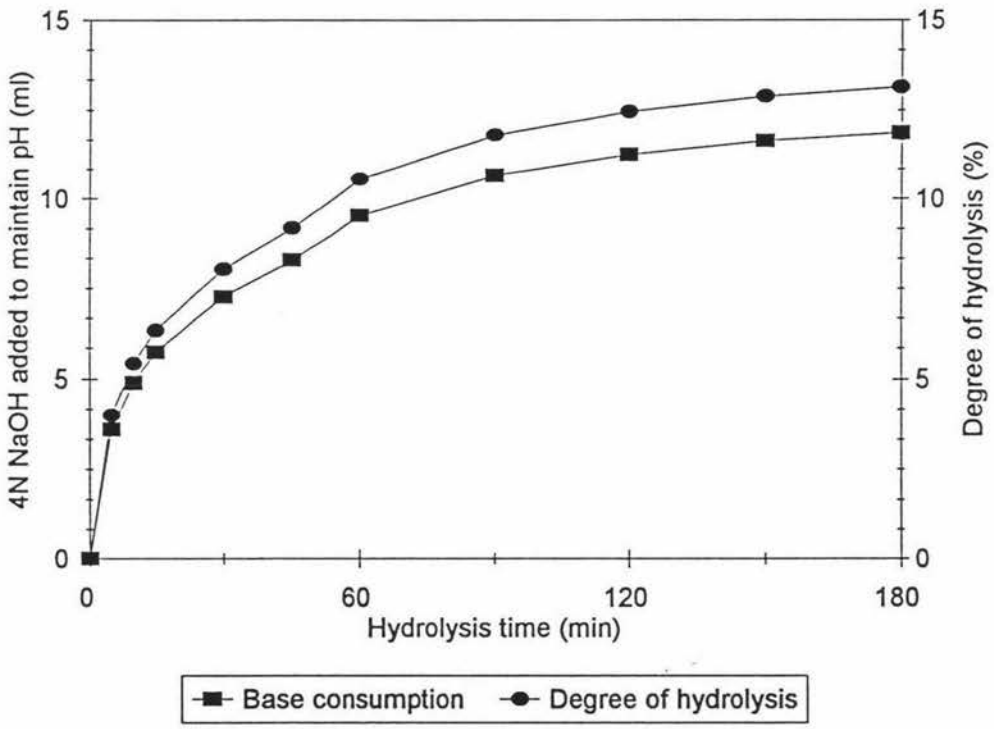


Figure 3.12 Base consumption and DH curves for Amano Protease A.

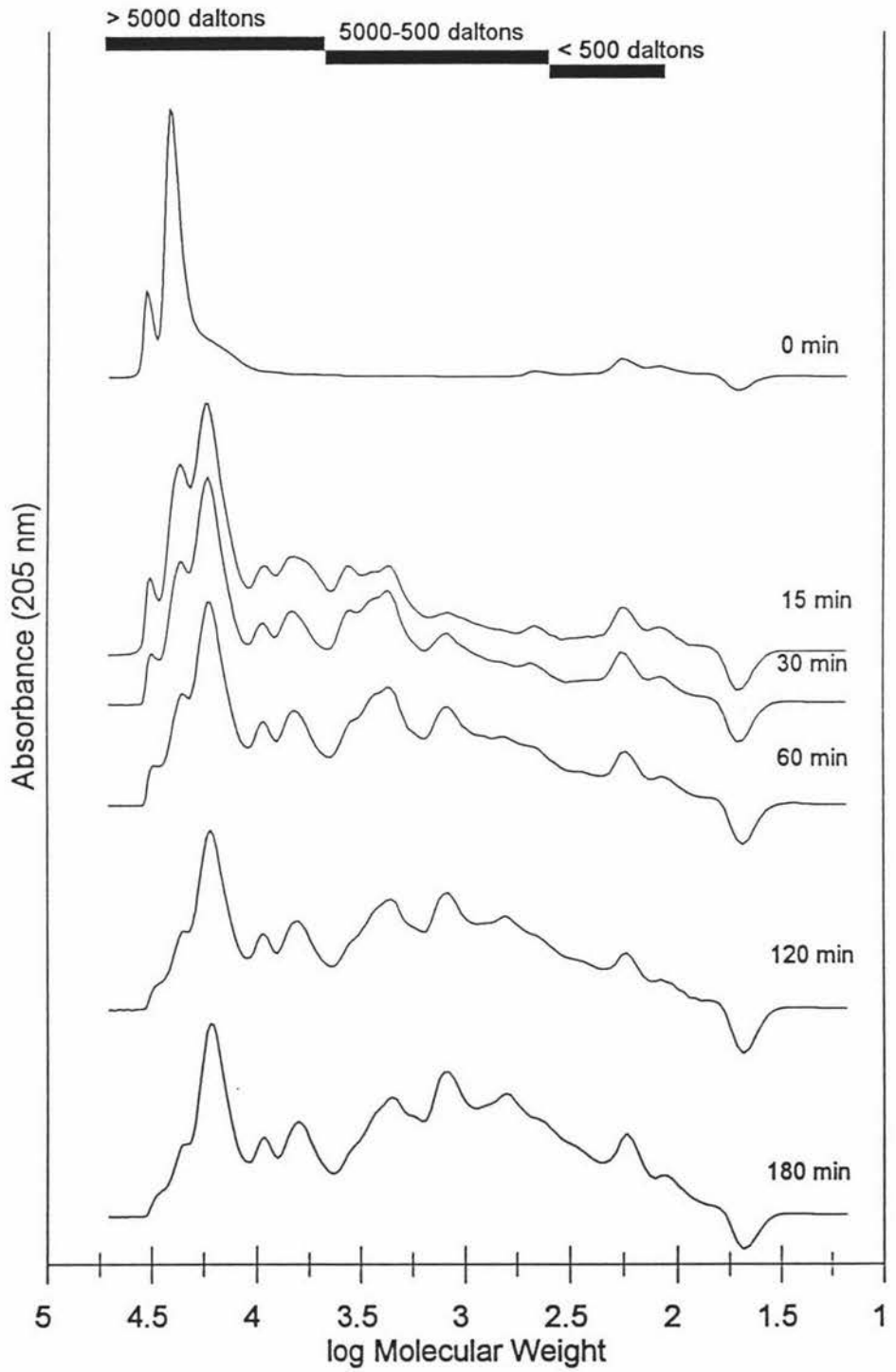


Figure 3.13 Time series MWP for Amano Protease B.

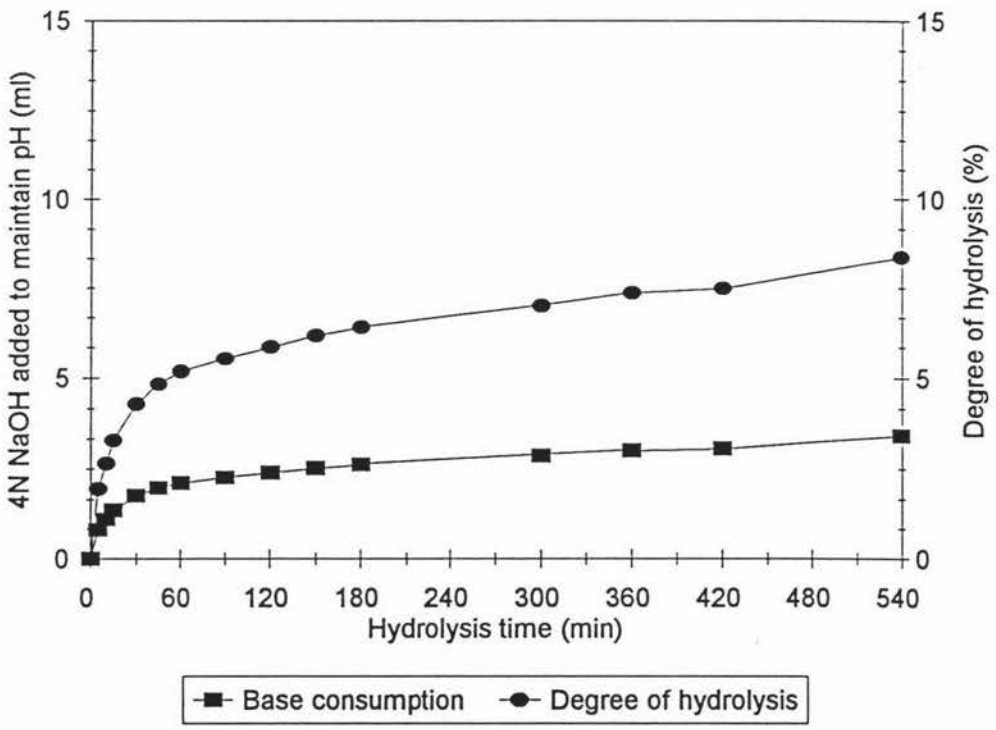


Figure 3.14 Base consumption and DH curves for Amano Protease B.

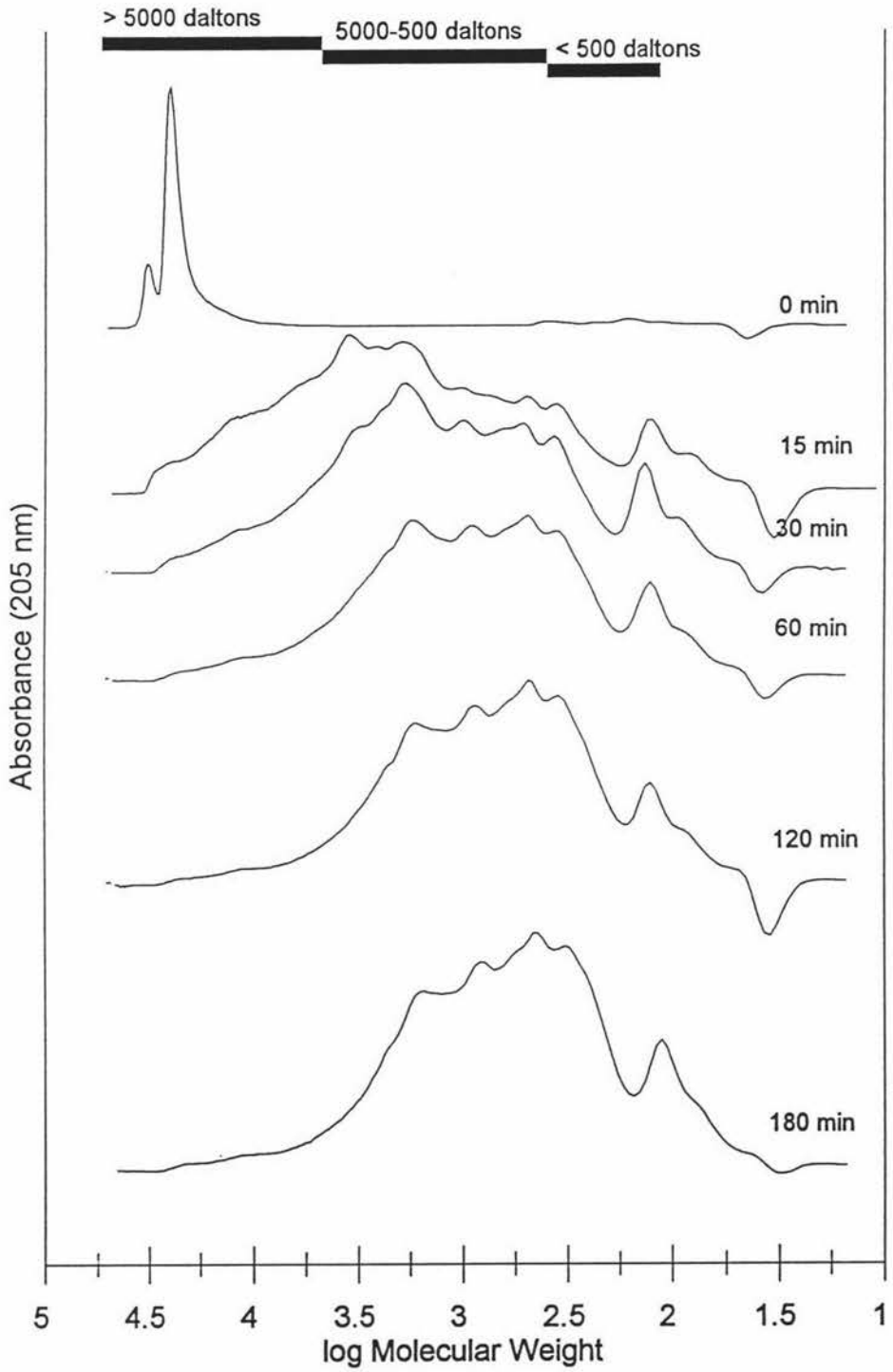


Figure 3.15 Time series MWP for Amano Papain.

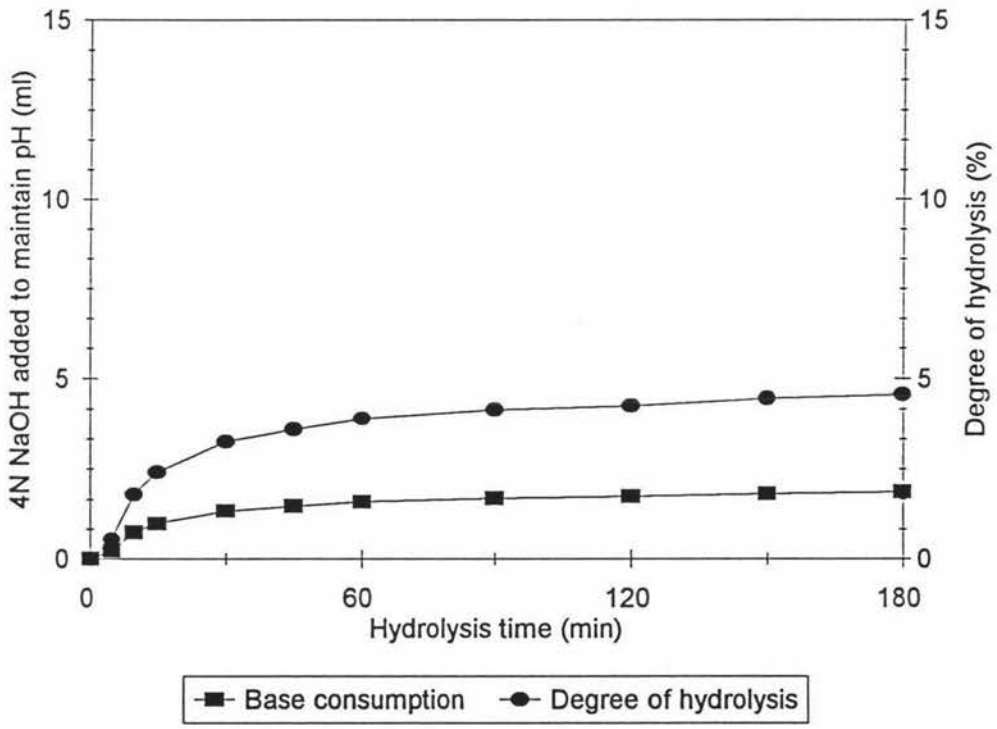


Figure 3.16 Base consumption and DH curves for Amano Papain.

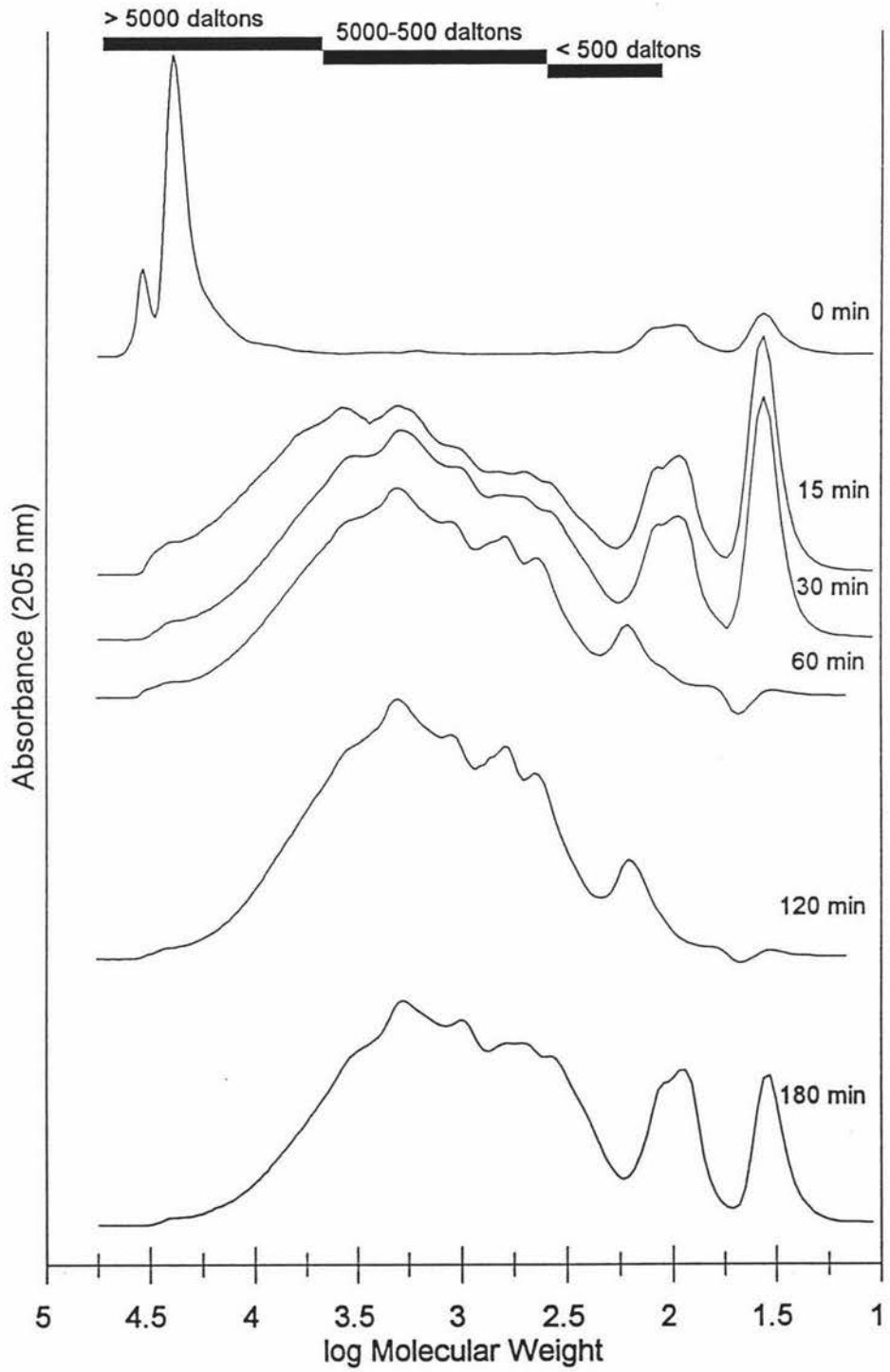


Figure 3.17 Time series MWP for Rohm Corolase S50.

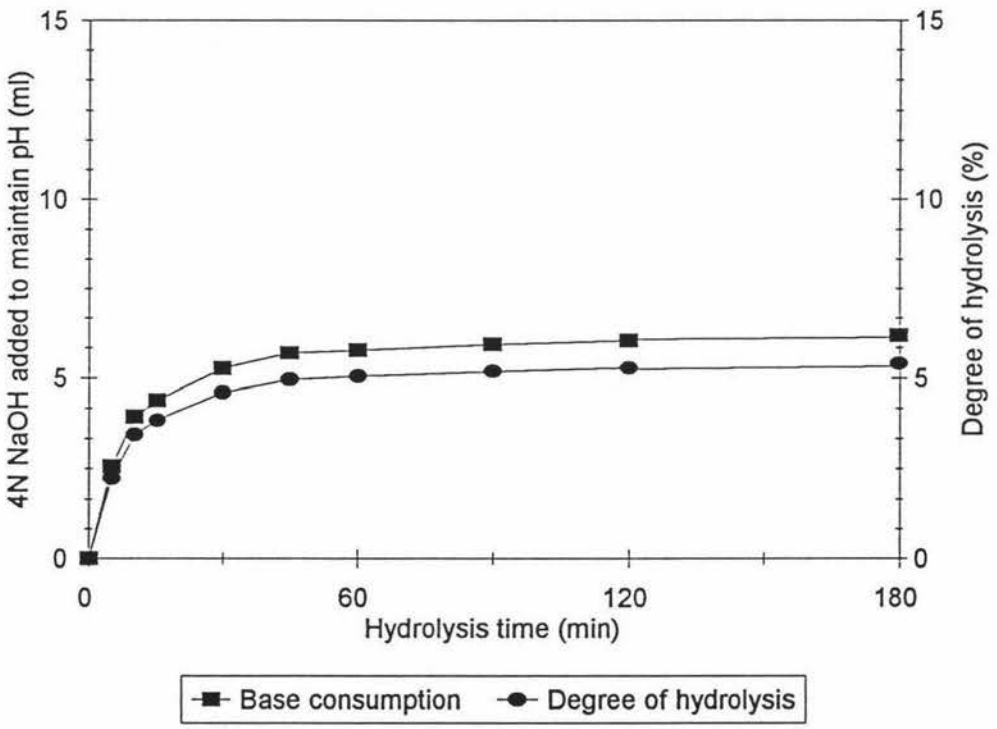


Figure 3.18 Base consumption and DH curves for Rohm Corolase S50.

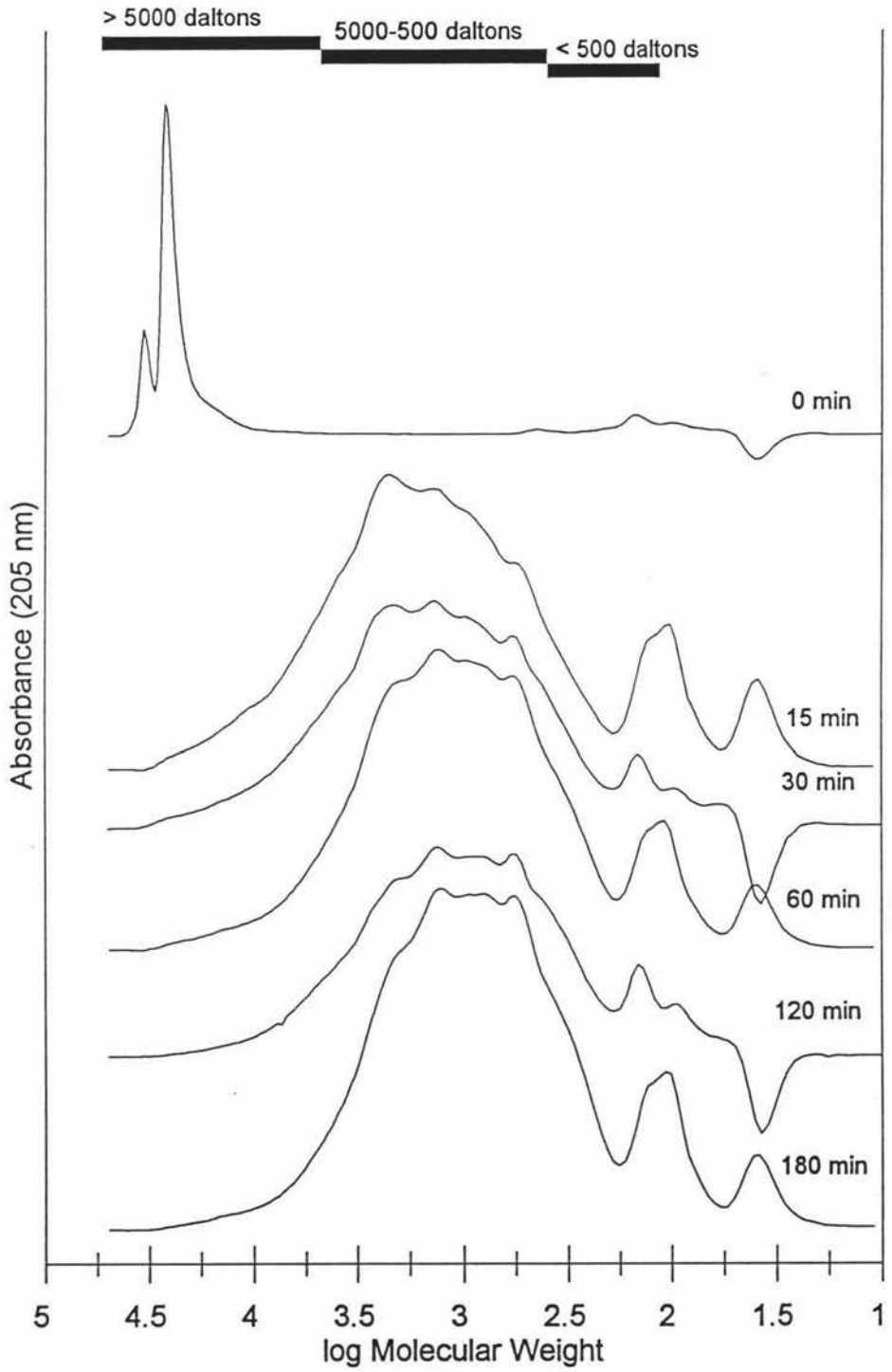


Figure 3.19 Time series MWP for Rohm Bromelain.

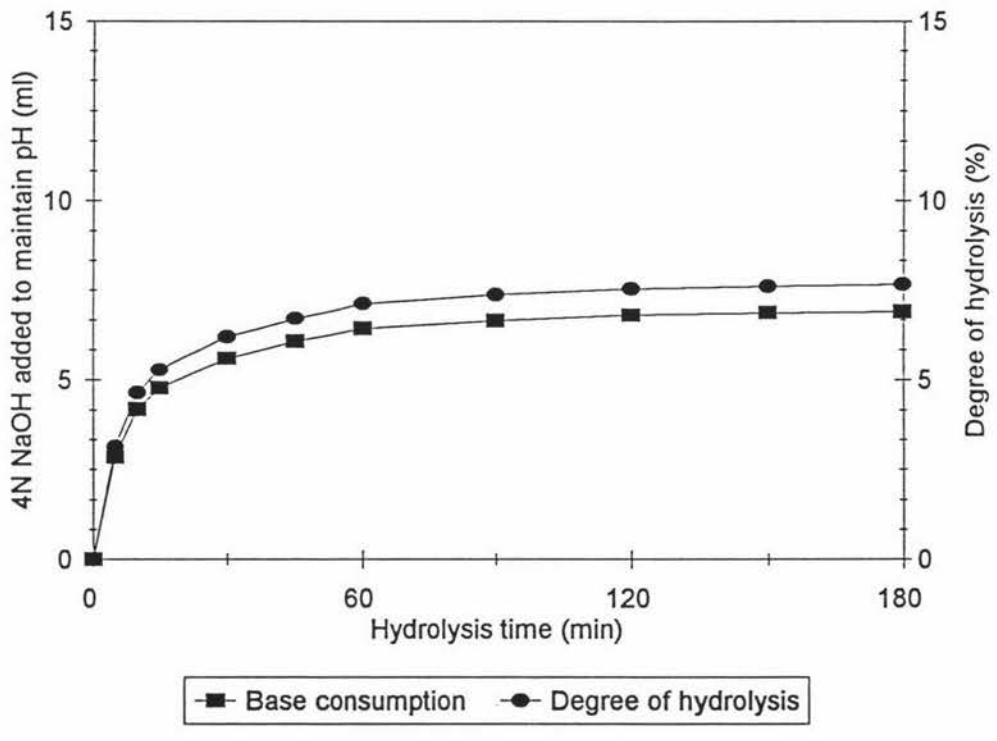


Figure 3.20 Base consumption and DH curves for Rohm Bromelain.

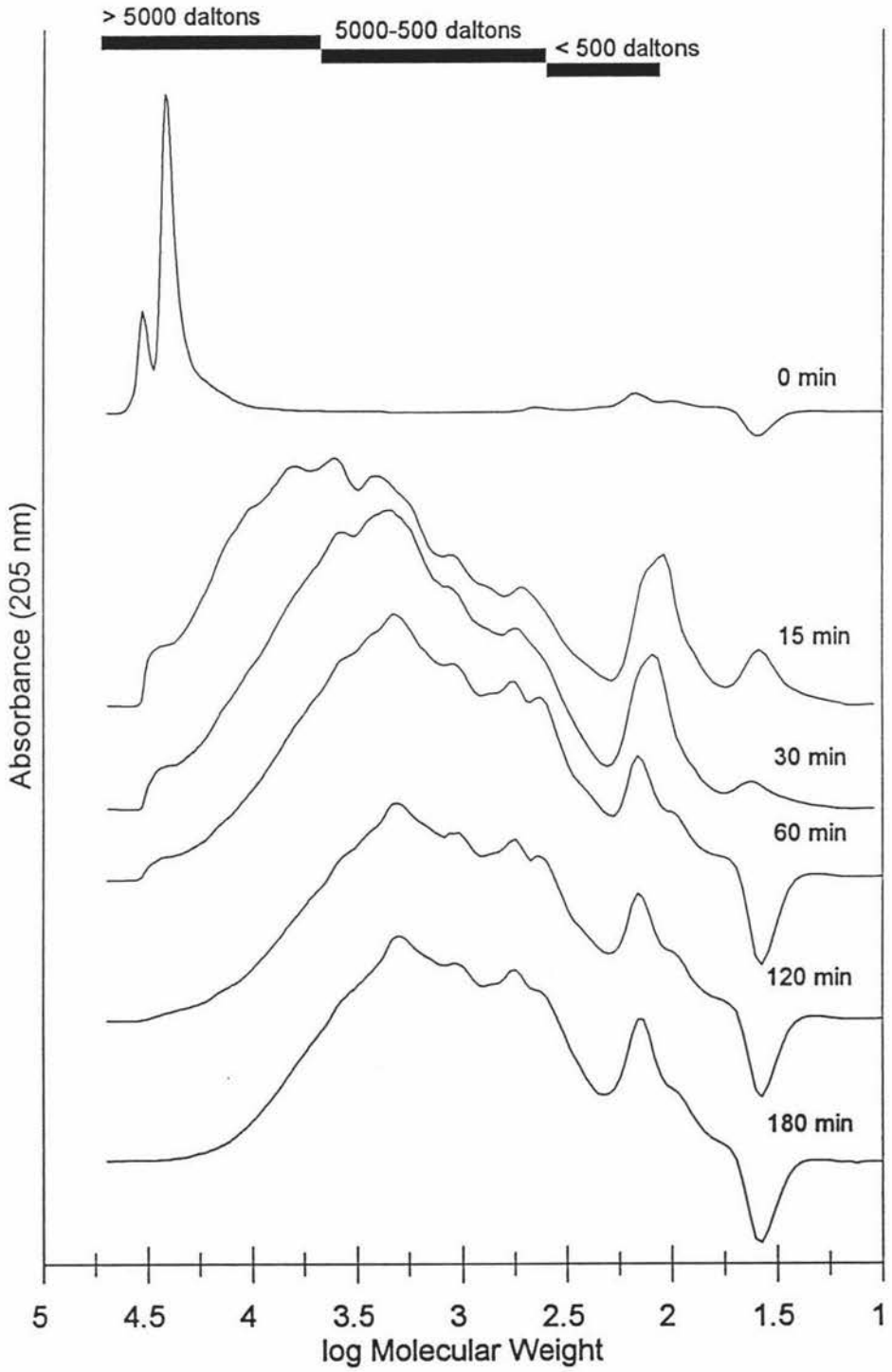


Figure 3.21 Time series MWP for Amano Bromelain.

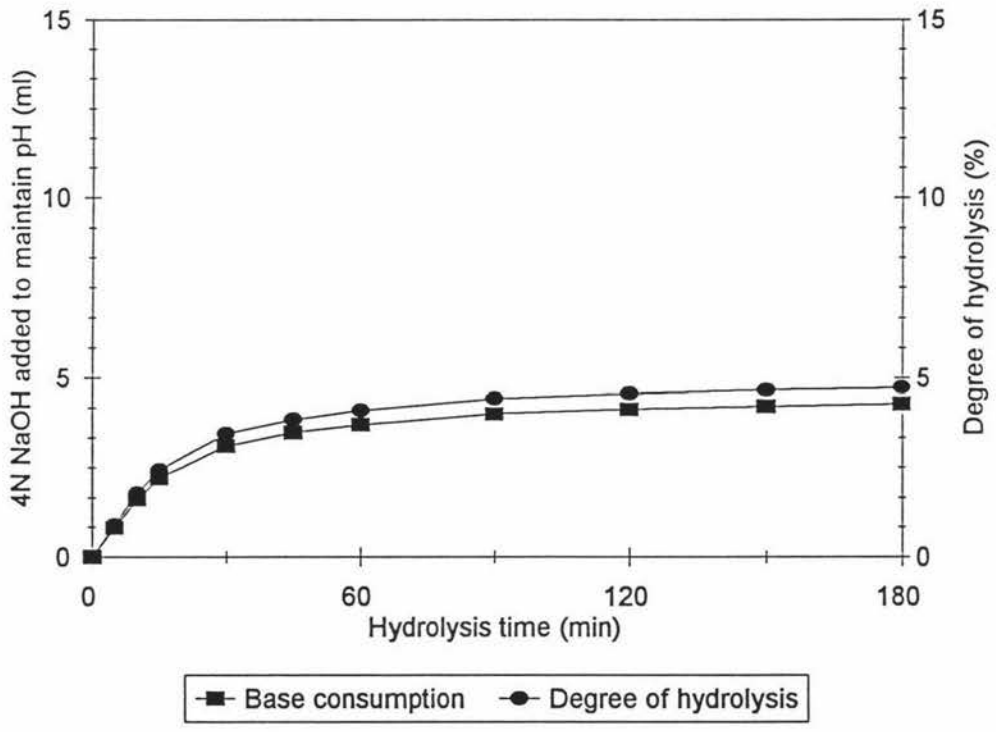


Figure 3.22 Base consumption and DH curves for Amano Bromelain.

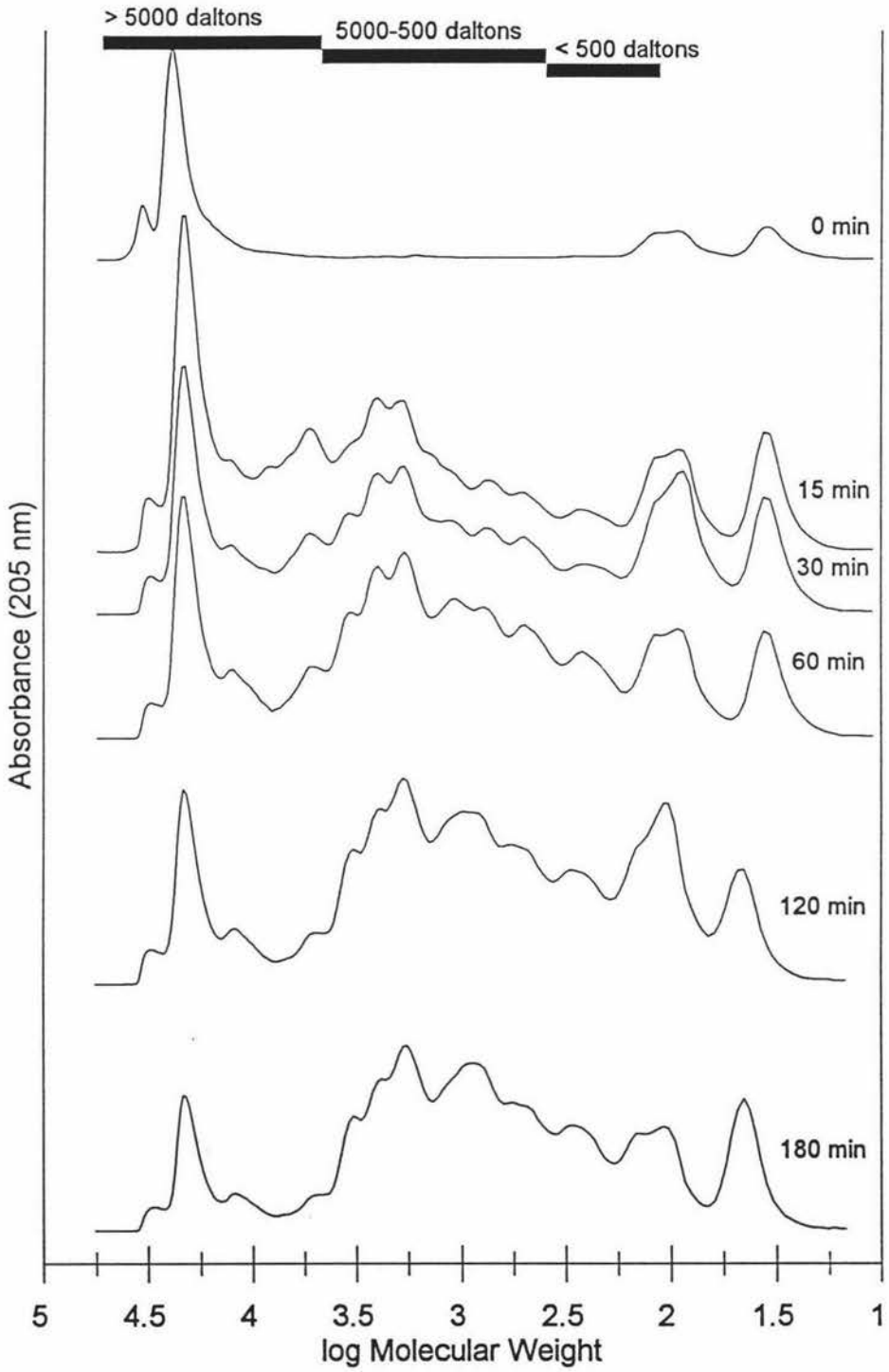


Figure 3.23 Time series MWP for Rhozyme P64.

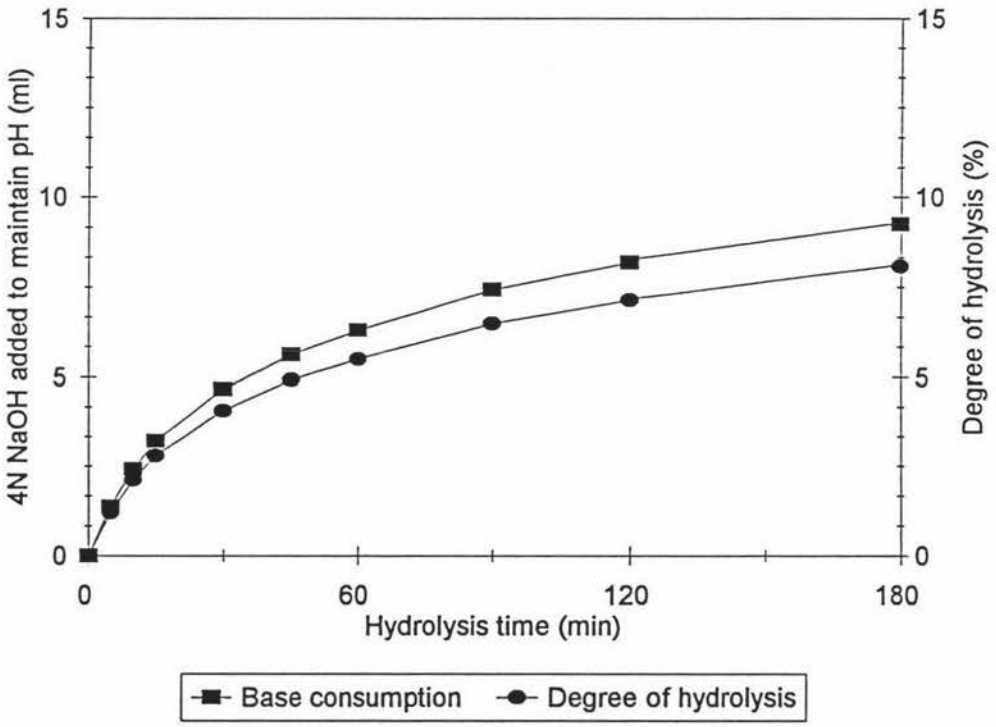


Figure 3.24 Base consumption and DH curves for Rhozyme P64.

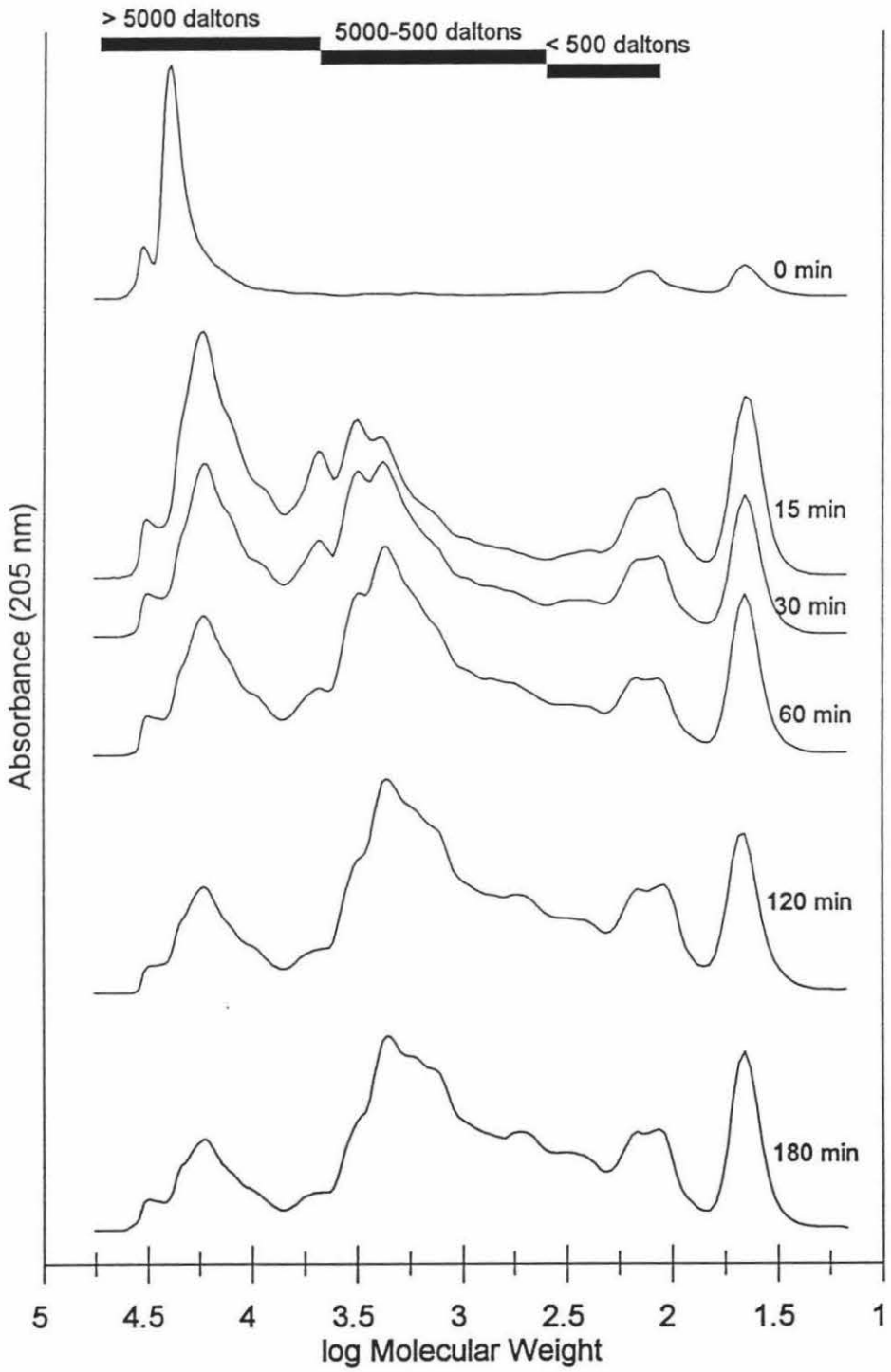


Figure 3.25 Time series MWP for Rhozyme P41.

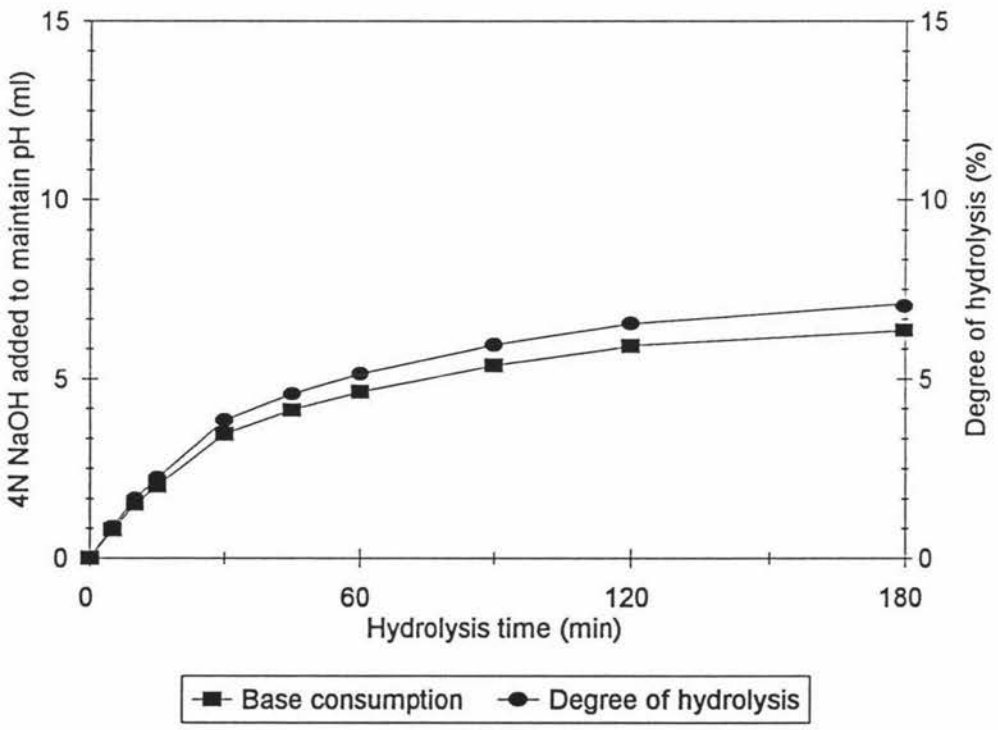


Figure 3.26 Base consumption and DH curves for Rhozyme P41.

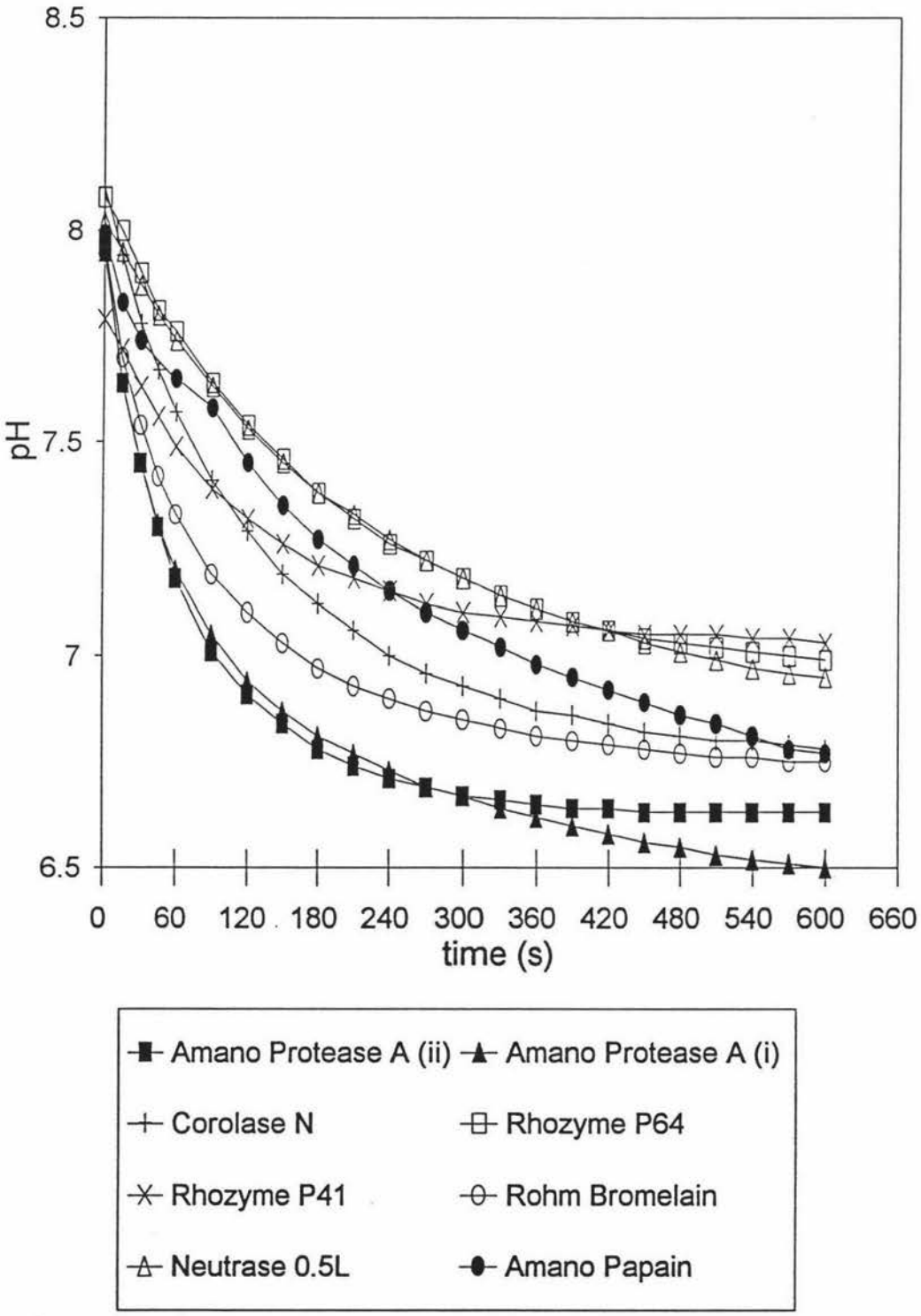


Figure 3.27 pH-drop curves for the enzymes screened (Part 1).

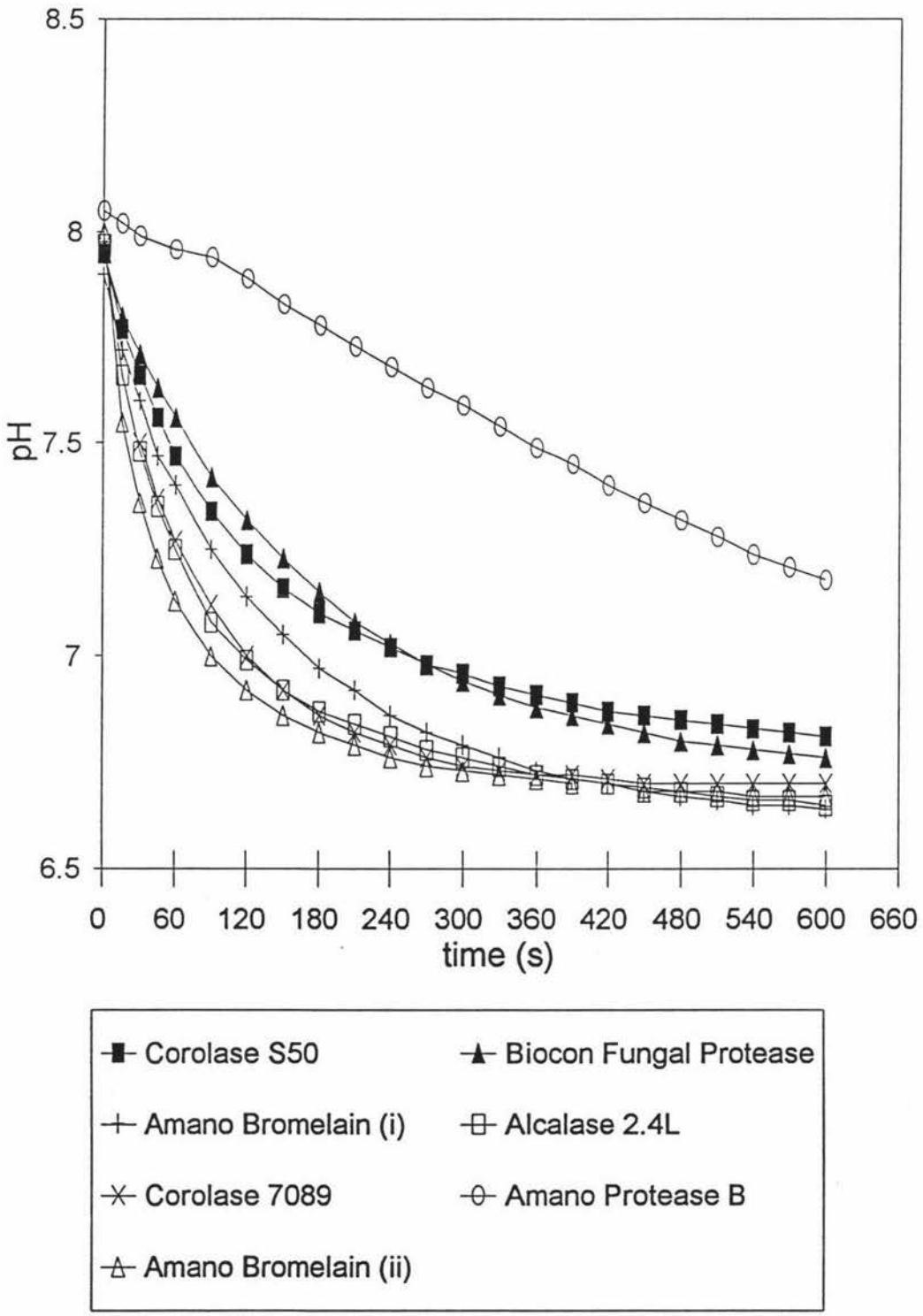
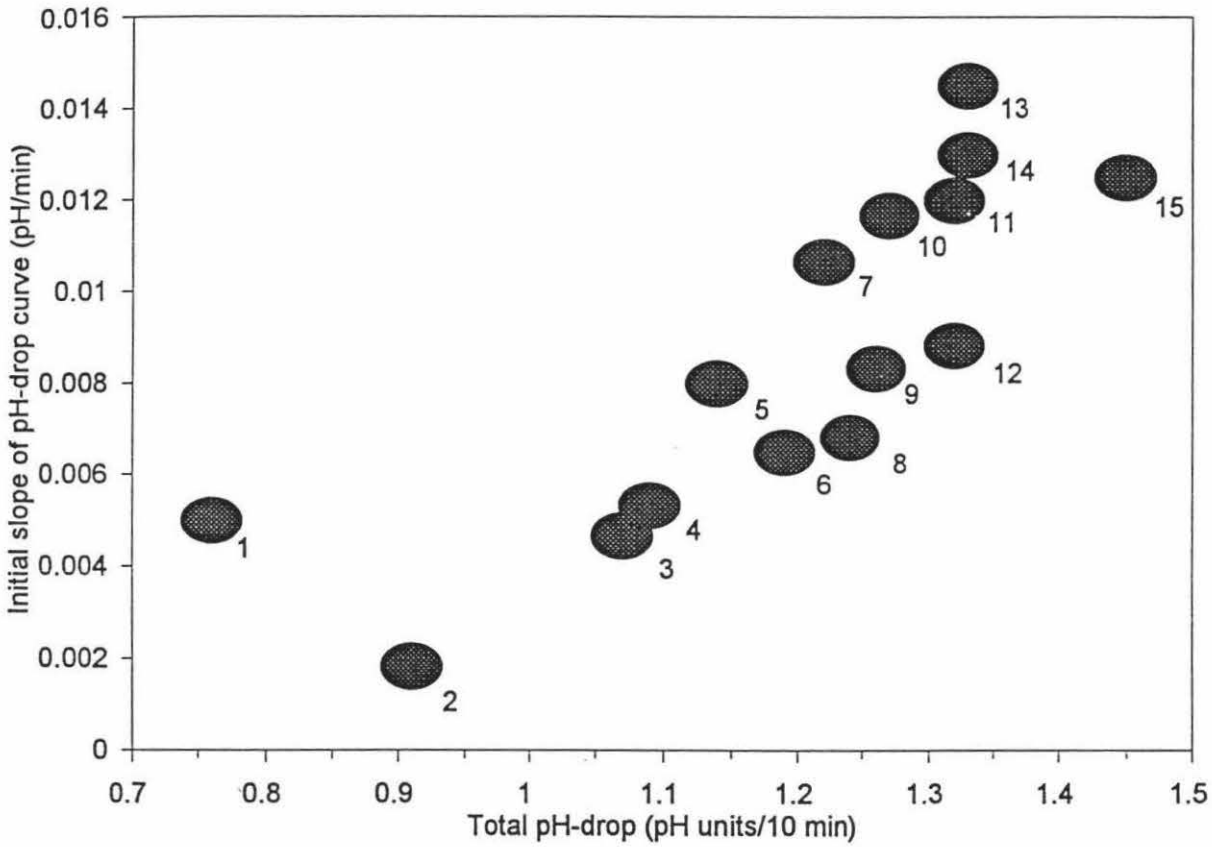


Figure 3.28 pH-drop curves for the enzymes screened (Part 2).



KEY

1 Rhozyme P41	6 Biocon Fungal Protease	11 Corolase N
2 Amano Protease B	7 Rohm Bromelain	12 Alcalase 2.4L
3 Neutrase 0.5L	8 Amano Papain	13 Amano Bromelain (ii)
4 Rhozyme P64	9 Amano Bromelain (i)	14 Amano Protease A (i)
5 Corolase S50	10 Corolase 7089	15 Amano Protease A (ii)

Figure 3.29 Graph showing the relationship between pH-drop and initial reaction rate for each enzyme.

3.4 Discussion

Enzymes for further experiments using a bioreactor system were selected using time series MWPs. The characteristics of the various enzymes, as determined from the MWPs, are summarised in Table 3.4. The formation of free amino acids during hydrolysis is an important consideration. The aim of the bioreactor experiments is to produce a hydrolysate containing a minimum proportion of free amino acids. If free amino acids are produced late in the hydrolysis reaction, then it will be possible to separate the peptides from which they are derived from the enzyme before they can be hydrolysed further. It is important to select an enzyme that can break down all of the substrate within a reasonable time period so that there is not a build-up of unhydrolysed material in the hydrolysis reaction vessel of the bioreactor. Two enzymes which showed the desired characteristics were Alcalase 2.4L and Amano Protease A. Both enzymes were able to hydrolyse all of the starting material within three hours of hydrolysis. The hydrolysate produced using Alcalase 2.4L did not have a free amino acid peak. The time series MWP from the experiment using Amano Protease A showed that free amino acids were released by hydrolysis late in the hydrolysis reaction.

The pH-drop method of assessing the suitability of enzymes for bioreactor processes gives a selection process based on enzyme activity alone. If this method had been used to select enzymes for further study in a bioreactor system, then Rohm Bromelain would have been selected in addition to Novo Alcalase 2.4L and Amano Protease A. However, Rohm Bromelain is not suited to the production of hydrolysates with low proportions of free amino acids. Examination of Figure 3.19 shows that this enzyme produces free amino acids from casein very early in the hydrolysis reaction.

Table 3.4 Summary of the characteristics of the action of various proteases on casein as determined from MWP analysis

Enzyme	Starting material remaining after 180 minutes of hydrolysis (%)	Free Amino Acid Peak √ = moderate peak √√ = large peak x = no peak	Formation of free amino acids - early or late in the hydrolysis reaction
Rohm Corolase N	10	√	Early
Biocon Fungal Protease	10	√√	Early
Neutrase 0.5L	10	√	Early
Alcalase 2.4L	0	x	-
Rohm Corolase 7089	10	√	Early
Amano Protease A	0	√√	Late
Amano Protease B	30	√	Early
Amano Papain	2.5	√√	Early
Corolase S50	5	√√	Early
Rohm Bromelain	2.5	√√	Early
Amano Bromelain	5	√√	Early
Rhozyme P64	17	√√	Early
Rhozyme P41	18	√√	Early

3.5 Conclusions

Amano Protease A and Novo Alcalase 2.4L were selected for bioreactor trials because, of the enzymes screened, these enzymes had a high activity for casein and either did not produce free amino acid formation (Alcalase 2.4L) or produced free amino acids late in the hydrolysis reaction (Amano Protease A). These enzymes have the required properties to produce a hydrolysate, from a CSTMR, with all material less than 5000 daltons molecular weight and low levels of free amino acids.

CHAPTER 4
MEMBRANE SCREENING

4.1 Introduction

Two different membrane configurations, hollow fibre and spiral wound, were assessed for their effect on the separation of a casein hydrolysate. In addition, the use of two different nominal MWCO values was evaluated for each membrane configuration. For the purposes of the present study, the maximum molecular weight of peptides in the final hydrolysate product was to be no greater than 5000 daltons. Therefore, it was necessary to assess the actual operating MWCO of the various membranes. Another important criterion for membrane selection was that a reasonable flux rate be obtained. If the flux was very low, then CSTMR runs with short hydraulic residence times (HRT) would be difficult to achieve in practice. This is because the reaction vessel volume would be very small with the consequence that the measurement and maintenance of the pH would be impossible. It would also be difficult to keep the reaction volume constant by matching the feed flowrate with the permeate flowrate with the equipment that was used. The relationship between reaction volume and permeate flowrate is given by the following equation for the HRT:

$$\text{HRT (min)} = \frac{\text{reaction volume (ml)}}{\text{permeate flowrate (ml/min)}} \qquad \text{Equation 4.1}$$

To test the effects of different membrane configurations and MWCOs, a hydrolysate was prepared under batch conditions, the enzyme was inactivated by heat and the resultant hydrolysate mixture was then ultrafiltered using each of the configurations/MWCOs. The hydrolysis conditions were selected using the MWPs obtained from the enzyme screening experiments. A hydrolysate for which the MWP showed

approximately one-third of the total material to be in each of the ranges > 5000 daltons, 1000-5000 daltons, and < 1000 daltons was selected.

4.2 Materials and Equipment

4.2.1 Enzyme

The enzyme used to produce the test hydrolysate was Rhozyme P64, Batch 18-91093-01. The characteristics of the casein hydrolysate produced using this enzyme have been described in Section 3.3.2.12.

4.2.2 Substrate

The substrate used was Alacid Acid Casein as described previously in Section 3.2.1.2.

4.2.3 Chemicals

"Analar" grade sodium hydroxide, acetonitrile and trifluoroacetic acid as described in Section 3.2.1.3 were used.

All water used for reagent and solution preparation and for cleaning the membranes was de-ionized, carbon-treated and filtered through a 0.22 μm membrane (MilliQ, Millipore Associates, Bedford, Massachusetts, USA).

4.2.4 Equipment

The following were employed:

Hollow fibre ultrafiltration cartridges H1P10-43 (Serial number DQ9083-6C) and H1P30-43 (Serial number DR8280-6B), fitted with a hollow fibre cartridge holder (Amicon Division, W R Grace and Co., Beverly, Massachusetts, USA). Both cartridges had a membrane surface area of 0.03 m^2 and nominal MWCOs of 10,000 and 30,000 daltons respectively.

Spiral wound ultrafiltration cartridges S1Y10 (Serial number 12956) and S1Y30 (Serial number 7684), membrane area 0.09 m^2, fitted with a spiral wound cartridge holder (Amicon Division, W.R. Grace and Co., Beverly, Massachusetts, USA). Both cartridges had a membrane surface

area of 0.09 m^2 and nominal MWCOs of 10,000 and 30,000 daltons respectively.

An Ismatech MV positive displacement pump, Type MV-Z (System Laboratoriumtechnic, Glattburg-Zurich, Switzerland).

4.3 Methods

4.3.1 Test hydrolysate preparation

The test hydrolysate selected resulted from the hydrolysis of casein with Rhozyme P64 for 60 min. The test hydrolysate was prepared using the same procedure and equipment as described in Section 3.2.2.2. A caseinate solution (1 L at 10% total solids) was prepared by adding casein to demineralized water, adjusting the pH of the slurry to approximately pH 7 with 4 N sodium hydroxide, heating the slurry to 60°C and holding for approximately 1 h. The resulting solution was then cooled in a water bath to 50°C. The pH of the solution was checked and adjusted with 4 N sodium hydroxide to pH 7 before the enzyme was added to give an enzyme to substrate ratio of 1:100 (w/w). The pH was maintained during hydrolysis by the addition of 4 N sodium hydroxide, using the autotitrator system as described in Section 3.2.2.2. The hydrolysis reaction was allowed to continue until 6 ml of 4 N sodium hydroxide had been added to the reaction mixture, this being the amount previously determined to give the DH required. The reaction was terminated by heating the hydrolysate to 90°C and holding it at that temperature for 15 min. The resulting hydrolysate was diluted 1:1 with water to give a solution of approximately 5% total solids for the membrane evaluations.

4.3.2 Membrane cleaning and storage

Before and after each use, the ultrafiltration cartridges were cleaned using the following regime.

1 Approximately 2 L of water was circulated through the membrane cartridge at 50°C and an inlet pressure of 172 kPa (25 psi) for 15 min. The first 200 ml of permeate was

discarded and the rest was recycled. This step was then repeated with fresh water.

2 Approximately 2 L of 0.1 N sodium hydroxide was circulated through the membrane cartridge at 50°C and an inlet pressure of 172 kPa for 15 min. The first 200 ml of permeate was discarded and the rest was recycled.

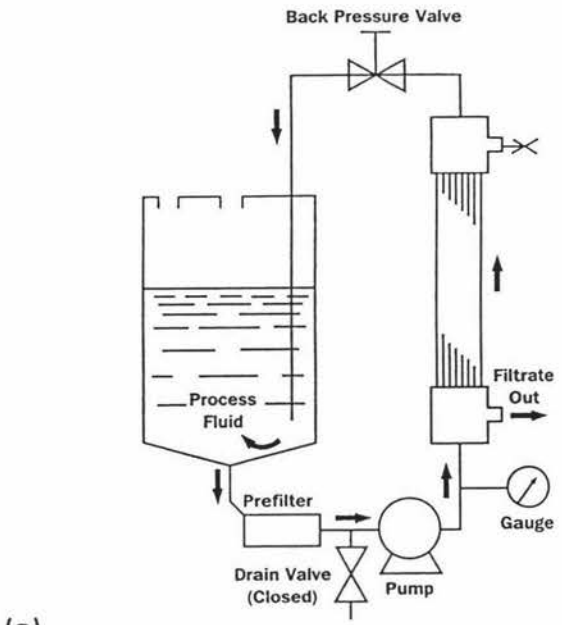
3 Step 1 was repeated.

4 The clean water flux of the membrane was checked to ensure that the membrane flux had been restored back to its original level, indicating that the membrane had been sufficiently cleaned.

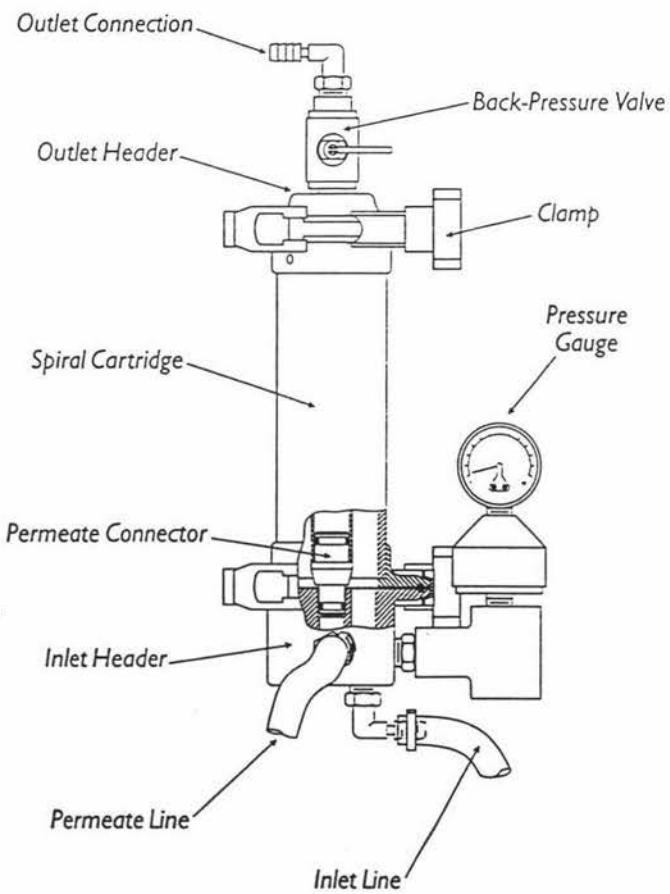
While they were not in use, the membrane cartridges were stored in 0.2% w/v sodium azide in water, at approximately 4°C, to prevent microbial growth.

4.3.3 Membrane evaluation method

Schematic diagrams of the two membrane configurations are shown in Figure 4.1. The test hydrolysate (1 L) was heated to 50°C in a water bath. The hydrolysate was then pumped to the membrane and the membrane back-pressure adjusted so that the inlet pressure to the membrane was 137-172 kPa (20-25 psi). The same recirculation rate, which was controlled by the pump speed, was used for all membranes. The retentate was recycled back to the feed beaker; the permeate was collected in a separate beaker. The permeate flowrate was determined at regular intervals using a measuring cylinder and stopwatch. Once the permeate flowrate remained steady for two consecutive readings, the feed, retentate and permeate were sampled for MWP (as detailed in Section 3.2.2.3).



(a)



(b)

Figure 4.1 Schematic diagrams of the hollow fibre and spiral wound membrane configurations: (a) Hollow fibre membrane module (Amicon, 1994) and (b) spiral wound membrane module (Amicon, 1992).

4.3.4 Analytical methods

HPLC analysis to produce MWPs has been described previously in Section 3.2.2.4. The analysis was carried out at Tatua Biologics Ltd. using the same HPSEC column as was used at NZDRI for analysis of samples from the enzyme screening experiments (Chapter 3). Total solids concentrations of the feed, permeate and retentate were estimated from the area of the respective MWPs.

4.4 Results

The raw data and flux calculations from the membrane screening experiments are given in Appendix IV. The permeate flowrates are plotted in Figure 4.2. The MWPs of the feed, permeate and retentate from each membrane are shown in Figures 4.3 to 4.7. The permeate flowrates, permeate and retentate total solids and flux (after 30 minutes) for each membrane are given in Table 4.1.

Table 4.1 Permeate flowrates, permeate and retentate total solids and fluxes for hollow fibre and spiral wound membranes

Membrane	Permeate flowrate after 30 minutes (ml/min)	Permeate total solids (% w/w)	Retentate total solids (% w/w)	Membrane flux ($L/m^2/h$)
Hollow fibre, 10,000 MWCO	6	1.2	4.8	12
Hollow fibre, 30,000 MWCO	13	1.2	3.7	26
Spiral wound, 10,000 MWCO	50	2.1	5.5	33
Spiral wound, 30,000 MWCO	65	2.2	5.7	43

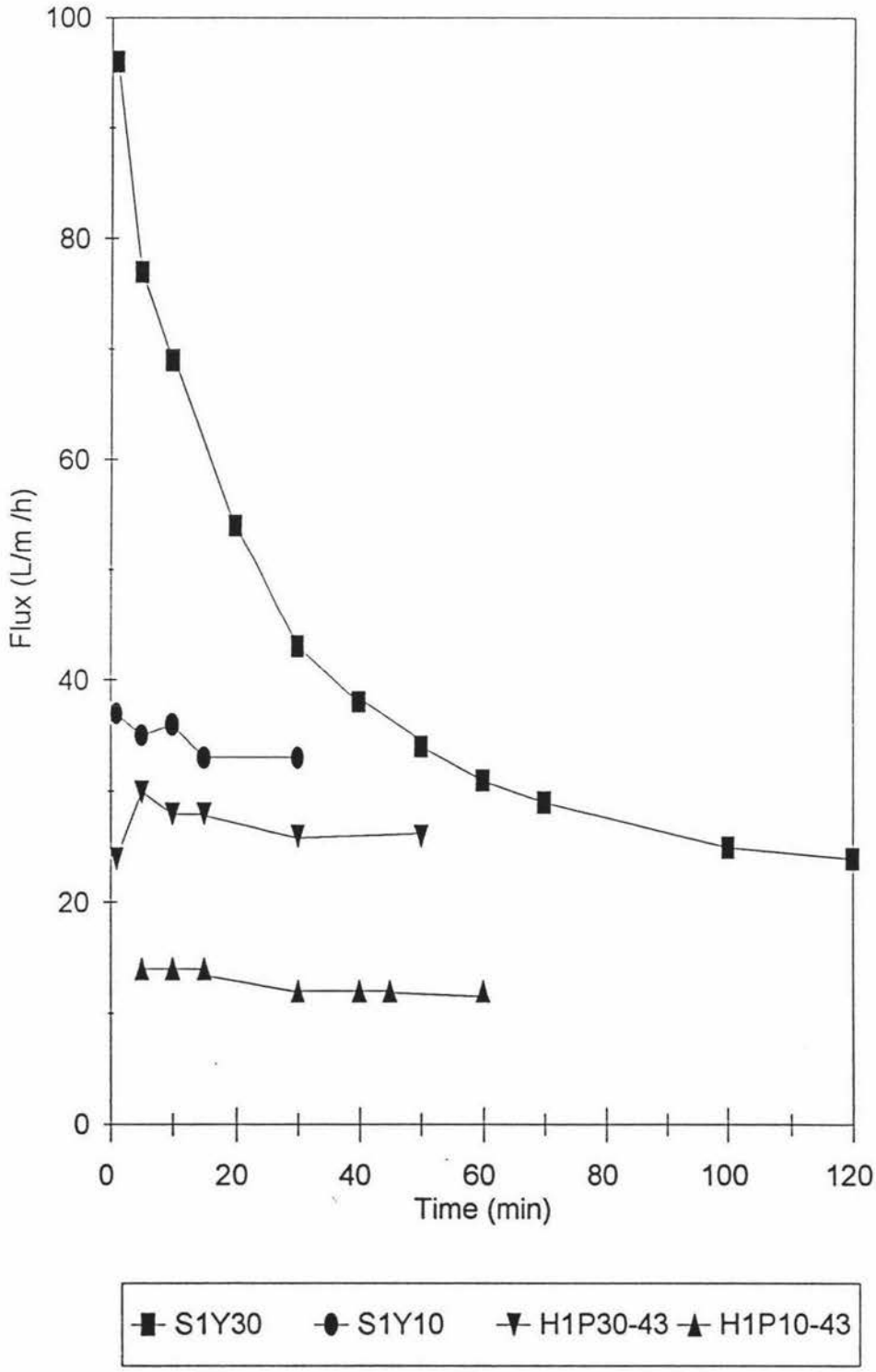


Figure 4.2 Ultrafiltration membrane fluxes.

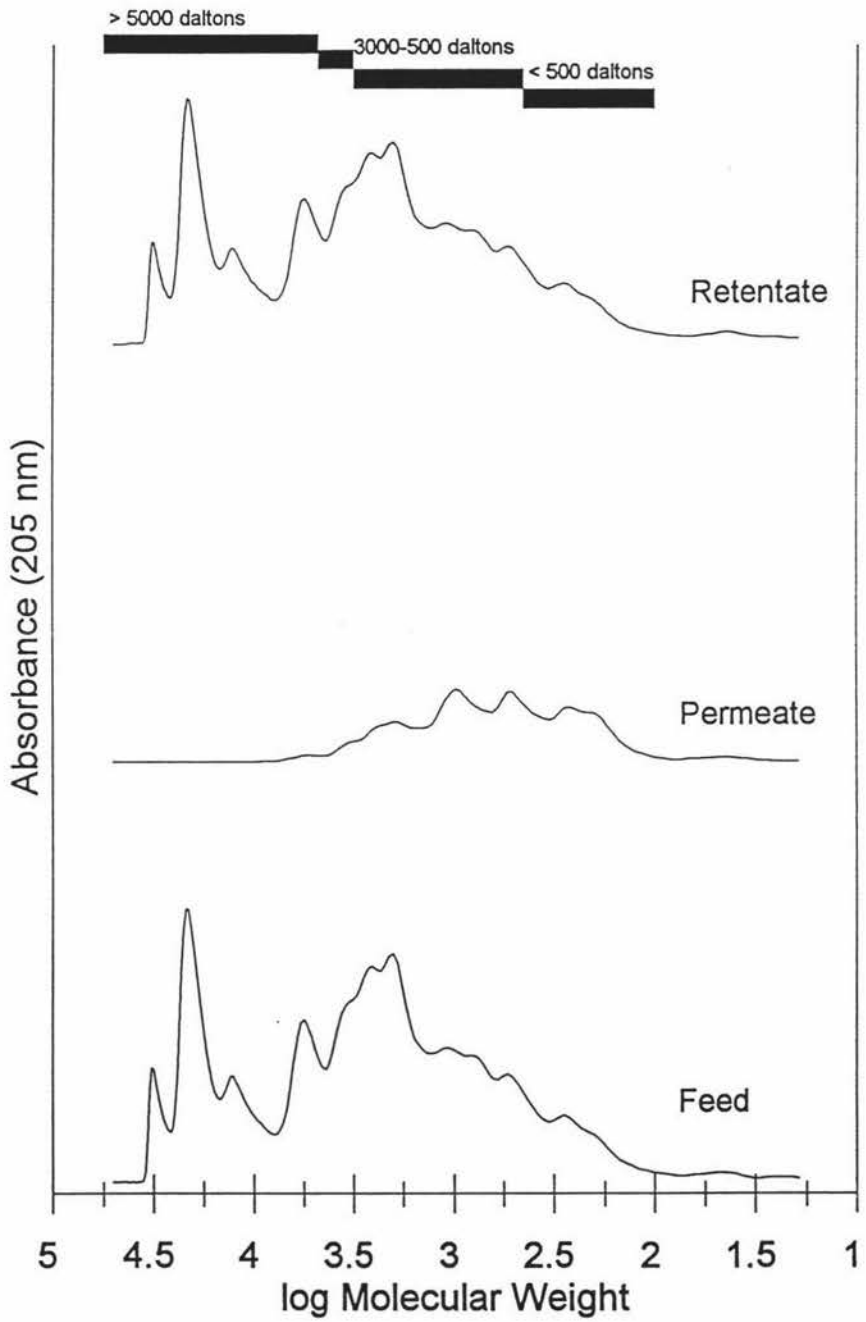


Figure 4.3 Feed, retentate and permeate MWPs for the 10,000 daltons MWCO hollow fibre membrane.

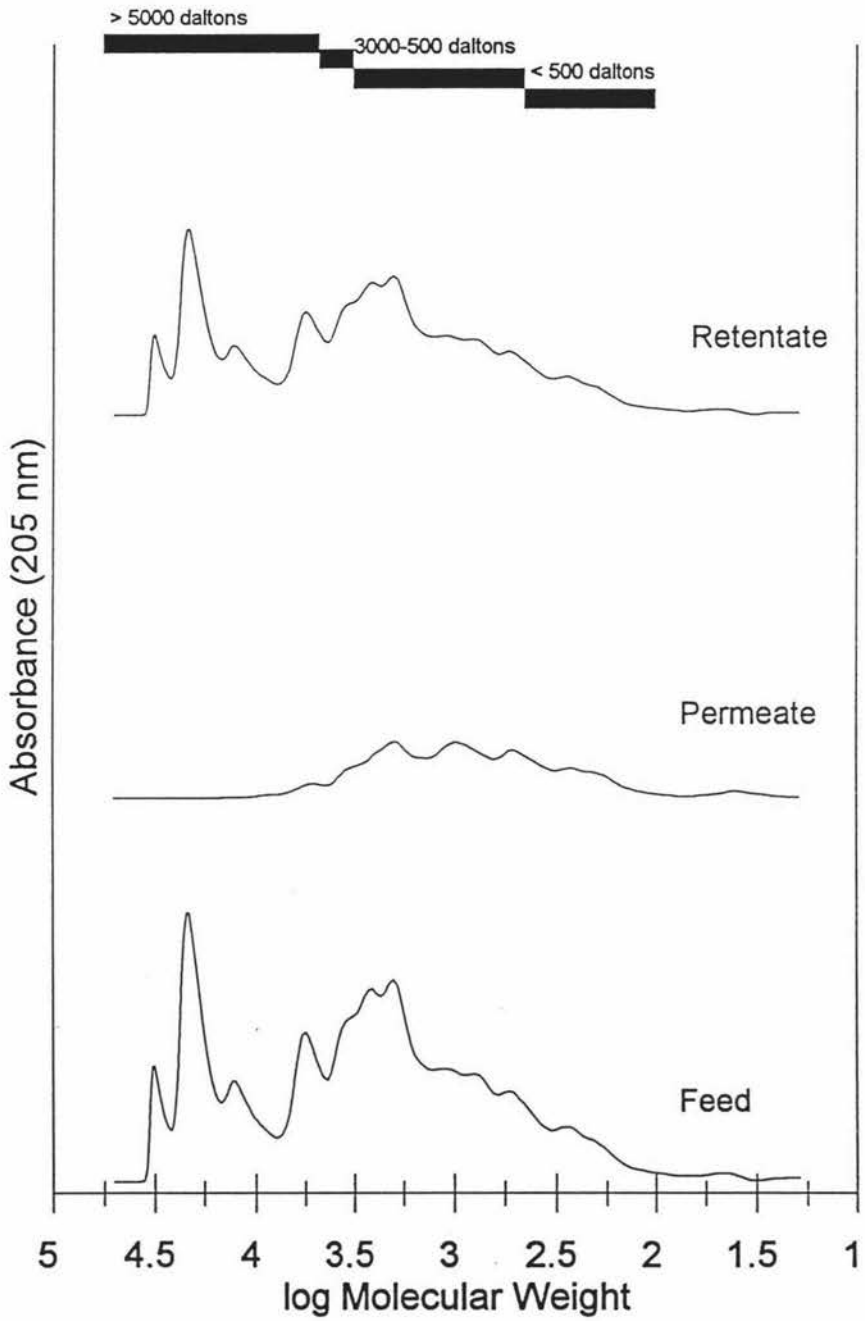


Figure 4.4 Feed, retentate and permeate MWPs for the 30,000 daltons MWCO hollow fibre membrane.

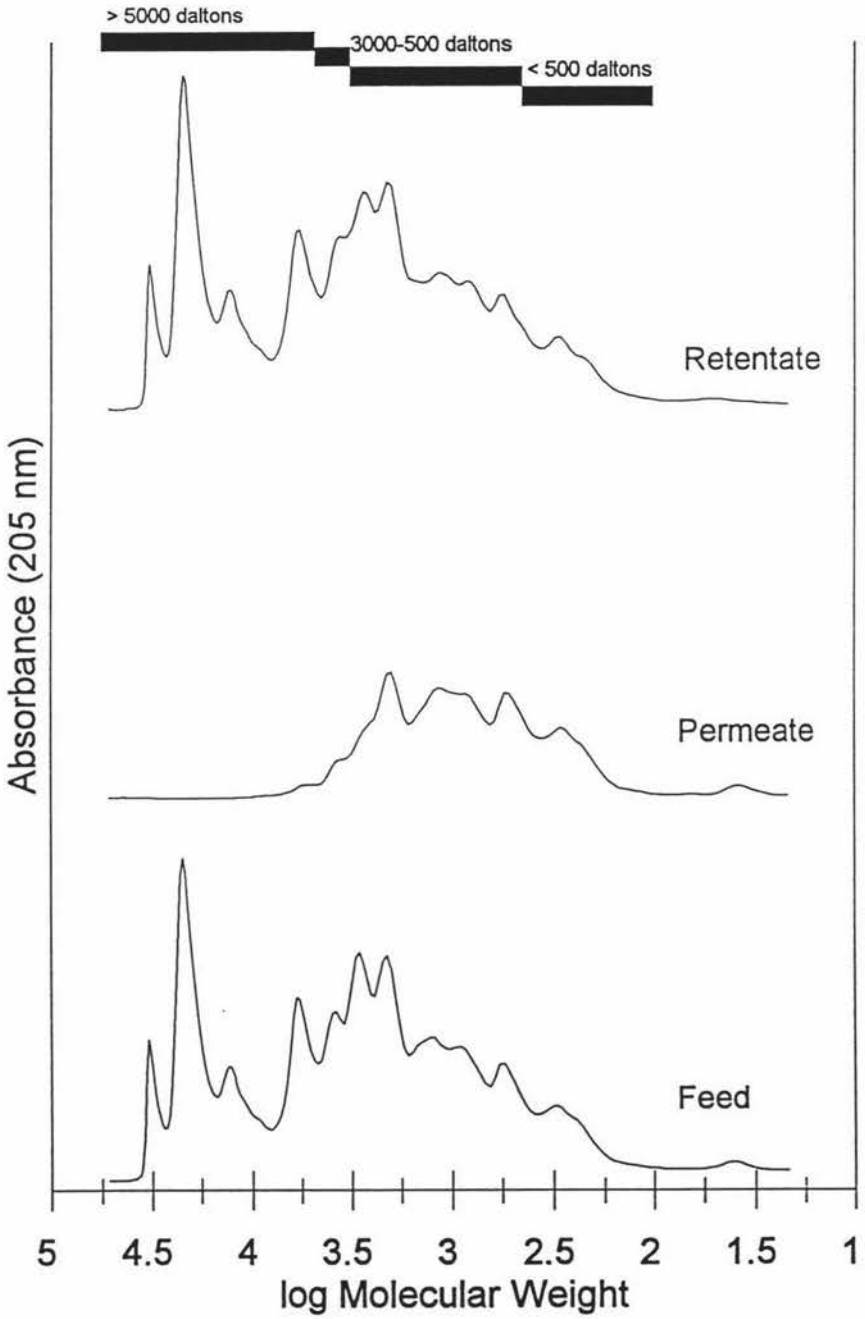


Figure 4.5 Feed, retentate and permeate MWPs for the 10,000 daltons MWCO spiral wound membrane.

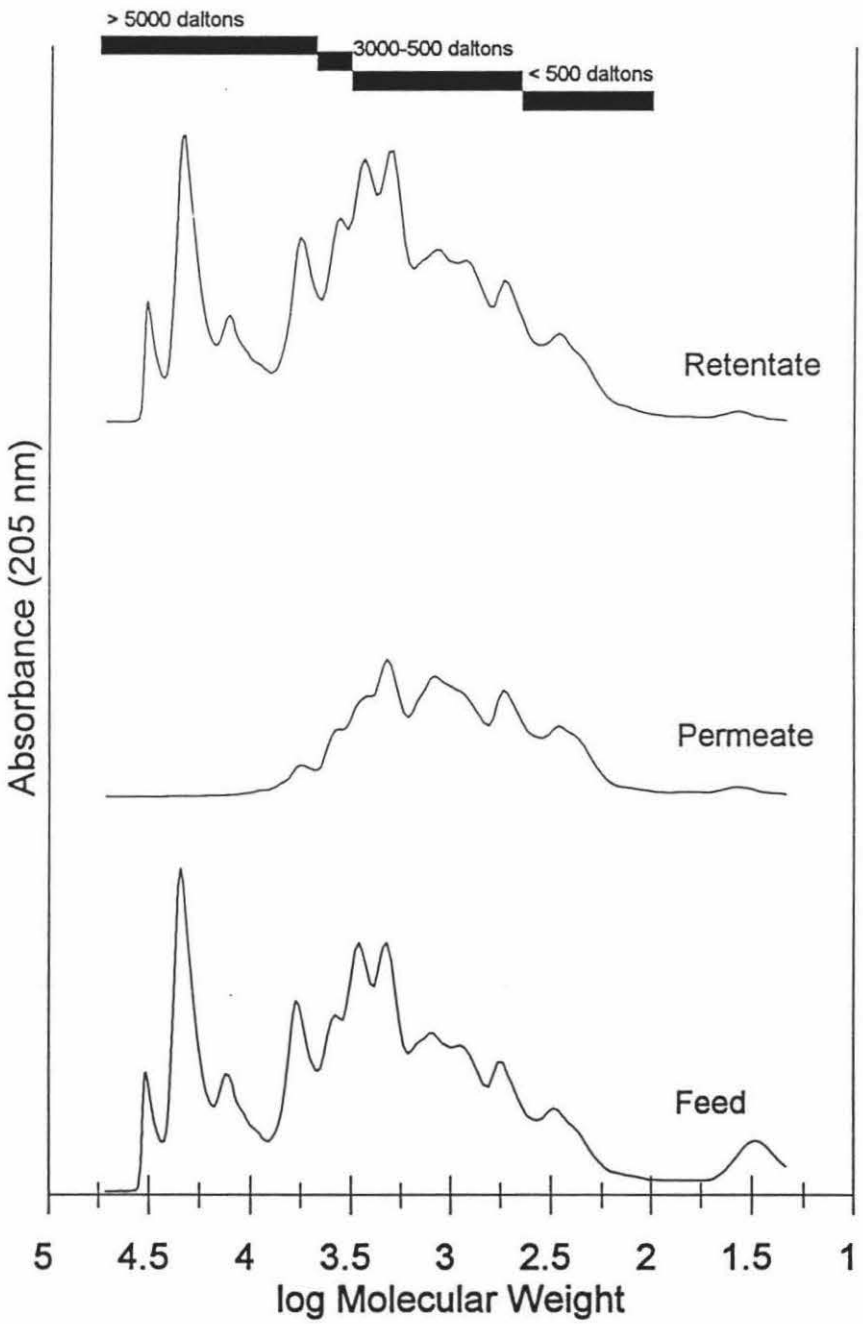
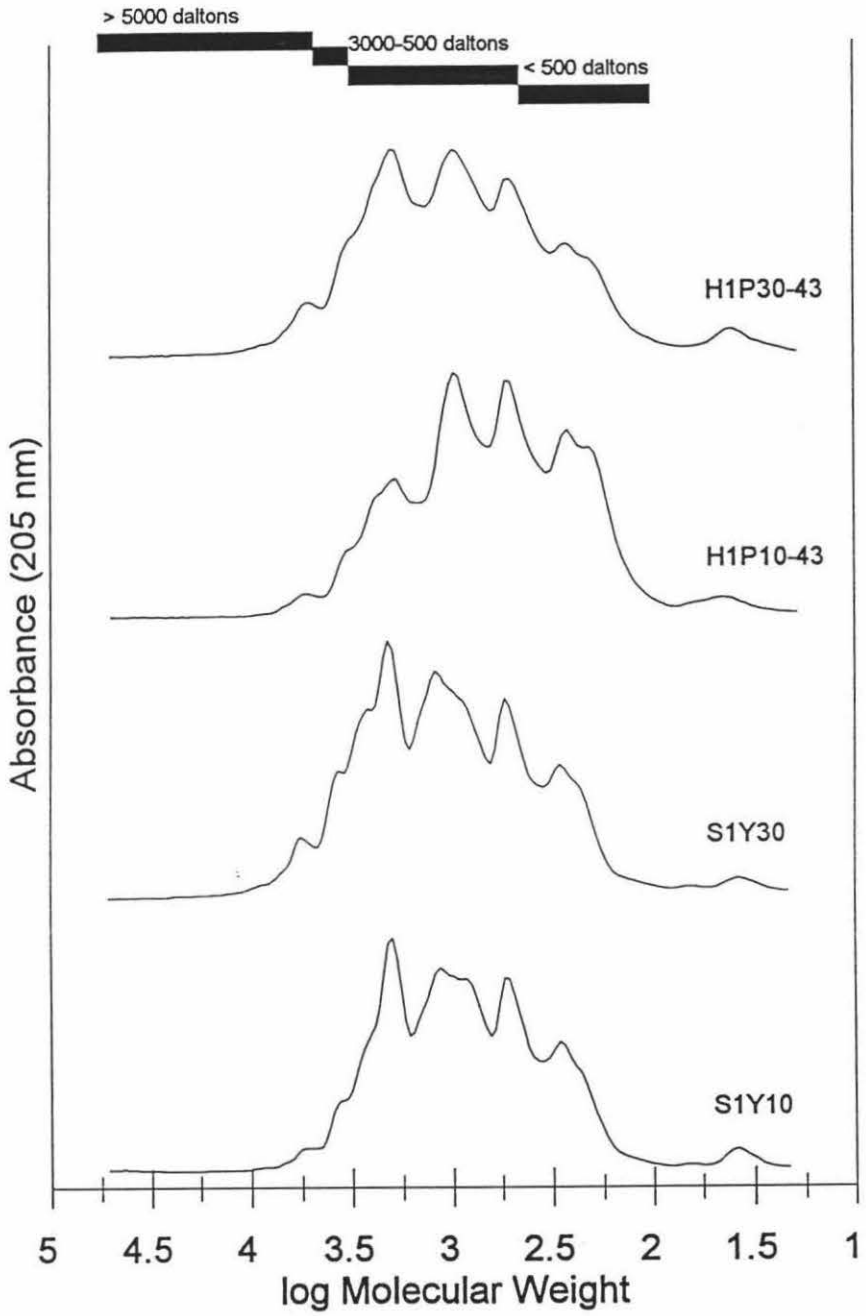


Figure 4.6 Feed, retentate and permeate MWPs for the 30,000 daltons MWCO spiral wound membrane.



KEY

S1Y10	Spiral wound membrane, 10,000 daltons MWCO
S1Y30	Spiral wound membrane, 30,000 daltons MWCO
H1P10-43	Hollow fibre membrane, 10,000 daltons MWCO
H1P30-43	Hollow fibre membrane, 30,000 daltons MWCO

Figure 4.7 Permeate MWPs for all membranes (scaled).

4.4.1 Hollow fibre membranes

The fluxes for the hollow fibre membranes were much lower than those of the spiral membranes and this was especially so for the H1P10-43 membrane (10,000 daltons nominal MWCO membrane). Examination of the MWPs shown in Figure 4.7 shows that the apparent MWCO of both the H1P30-43 membrane (30,000 dalton nominal MWCO and the H1P10-43 membrane was approximately 8,000 daltons. A larger proportion of material in the molecular weight range 1,500-5,000 daltons permeated the H1P30-43 membrane. The membrane feed concentration was approximately 5% total solids, however, the concentration of the permeate from both membranes was only 1.2% total solids. The inlet pressure of the hollow fibre membrane system fluctuated rapidly (over a range of 35 kPa) during both runs.

4.4.2 Spiral wound membranes

The spiral wound membranes had the highest fluxes, with the S1Y30 membrane (30,000 daltons nominal MWCO) having the highest flux of all the membranes tested after thirty minutes of operation. The flux of this membrane declined over time however (Figure 4.6), until after 120 minutes of operation it had the same flux as the S1Y10 membrane (10,000 daltons nominal MWCO). The apparent MWCO for both of the spiral wound membranes was the same as that obtained from both of the hollow fibre membranes, approximately 8,000 daltons molecular weight. As for the hollow fibre membranes, the MWPs of the permeates (Figure 4.7) show that the permeate from the 30,000 daltons nominal MWCO membrane contains a higher proportion of peptides in the molecular weight range 1,500-5000 daltons than does the 10,000 daltons nominal MWCO membrane. Both the spiral wound membranes allow a larger proportion of material in this molecular weight range to permeate the membrane than do the hollow fibre membranes. The concentration of the permeate from both spiral wound membranes was approximately 2% total solids.

4.5 Discussion

Two factors must usually be considered when choosing a membrane for the ultrafiltration of protein solutions, particularly protein hydrolysates: the level of particulate matter present in the feed stream and the affinity of the membrane material for the feed material. Hollow fibre membranes with wide channels (1.1 mm) are recommended for feedstocks containing particulate material, agglomerates or precipitates ranging up to 30% solids content (Amicon, 1994). This is an important consideration because an insoluble precipitate is often formed during the early stages of the hydrolysis of casein. However, the pressure fluctuations observed in the present study show that clogging of the hollow fibre membrane channels occurred. The pressure fluctuations were caused by particulate matter in the hydrolysate blocking the channels of the hollow fibre membrane and later being dislodged. This problem did not occur when the spiral wound membranes were used. Although the spiral membranes have smaller channels (0.8 mm) than the hollow fibre membranes, the fluid flow through the membrane is more turbulent and consequently there may be less tendency towards particulate fouling. Amicon's suggestion that hollow fibre membranes with wide channels are better able to handle particulate material did not prove valid for the casein hydrolysate feedstock used for the experiments described in the present work.

The affinity of feed material for the membrane is governed by the composition of the membrane. A high affinity for the substrate can lower yields and may also cause fouling of the membrane. Amicon spiral wound membranes are made of a hydrophillic cellulosic material that is less absorptive than is the hollow fibre polysulphone membrane and are, therefore, especially recommended for the ultrafiltration of proteins. In the present experiment, the fluxes obtained were higher for the spiral wound membranes than for the hollow fibre membranes. There was little evidence of membrane fouling; the fluxes remained constant over time for all of the membranes except the S1Y30 membrane. The concentration of the permeates obtained from the spiral wound membranes was approximately

double that obtained for the hollow fibre membranes, regardless of which nominal MWCO was used. These observations suggest that the polysulphone material from which the hollow fibre membranes is made may have adsorbed some of the proteins or peptides present in the feedstock. This interpretation is confirmed by the total solids measurements of the retentates. The total solids results for the permeates from the hollow fibre membranes are lower than expected and, hence, some of the peptide material cannot be accounted for. A similar observation was made by Mannheim & Cheryan (1990). These authors conducted experiments designed to determine the fate of the enzyme (Alcalase 2.4L) in their membrane system, which consisted of polysulphone hollow fibre membranes. They operated the CSTMR system using a solution of enzyme only and found that they could not detect as much enzyme activity as they expected. They described this effect of the membrane on the enzyme as "membrane poisoning". The results obtained from the membrane screening experiments carried out in the present study suggest that the enzyme may have been adsorbed by the polysulphone membrane. It is also possible that the enzyme was denatured by contact with the hydrophobic membrane surface.

The MWPs of the feed, retentate and permeate for each membrane showed that the actual MWCO for all membranes, regardless of nominal MWCO, was about 8000 daltons. A small amount of the material present in the permeates, 2-5% (estimated from the MWPs), was greater than 5000 daltons molecular weight. However, the amount of material present was very small and specialised allergenicity testing, which was not carried out as part of the present study, would be required to determine the effect of this on the allergenicity of the hydrolysate. The spiral wound membranes, especially the S1Y30, allowed more of the material in the range 1,500-5,000 daltons molecular weight to permeate than did the hollow fibre membranes. This was desirable in terms of the target MWP.

4.6 Conclusions

The spiral wound membrane with a nominal MWCO of 30,000 daltons (S1Y30) was chosen for the bioreactor experiments because it had a high flux, gave the highest recovery of peptides, was not susceptible to particulate fouling and, most importantly, gave the most desirable MWP relative to the target product.

CHAPTER 5
CONTINUOUS BIOREACTOR EXPERIMENTS

5.1 Introduction

Much has been published on the use of membrane bioreactors or CSTMRs to produce protein hydrolysates. Published work has mostly focused on optimizing the CSTMR to give maximum possible conversion of intact protein to trichloroacetic-acid-soluble peptides. Few authors have examined the effect of CSTMR HRT on the molecular weight distribution of the final product. There have been few attempts to link the fundamentals of protease specificity with the engineering aspects of the process to enhance product properties. For example, it should be possible to utilize a CSTMR process to produce hydrolysates with defined molecular weight distributions by judicious enzyme and HRT selection.

The target of the present study was to use the CSTMR system to produce a hydrolysate that was low in free amino acids and had a maximum molecular weight of 5000 daltons. Two enzymes were selected, on the basis of time series MWPs, to achieve this aim (Chapter 3). Alcalase 2.4L produces casein hydrolysates that are low in free amino acids. Amano Protease A produces casein hydrolysates with MWPs similar to Alcalase but forms a significant proportion of free amino acids late in the reaction. However, if the bioreactor HRT is short enough, then the peptides produced by Amano Protease A should pass through the ultrafiltration membrane before they can be hydrolysed further to free amino acids. Both enzymes have a high activity for casein, a necessary requirement to prevent the build-up of unhydrolysed protein, and subsequent fouling of the membrane, in the CSTMR.

The type of ultrafiltration membrane used for CSTMR processes has been shown to be important to the long-term stability of the system (Mannheim & Cheryan, 1990). In the present study four ultrafiltration membranes were evaluated for their potential effectiveness in the CSTMR system (Chapter 4). The use of spiral wound ultrafiltration membranes

resulted in higher permeate flowrates and fluxes than did the hollow fibre membranes. There was evidence that the hydrophillic cellulosic material from which the spiral wound membranes are made was less absorptive to proteins and peptides (and possibly enzymes) than the polysulphone material from which the hollow fibre membranes are made. The S1Y30 membrane (spiral wound, 30,000 daltons nominal MWCO) gave the most desirable permeate MWP relative to the target product.

Four CSTMR experiments were conducted: two experiments per enzyme with two different HRTs each. In addition two experiments were carried out, one for each enzyme, using batchwise hydrolysis followed by ultrafiltration, to assess the products and the efficiency of their production compared to CSTMR-type hydrolyses.

5.2 Materials and Equipment

5.2.1 Enzymes

The following enzymes were used for continuous bioreactor and batch experiments:

Novo Alcalase 2.4L, Batch PMN 5028;

Amano Protease A, Batch PRR11502A.

5.2.2 Substrate

The substrate used was Alacid Acid Casein as described previously in Section 3.2.1.2.

5.2.3 Chemicals

"Analar" grade sodium hydroxide, acetonitrile and trifluoroacetic acid as described in Section 3.2.1.3 were used.

Demineralised water was used to prepare all solutions and reagents. MilliQ water was used to prepare HPLC reagents.

5.2.4 Equipment

The following were employed:

Spiral wound ultrafiltration cartridge S1Y30 (nominal MWCO 30,000 daltons) and fittings (Amicon) and Ismatech MV pump (as described in Section 4.2.4);

Two Masterflex peristaltic pumps with Masterflex speed controllers (Cole-Parmer Instrument Company, Chicago, Illinois, USA).

The pH was maintained during hydrolysis using an autotitrator system as described previously in Section 3.2.2.2.

Water baths were set at 50°C and 95°C, as required.

5.3 Methods

5.3.1 CSTMR experiments

The set-up for the CSTMR experiments is shown in Figure 5.1. Experiments were conducted by filling the reaction vessel with the appropriate volume of caseinate solution, allowing this to equilibrate to 50°C, and adding sufficient enzyme to give an enzyme to substrate ratio of 1:100 (v/w) for Alcalase 2.4L and 1:50 (w/w) for Amano Protease A. The pump to the ultrafiltration unit and the pump from the feed reservoir to the reaction vessel were started as soon as the enzyme was added. The flowrate of the feed pump was continually adjusted to match the permeate flowrate. The permeate was collected in a beaker placed in a 95°C water bath to inactivate any enzyme that may have leaked into the permeate. The pH of the reaction vessel contents was maintained using the autotitrator system and 4N NaOH. The hydrolysis conditions for all of the CSTMR experiments were the same: temperature, 50°C; pH 8 and substrate concentration, 5% w/w.

Four experiments were carried out: two using Alcalase 2.4L and two using Amano Protease A. The HRT of the experiments was controlled by adjusting the reaction volume used. A "short" and a "long" HRT were used for each enzyme. An attempt was made to calculate the reaction vessel volume required from the permeate flowrate data obtained in Chapter 4. In

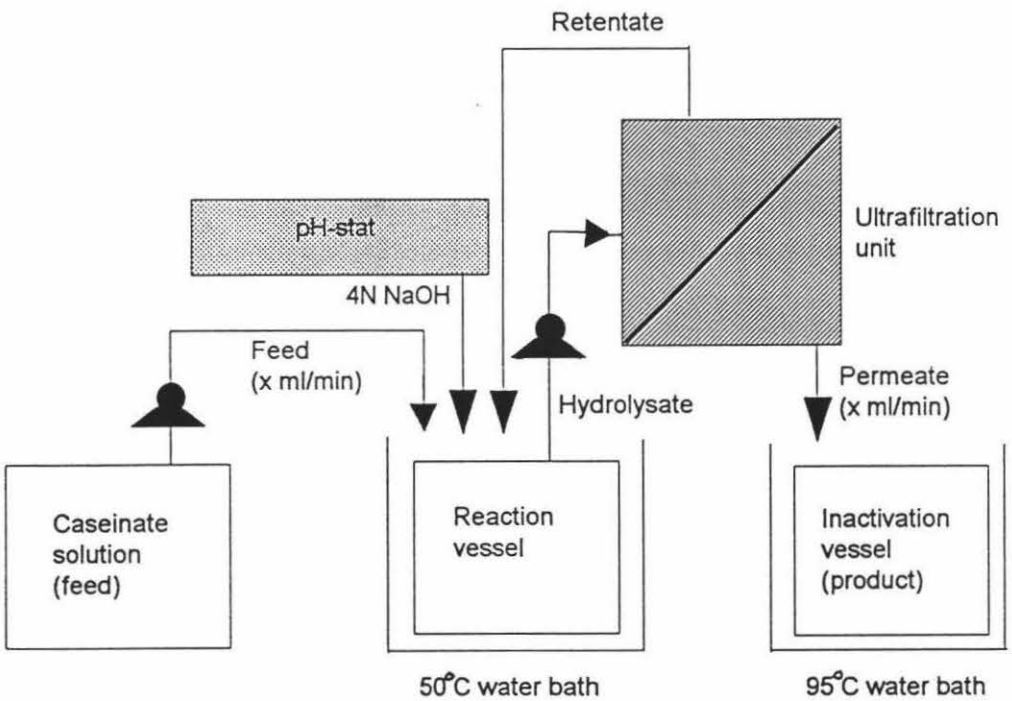


Figure 5.1 Sketch of the bioreactor set-up.

practice it was found that the permeate flowrates obtained in Chapter 4 were not accurate predictors for the CSTMR system, presumably because the peptide mixture produced from the CSTMR experiments would have a different peptide composition to the test hydrolysate. Hence, the HRTs used were dependent upon the permeate flowrate achieved in practice.

Samples of the permeate were collected at 15, 30, 45 and 60 min and then at half hourly or hourly intervals for residual active protease, total solids, total nitrogen and MWP analysis (using the sampling method described in Section 3.2.2.3). The samples for total solids and total nitrogen analysis were inactivated by placing them in a 95°C waterbath for at least 15 minutes. Samples for MWP and residual active protease analysis were placed directly into MWP eluent or onto the agar plates, as appropriate. After 6-10 h of continuous hydrolysis (when the membrane started to foul) or after 10h, whichever came first, samples of the retentate, the permeate, the reaction vessel contents and the bulk permeate were also taken for residual active enzyme, total solids, total nitrogen and MWP analysis. The permeate flowrate was measured at the time intervals described above using the method described in Section 4.3.3.

The membrane was cleaned and stored as described previously in Section 4.3.2.

5.3.2 Batch experiments

Hydrolysates were prepared in a batchwise fashion, as described in Section 3.2.2.2. Casein was hydrolysed with Alcalase 2.4L for 150 min in one experiment and with Amano Protease A for 120 min in another experiment. The hydrolysis time used approximated the longest HRT achieved for each enzyme in the CSTMR experiments. Both hydrolyses were carried out using a substrate concentration of 5%, temperature of 50°C, pH 7 and an E:S of 1:100 for Alcalase 2.4L and 1:50 for Amano Protease A. The higher concentration of Amano Protease A was required because the batch of Amano Protease A used for these experiments had half the activity of the batch used in the enzyme screening experiments

(manufacturer's information). The hydrolysates were then ultrafiltered as described in Section 4.3.3. using the S1Y30 spiral wound membrane. The ultrafiltration process was continued until the retentate had been reduced to about half of its original volume. Samples of the resulting permeate and retentate were taken and analyzed for MWP, total nitrogen and total solids as described previously. The permeate was tested for residual protease activity.

5.3.3 Analytical methods

HPLC analysis to produce MWPs has been described previously in Section 3.2.2.4.

Total solids and total nitrogen analyses were carried out by the NZDRI Analytical Chemistry Section using the methods outlined by Crofts & Gray (1991).

The residual active protease method has been described by Armstrong (1991). A thin layer of agar containing milk protein was used as the substrate for enzyme action. The solution to be tested was placed in small wells in the agar which was then incubated (37°C, 48 h). Active enzyme was indicated by a zone of precipitation around the well. A qualitative estimate of the amount of active enzyme present was obtained from the size of the precipitation zone.

5.4 Results

5.4.1 CSTMR experiments

The permeate flowrates, hydraulic residence times, total solids, total nitrogen, residual active protease and % conversion results are given in Appendix V. The % conversion was calculated as the ratio of total nitrogen in the permeate to total nitrogen in the feed. The HRT for each experiment was calculated from the reaction volume, which included the volume of the tubing and the membrane cartridge, and the average permeate flowrate over the whole experiment, using Equation 4.1.

The CSTMR experiments are summarised in Table 5.1. Figures 5.2

to 5.9 show the MWPs of the permeate samples taken during each experiment and the MWPs of the reaction vessel and bulk permeate samples taken at the end of each experiment. The instantaneous % conversion and the permeate flowrate are plotted against time in Figures 5.10 and 5.11. The observations which follow are based on Figures 5.2 to 5.11.

Table 5.1 Summary of CSTMR experiments

Experiment	Enzyme	Reaction Volume (ml)	Average permeate flowrate (ml/min)	HRT (min)	Conversion (% total nitrogen)
CSTMR 1	Alcalase 2.4L	950	6	160	69
CSTMR 2	Alcalase 2.4L	960	10	100	39
CSTMR 3	Amano Protease A	950	9	110	84
CSTMR 4	Amano Protease A	200	4	50	69

5.4.1.1 CSTMR 1 (Alcalase 2.4L, HRT = 160 min)

The permeate MWPs for CSTMR 1 (Figure 5.2) show that the permeate MWP remained constant from 150 min to 600 min (when the experiment was stopped). This could be taken as an indication that steady state had been reached and maintained for this experiment. However, examination of Figure 5.10 shows that the productivity of the CSTMR, as measured by the % total nitrogen converted, was increasing throughout the course of the experiment. This observation indicates that steady state had not been reached. The maximum instantaneous conversion reached in this experiment was 93% total nitrogen at 600 min. Figure 5.11 shows that, after an initial small loss of permeate flowrate, there was no evidence of membrane fouling over the 10 h of CSTMR operation. The MWPs shown in Figure 5.3 reveals that nearly all of the reaction vessel contents are hydrolysed. The residual active protease results were positive for all of the instantaneous permeate samples.

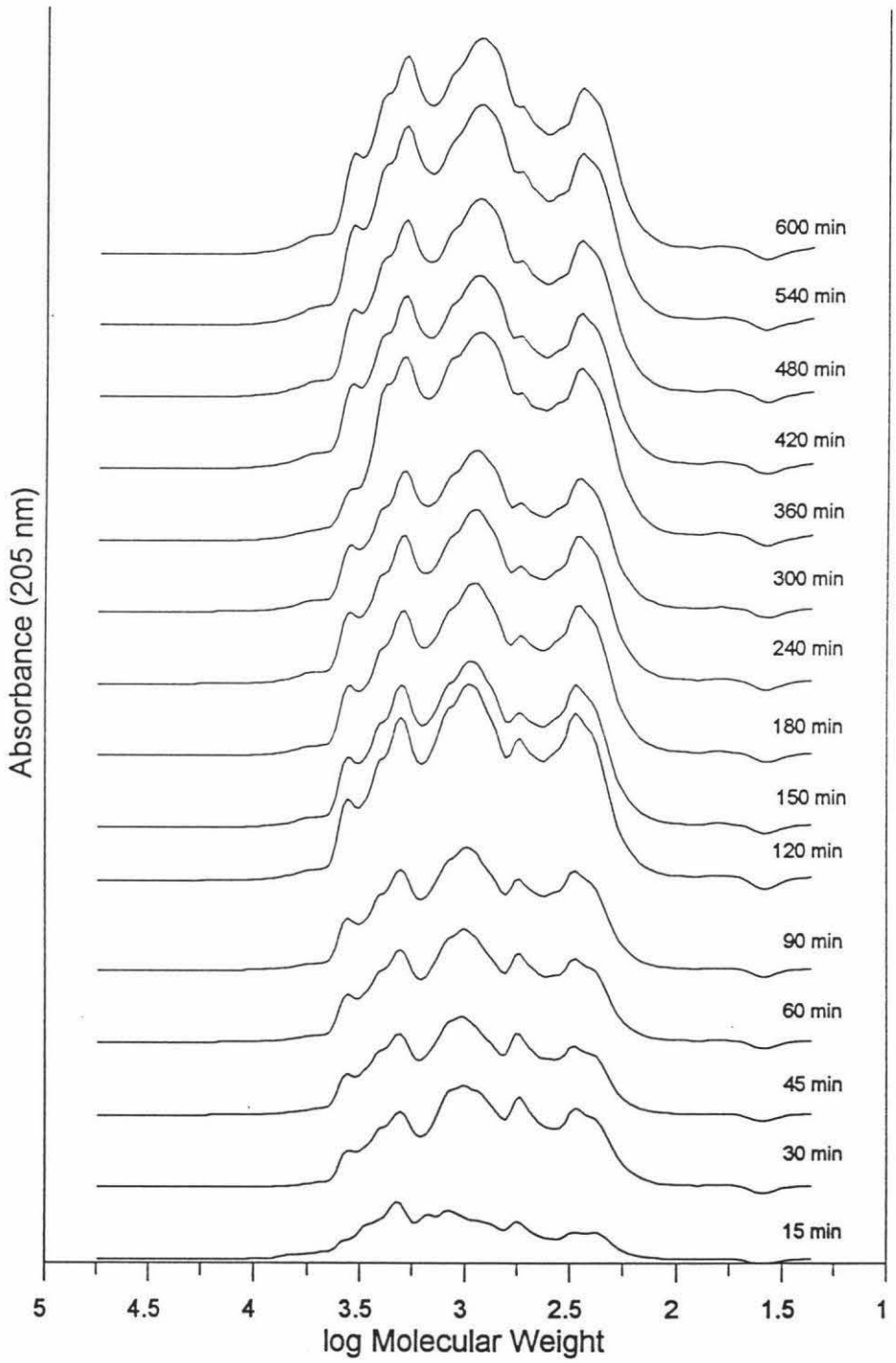


Figure 5.2 MWPs of permeate samples taken during CSTMR 1 (HRT = 160 min).

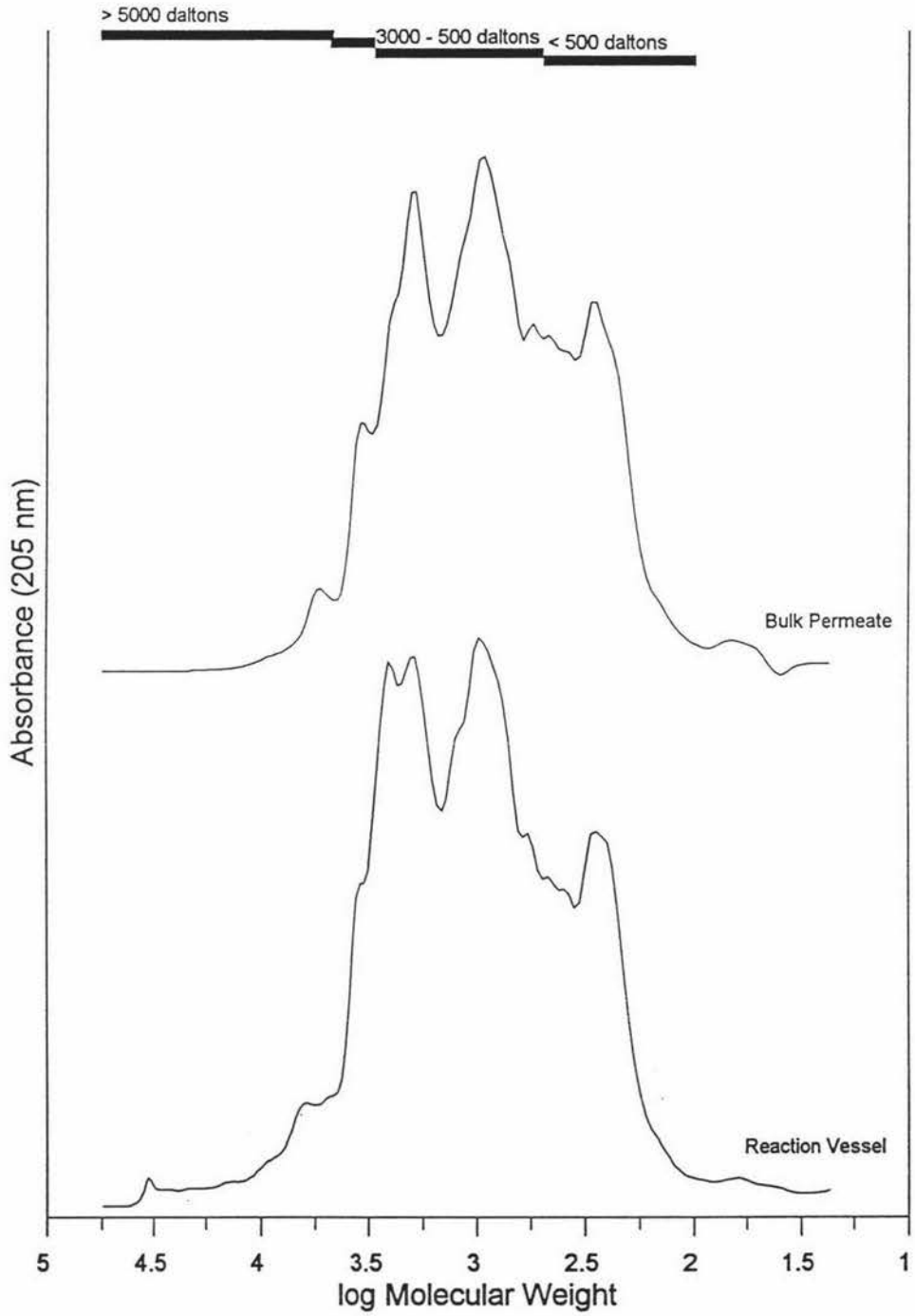


Figure 5.3 MWPs of reaction vessel and bulk permeate samples taken at the end of CSTMR 1 (HRT = 160 min).

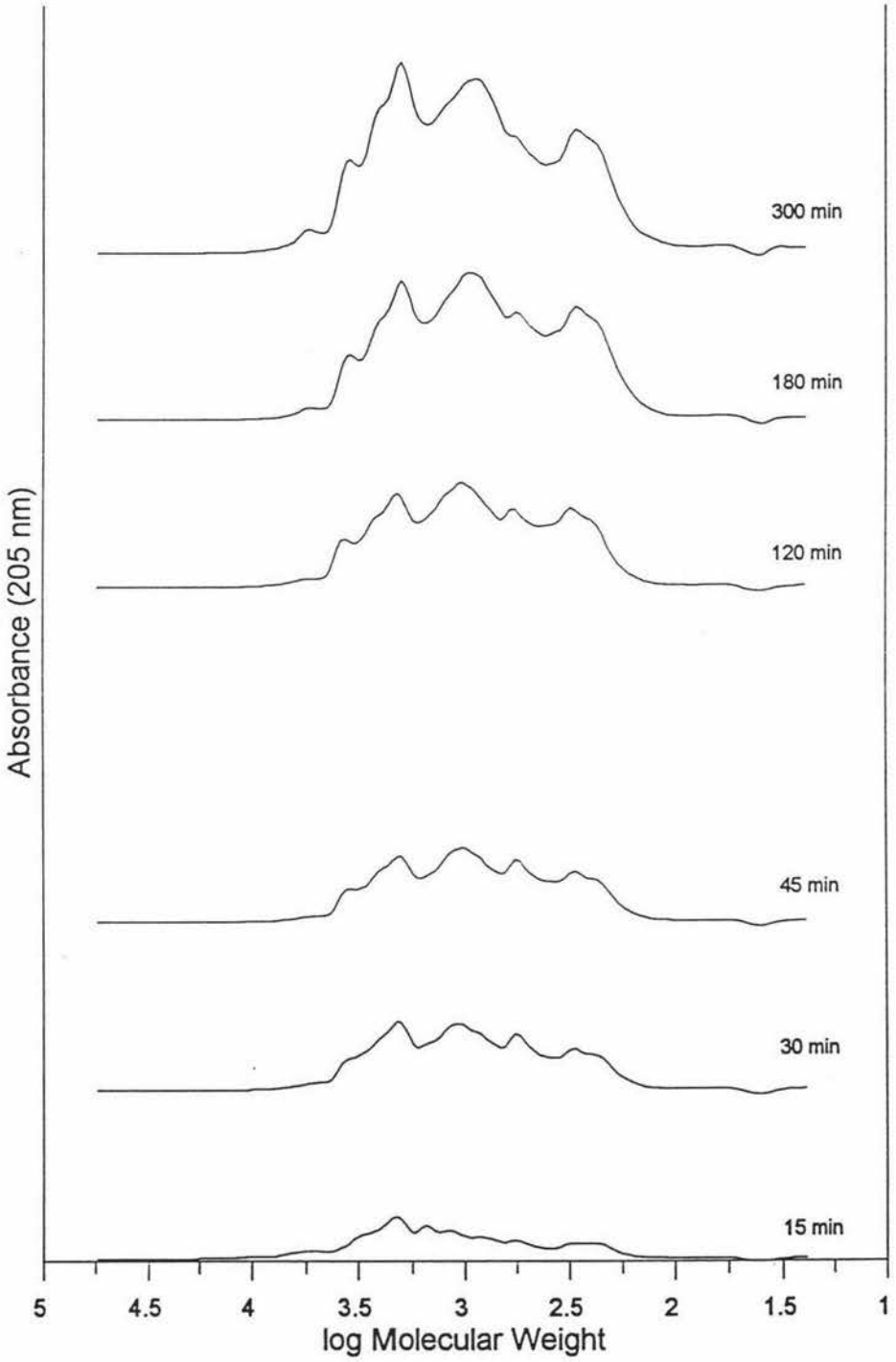


Figure 5.4 MWPs of permeate samples taken during CSTMR 2 (HRT = 100 min).

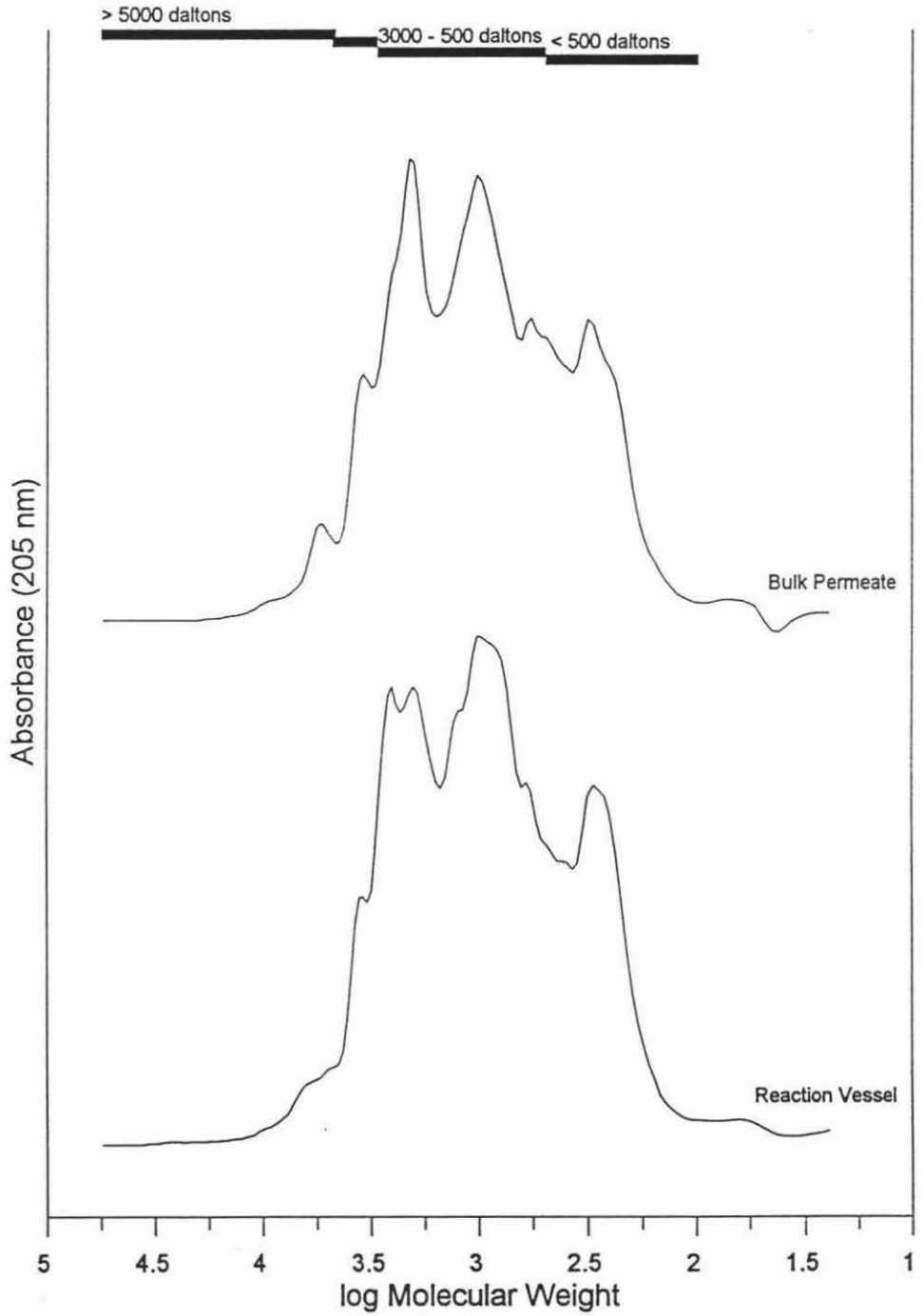


Figure 5.5 MWPs of reaction vessel and bulk permeate samples taken at the end of CSTMR 2 (HRT = 100 min).

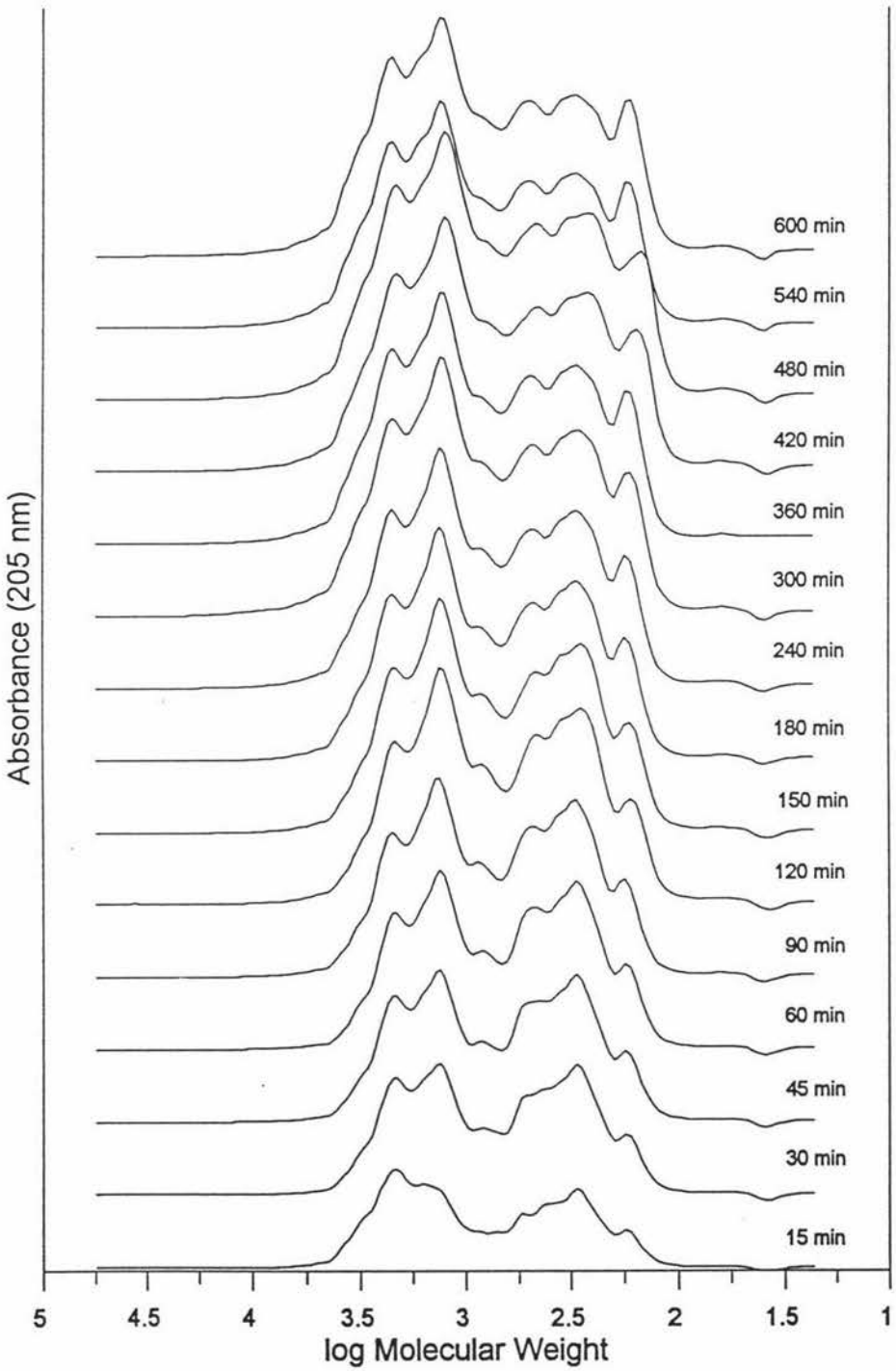


Figure 5.6 MWPs of permeate samples taken during CSTMR 3 (HRT = 110 min).

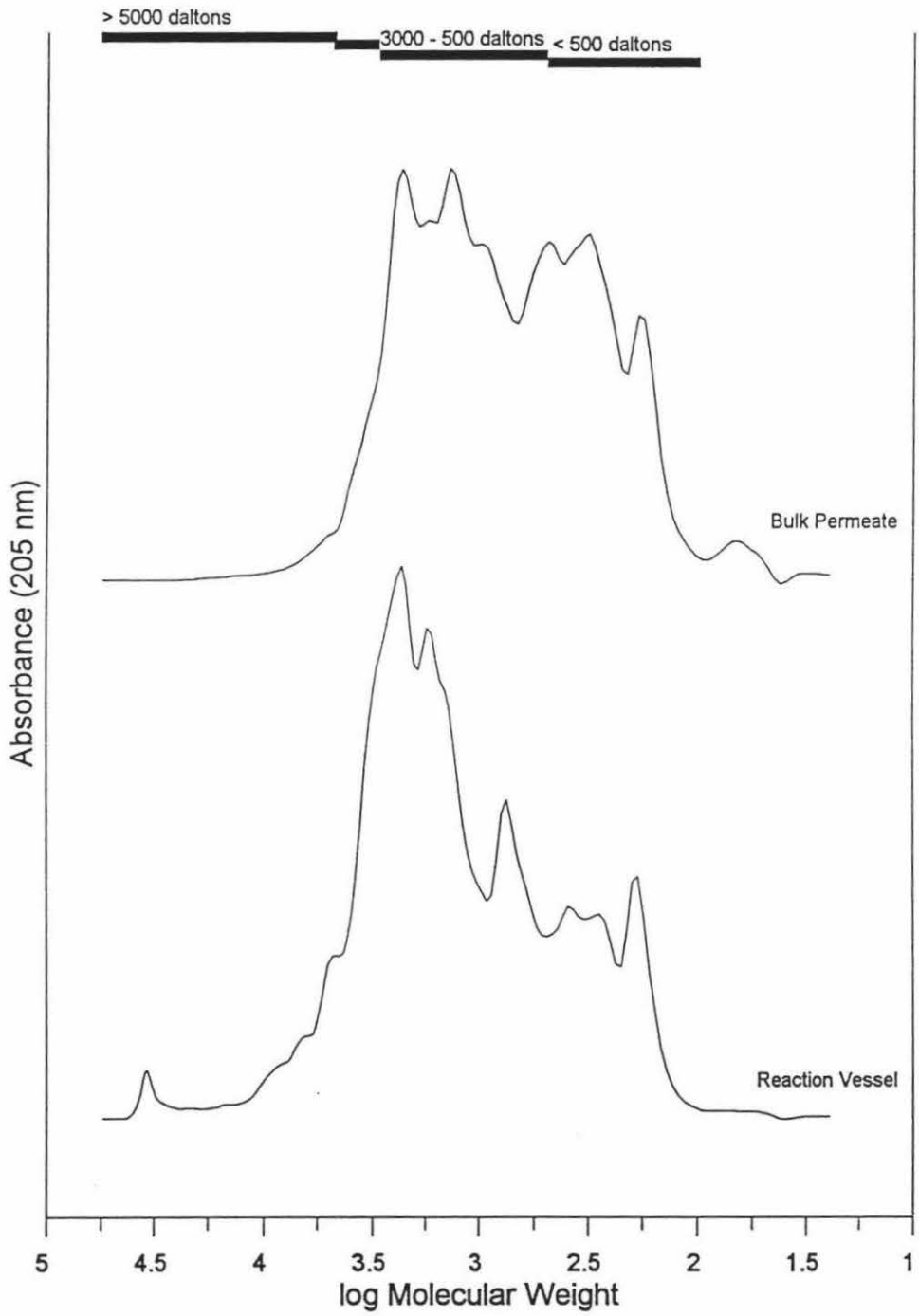


Figure 5.7 MWPs of reaction vessel and bulk permeate samples taken at the end of CSTMR 3 (HRT = 110 min).

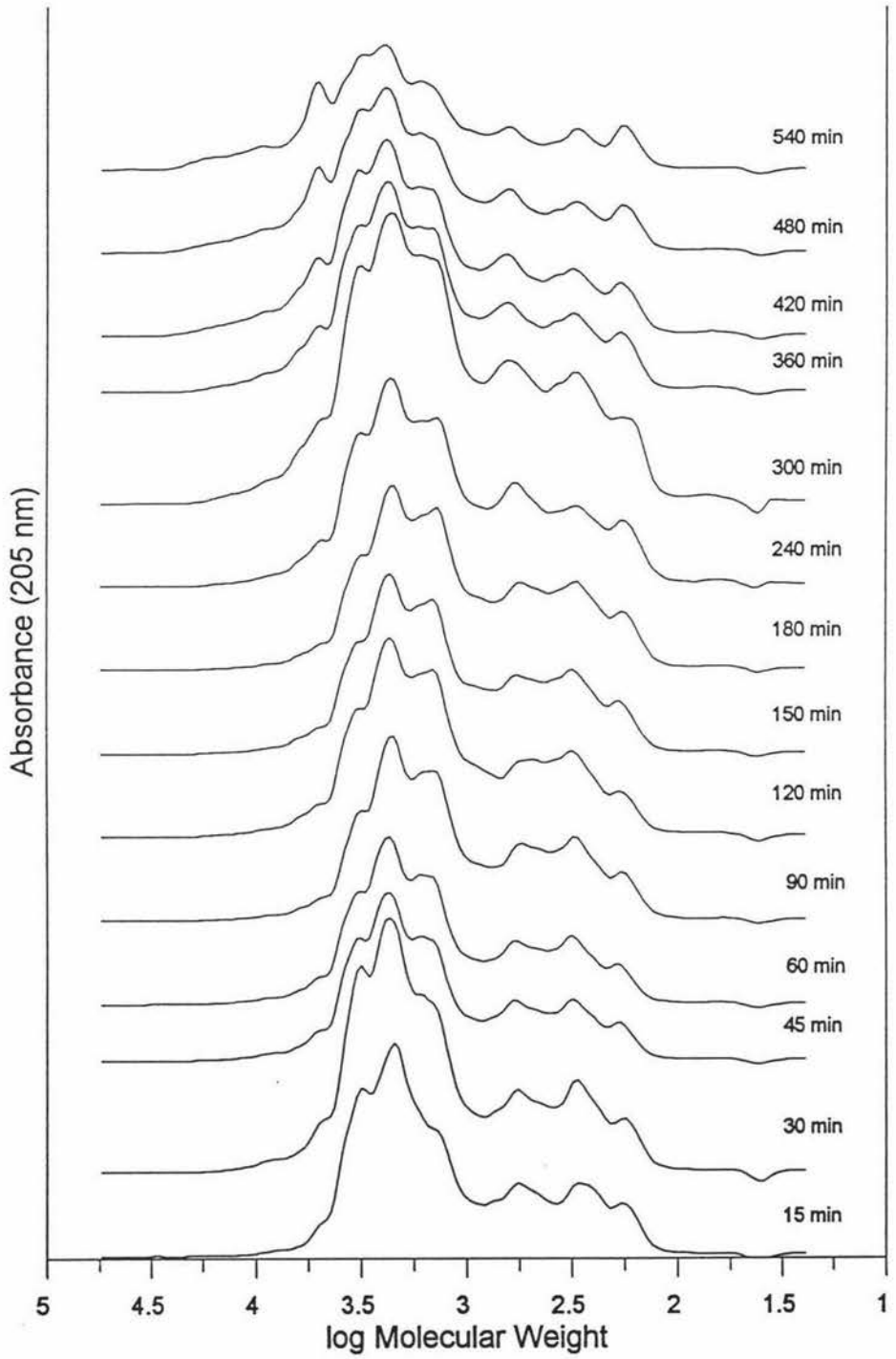


Figure 5.8 MWPs of permeate samples taken during CSTMR 4 (HRT = 50 min).

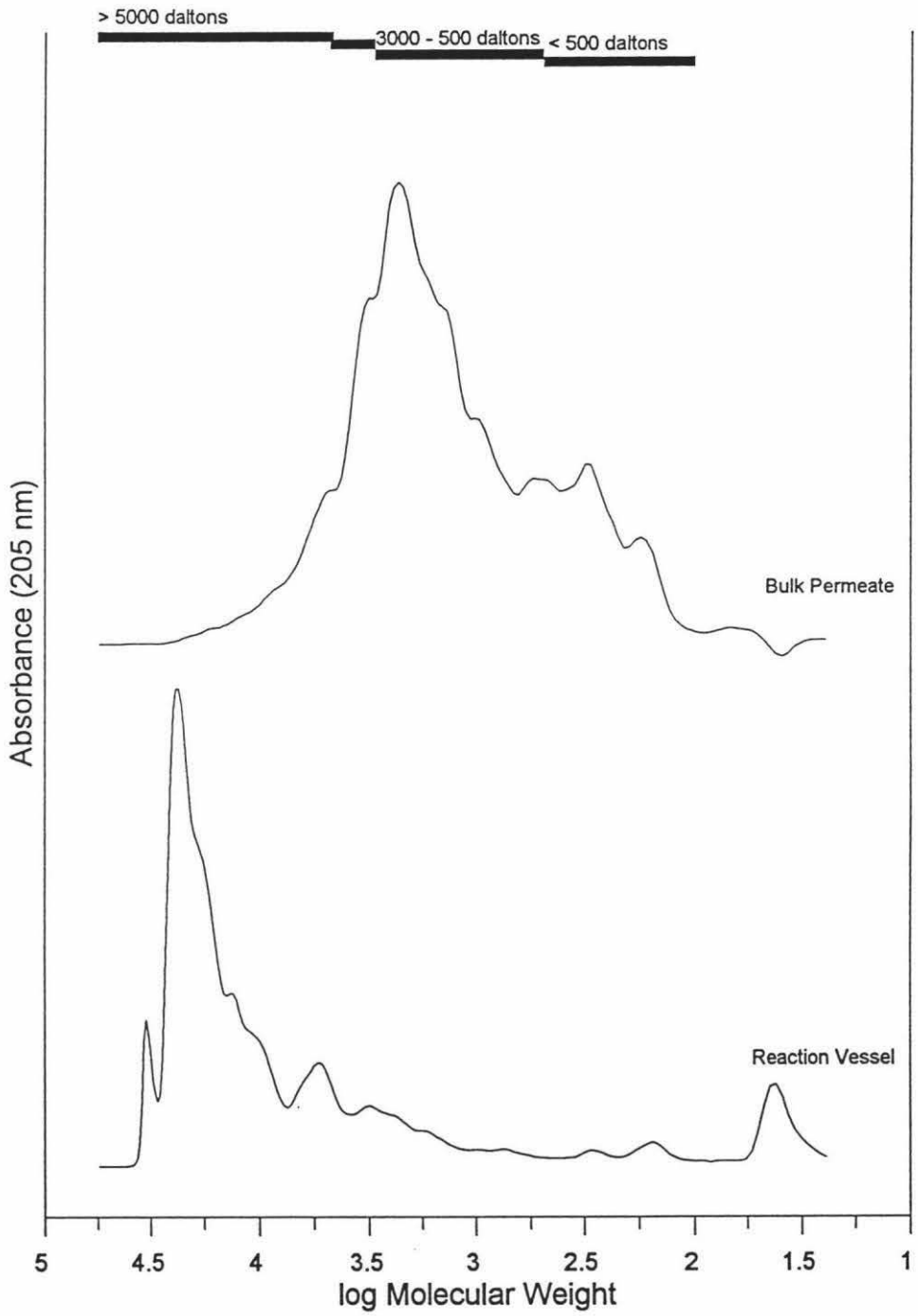


Figure 5.9 MWPs of reaction vessel and bulk permeate samples taken at the end of CSTMR 4 (HRT = 50 min).

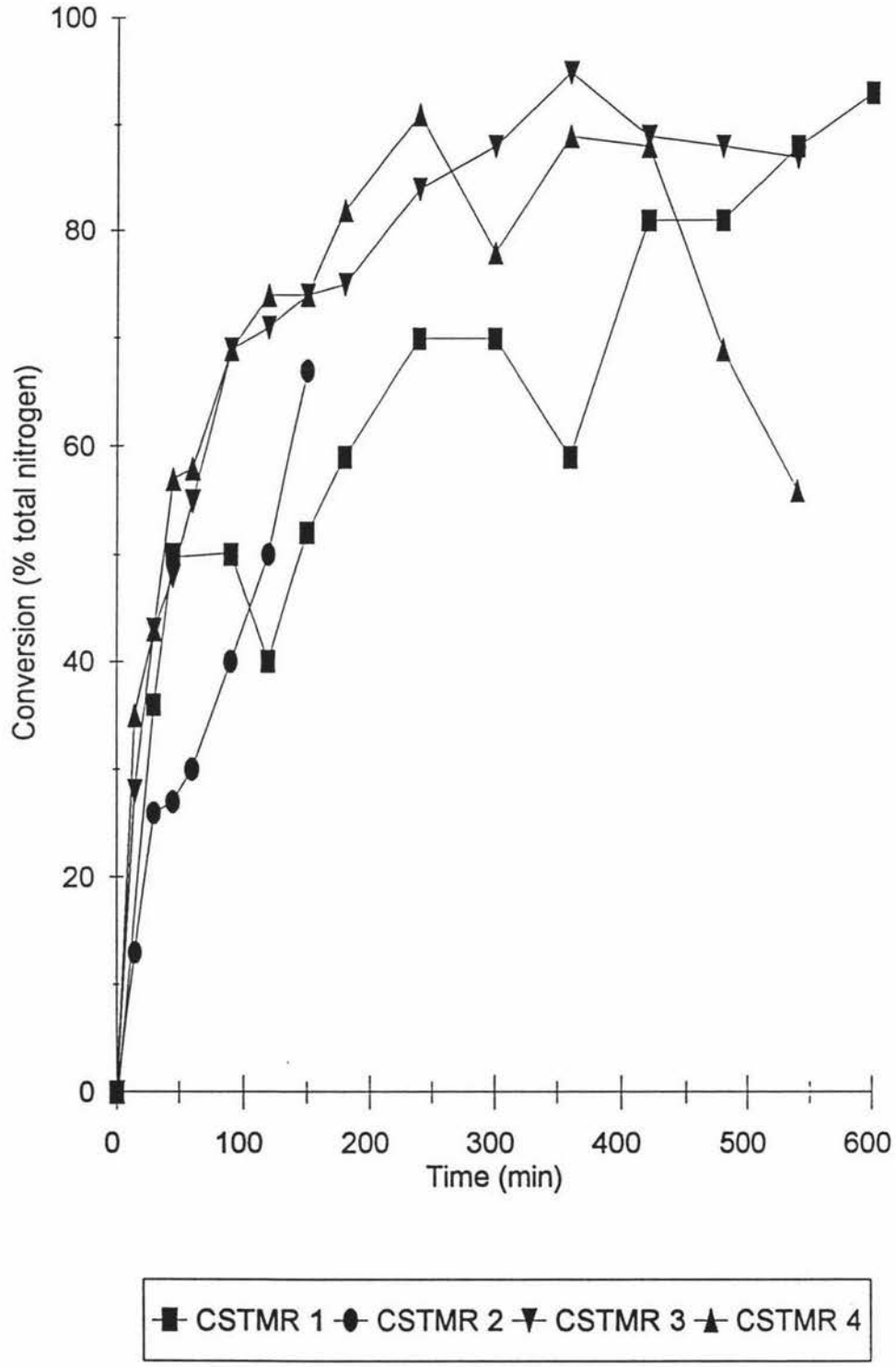


Figure 5.10 % Conversion of total nitrogen for the CSTMR experiments.

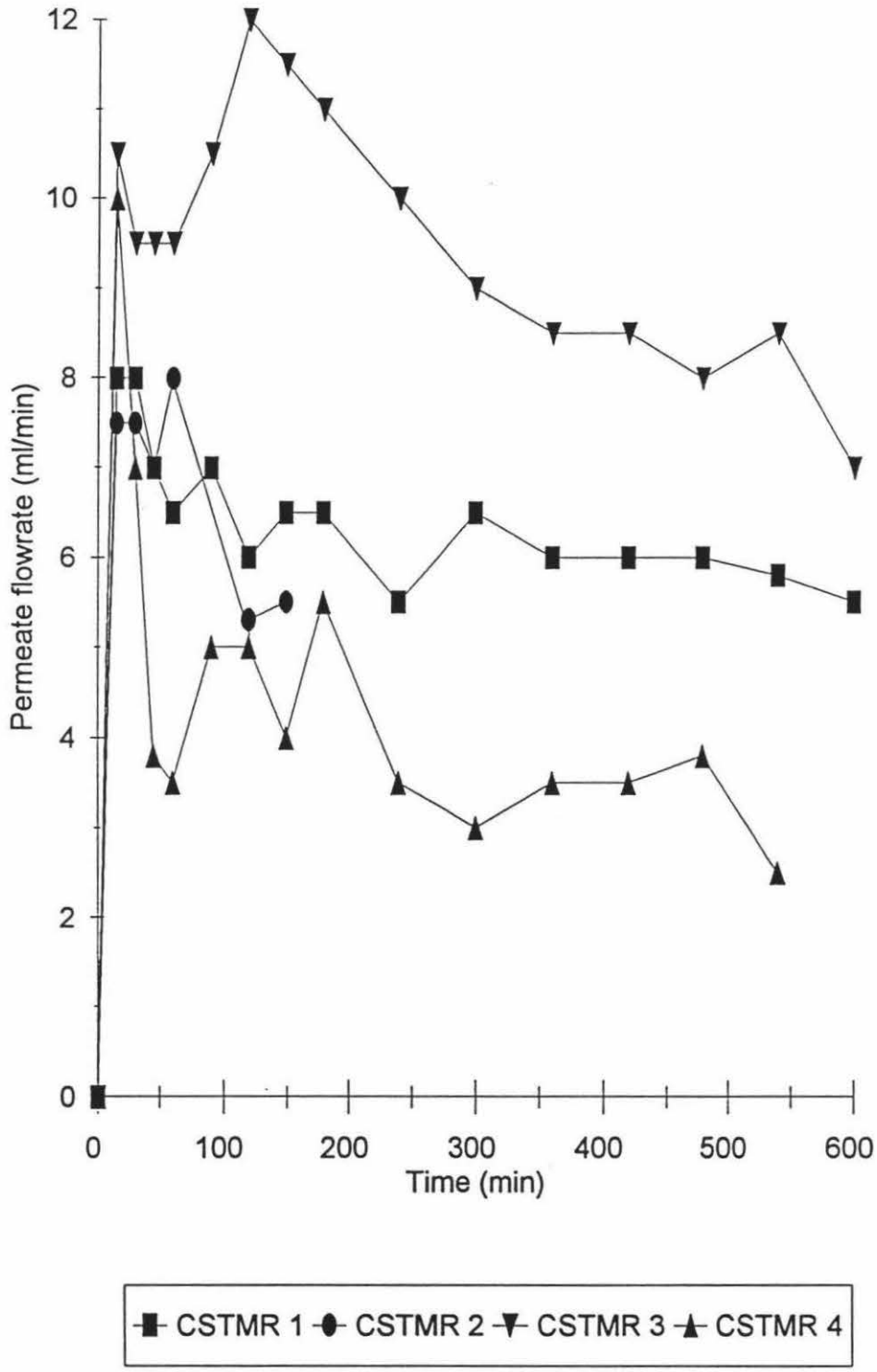


Figure 5.11 Permeate flowrates for the CSTMR experiments.

5.4.1.2 CSTMR 2 (Alcalase 2.4L, HRT = 100 min)

The CSTMR 2 experiment was shortened due to an insurmountable reduction in the permeate flowrate. In this experiment a peristaltic pump was used to pump the hydrolysate to the membrane; a positive displacement pump was used in all other experiments. After 300 min of operation the membrane blocked. Examination of Figure 5.4 shows that the permeate MWPs at 180 min and 300 min are very similar although the area of the 300 min MWP is greater. Figure 5.10 shows that reactor conversion was still increasing. These two observations indicate that steady state had not been achieved in this experiment. Figure 5.11 shows evidence of fouling of the membrane. Once again the MWPs of the reaction vessel contents and the bulk permeate, Figure 5.5, show that the reaction vessel contents were almost completely hydrolysed. The maximum instantaneous conversion obtained in this experiment was 67 % total nitrogen at 300 minutes. The residual active protease results were positive for all of the instantaneous permeate samples.

5.4.1.3 CSTMR 3 (Amano Protease A, HRT = 110 min)

The results obtained for experiment CSTMR 3 show that for this experiment steady state was achieved. The MWPs of the permeates (Figure 5.6) are almost identical from 60 min to 600 min. Figure 5.10 shows that the % conversion remained relatively constant after 300 min. However, Figure 5.11 shows evidence of increasing fouling. The MWPs of the reaction vessel and the bulk permeate (Figure 5.7) once again show that little starting material is present in the reaction vessel, showing that starting material is not building up in the reaction vessel. The presence of a large peak at 3000 daltons molecular weight in the reaction vessel MWP shows that although the amount of proteolysis occurring in the reaction vessel is sufficient to use up all the casein substrate, the reaction is not going to completion. The maximum instantaneous conversion occurred at 360 min and was 95%. The residual active protease results were positive for all of the instantaneous permeate samples.

5.4.1.4 CSTMR 4 (Amano Protease A, HRT = 50 min)

Figure 5.8 shows that the MWPs of the permeate samples from CSTMR 4 were somewhat variable. The MWPs from 45 min to 480 min are similar. At 300 min there is a marked increase in the area of the MWP. At 540 min the area of the MWP decreases dramatically. The conversion (% total nitrogen), from Figure 5.10, is constant from 200-400 min followed by a sharp decrease. There is little evidence of fouling of the membrane (Figure 5.11) until 480 min and by 540 min the reactor became impossible to operate due to the declining permeate flowrate. Figure 5.9 shows that almost all of the reaction vessel contents remained unhydrolysed. The residual active protease results were positive for all of the instantaneous permeate samples.

5.4.2 Batch experiments

The results of total solids, total nitrogen, residual active protease and the base consumption data for the batch experiments are given in Appendix VI. The % conversion for the batch experiments was calculated from the total nitrogen results of the casein substrate and the bulk permeate. The results of the batch experiments are summarised in Table 5.2. The MWPs of the feed, permeate and retentate from the ultrafiltration step in each of the batch experiments are given in Figures 5.12 and 5.13.

Table 5.2 Summary of results from batch hydrolysis experiments

Experiment	Enzyme	Hydrolysis Time (min)	Conversion (% total nitrogen)
Batch 1	Alcalase 2.4L	150	87
Batch 2	Amano Protease A	120	93

The MWPs of the feed, permeate and retentate from Batch 1 (Alcalase 2.4L), shown in Figure 5.12, are all very similar, although the

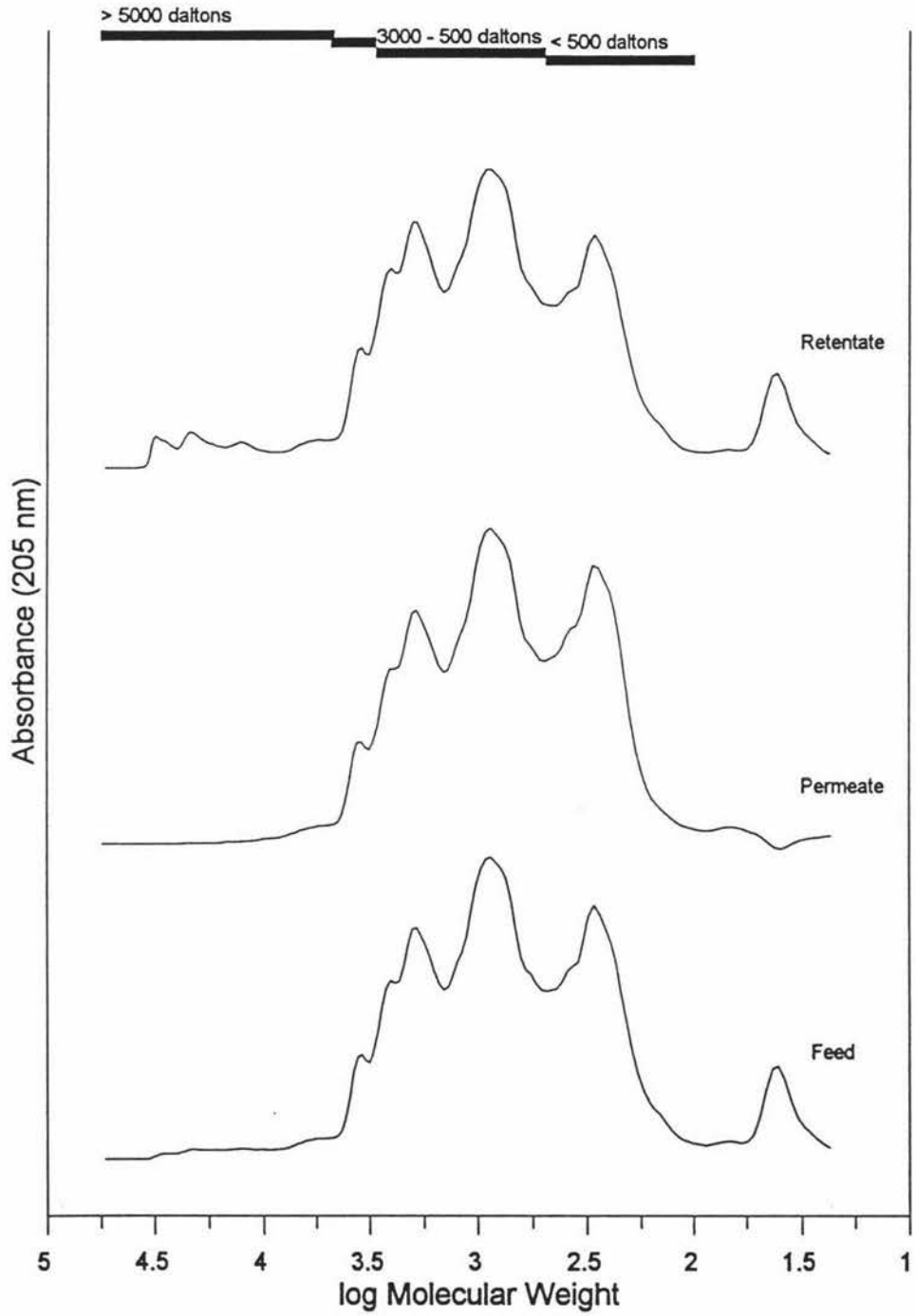


Figure 5.12 Batch 1 (Alcalase 2.4L, 150 min hydrolysis): Feed, permeate and retentate MWPs (scaled).

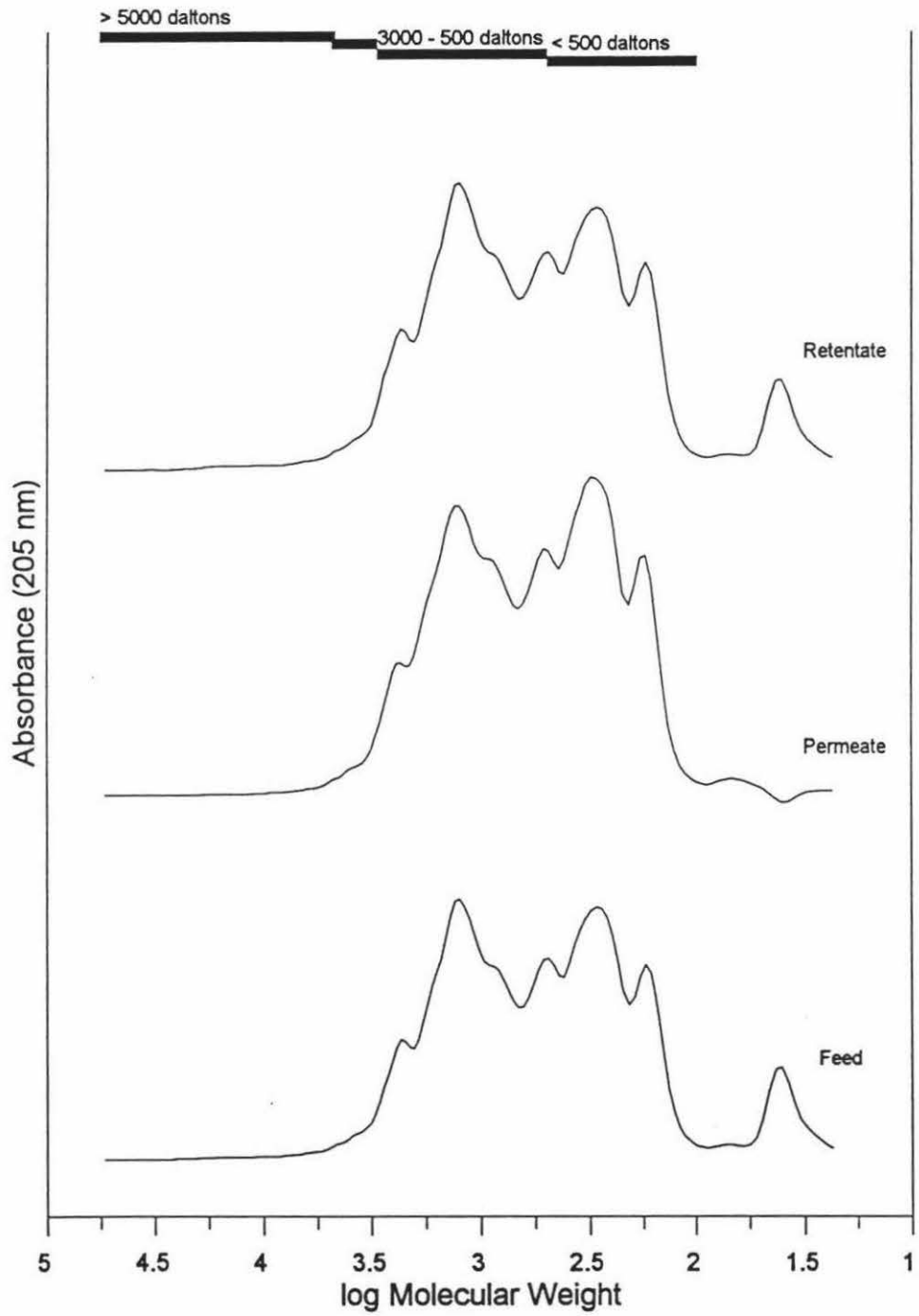


Figure 5.13 Batch 2 (Amano Protease A, 120 min hydrolysis): Feed, permeate and retentate MWPs (scaled).

retentate MWP does shows that there was some concentration of material > 5000 daltons molecular weight. Likewise, Figure 5.13 shows that the feed, permeate and retentate from Batch 2 (Amano Protease A) all had very similar MWPs. The total solids content of the three streams from each of the batch experiments ranged from 5.00-7.25 % w/w.

5.5 Discussion

The variability in some of the % conversion results (Figure 5.10) could be attributed to slight variations in the HRT which may have resulted from manual control of the caseinate feed pump (it was difficult to match the permeate flowrate exactly).

The type of pump used to pump the reaction vessel contents to the ultrafiltration unit was an important consideration. It was suspected that the fluid flow dynamics of the peristaltic pump used in CSTMR 2 encouraged the formation of larger precipitate particles that were able to block the membrane inlets.

The CSTMR and batch hydrolysis experiments are summarized in Table 5.3. The productivity (g hydrolysate produced/g enzyme used) was highest for all of the CSTMR experiments. The magnitude of the productivity results is similar to results reported by Mannheim & Cheryan (1990). The batch productivity results are based on the weight of hydrolysate produced after ultrafiltration. If the weight of the hydrolysate produced before ultrafiltration was used to calculate the batch experiment productivity then these results would be nearer to those obtained for the CSTMR experiments.

The experiment using Alcalase 2.4L and a long HRT (CSTMR 1) produced a good average conversion of protein to peptides (69%) and achieved a maximum instantaneous conversion greater than 90%. However, the MWP of the product produced from CSTMR 1 did not have the desired characteristics because of high amounts of material less than 500 daltons molecular weight.

Table 5.3 Summary of the CSTMR and batch hydrolysis experiments

Experiment	Enzyme	HRT (CSTMR) or Hydrolysis time (batch) (min)	Conversion (% total nitrogen)	Productivity (g hydrolysate produced/g enzyme used)
CSTMR 1	Alcalase 2.4L	160	69	280
CSTMR 2	Alcalase 2.4L	100	39	180
CSTMR 3	Amano Protease A	110	84	130
CSTMR 4	Amano Protease A	50	69	670
Batch 1	Alcalase 2.4L	150	87	60
Batch 2	Amano Protease A	120	93	30

The experiment using Alcalase 2.4L and a shorter HRT (CSTMR 2) was terminated before the full conversion capability could be measured. The MWP of the product of CSTMR 2 was also undesirable relative to the target MWP.

The MWPs of the reaction vessels for both CSTMR 1 and CSTMR 2 clearly show that almost all material is as hydrolysed as it possibly can be before it is separated by the membrane. In addition to this it appears that the peptides were concentrated in the reactor even though they should have been able to pass through the membrane. These observations indicate that at both the high and low HRTs the enzyme was able to hydrolyse all of the protein to small peptides. The accumulation of material in the reactor is not due to retention of peptides larger than 8000 daltons molecular weight, nor was it due to the accumulation of starting material as other

authors suspected (Cheryan, 1986). It was caused by the retention of peptides that would normally be expected to pass through the membrane easily. As these peptides accumulate the efficiency of the membrane to allow them though into the permeate appears to increase as shown by the increasing % conversion data. Although it is not obvious from the literature one author seems to have been aware of this effect. Cheryan's (1986) statement that "The belief that membrane bioreactors operated in the recycle mode are more productive because "inhibitory end-products can be removed as soon as they are formed" is incorrect due to the equilibrium partitioning nature of the membrane process" is in agreement with this interpretation.

As peptides accumulate above a certain concentration the effect of fouling may take over and lower reactor productivity. This may explain effects that others have noticed but attributed to decreasing activity of the enzyme. Mannheim & Cheryan (1990) showed that a CSTMR system using Alcalase 2.4L and casein under very similar conditions resulted in steady state operation from 120 min to 600 min. These authors automated their experiment so that the casein feed flowrate matched the permeate flowrate exactly. They investigated a number of factors that may have contributed to the gradual decline on reactor productivity that they observed. These authors discounted enzyme leakage as a cause of declining reactor productivity because they did not measure any enzyme activity in the permeate after 90 min of reactor operation. This observation appears to contradict observations made in the present study, where positive residual active protease results were obtained for all of the instantaneous permeate samples. The method used in the present study to detect residual enzyme activity has been shown to be able to detect very low levels of enzyme activity (O'Sullivan, unpublished results). Mannheim & Cheryan (1990) attributed the decline in reactor productivity to the "poisoning" of the enzyme by the polysulphone membrane that was used. They suggested that this effect could be overcome by adding more enzyme to the CSTMR reaction vessel during its operation. The results of the present work

suggest that the addition of more enzyme may not have any effect on the reactor productivity.

The experiment using Amano Protease A and a long HRT (CSTMR 3) produced the highest average conversion of protein to peptides (84%) and achieved a maximum instantaneous conversion greater than 90%. However, the MWP of the product produced from CSTMR 3 did not have the desired characteristics because of high amounts of material less than 500 daltons molecular weight. In particular, there was a high free amino acid peak.

The experiment using Amano Protease A and a short HRT (CSTMR 4) produced a good average conversion of protein to peptides (69%) and achieved a maximum instantaneous conversion greater than 90%. The MWP of the product produced from CSTMR 4 had the desired characteristics relative to the target product. However, Figure 5.11 shows that substantial fouling of the membrane occurred. Most of the fouling occurred very early in the experiment which suggests that the accumulation of total solids in the reaction vessel did not cause the fouling. It may be possible to optimise the HRT used to overcome this fouling effect without compromising the molecular weight profile. The MWP of the reaction vessel contents at the end of the CSTMR 4 experiment show that most of the material present is unhydrolysed casein. One explanation for this is that some or all of the enzyme present at the start of the experiment had either leaked through the membrane or had been inactivated in some way. An alternative explanation is that too little enzyme had been added initially and that as the concentration of the reaction vessel increased the enzyme was overwhelmed with substrate.

The results of the batch experiments show that the hydrolysate could have been ultrafiltered for a much longer time, which would have increased the yield considerably. However, the MWPs of the bulk permeates from Batch 1 and Batch 2 in particular, do not match the target MWP.

The MWPs of the permeate from the batch and CSTMR experiments using Alcalase 2.4L, given in Figure 5.14, show that the use of a CSTMR,

and its HRT, had an effect on the molecular weight distribution of the final hydrolysate product. It is clear from the MWPs that the use of a CSTMR resulted in hydrolysates that had a lower proportion of di-peptides and free amino acids than the batch processes and that the shortest HRT had an even greater effect in this direction. The effect was more obvious in the MWPs from the experiments using Amano Protease A (Figure 5.15). In this case, a reasonably short HRT (50 min) gave rise to a hydrolysate with a much lower level of free amino acids than the product from the batch hydrolysis. A comparison between the Amano Protease A CSTMR short HRT MWP and the Amano Protease A MWPs from the enzyme screening trials (Chapter 3) reveals that the peptides that were smaller than 5000 daltons permeated the membrane before they could be hydrolysed further to di- and tri-peptides and free amino acids. In this case, the CSTMR makes it possible to take advantage of the way in which the enzyme hydrolyses the protein in order to produce hydrolysates with low levels of free amino acids.

The greater success of Amano Protease A at short HRT (CSTMR 4) to produce the desired molecular weight profile probably results from the fact that the HRT was not long enough to allow the reaction to go to completion. Alcalase 2.4L may work just as well if a lower HRT or a lower E:S was used.

The products from the CSTMR experiments described here had higher proportions of peptide material in the range 3000-5000 daltons molecular weight than did the equivalent batch hydrolyses. The relatively high amounts of peptides in the range 3000-5000 daltons in the CSTMR products made in this study may confer some functional advantages (*e.g.* emulsification capacity) on these products. The functional properties of the final hydrolysates were not examined in the present study.

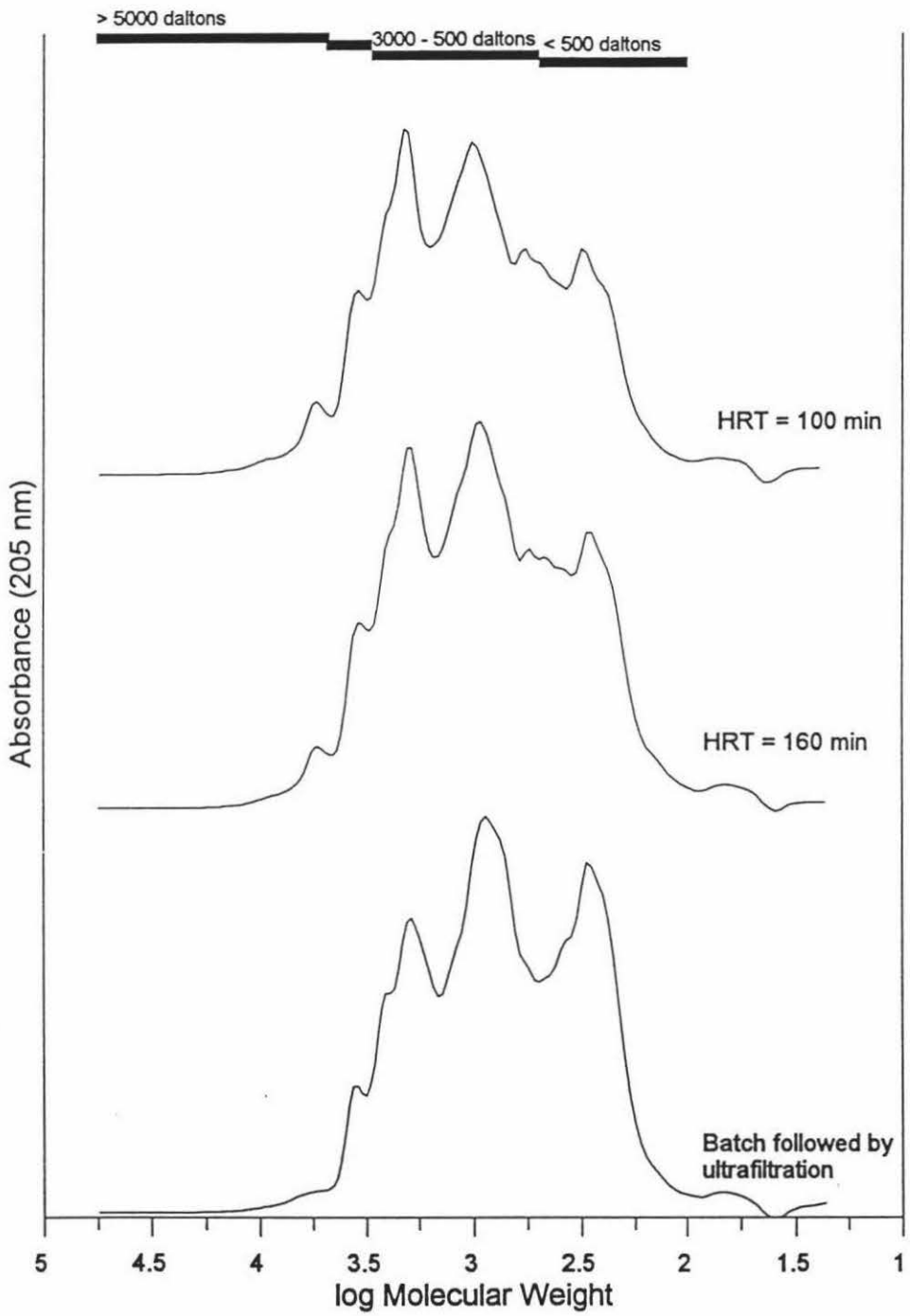


Figure 5.14 MWPs of the batch hydrolysis and CSTMR products made with Alcalase 2.4L.

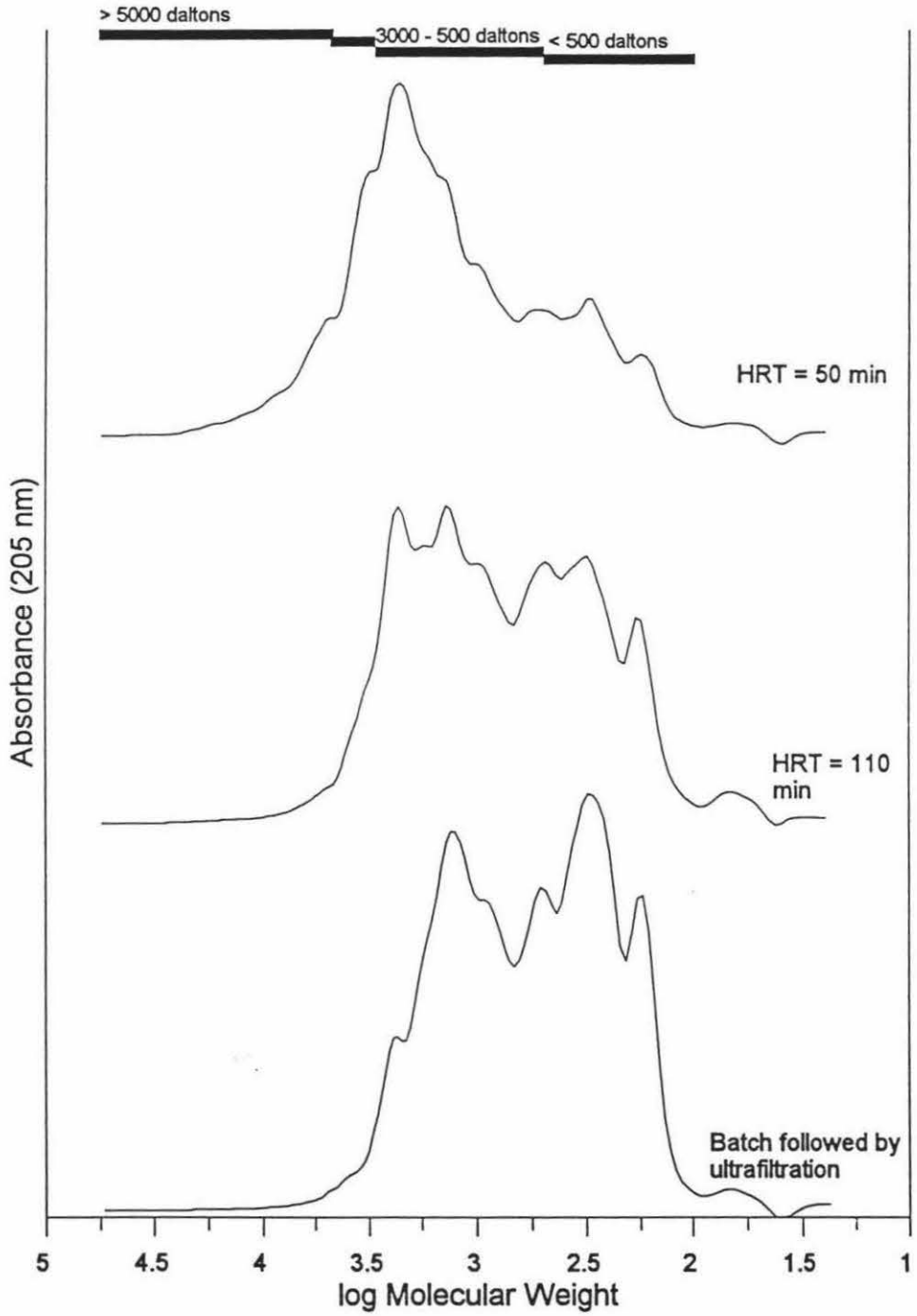


Figure 5.15 MWPs of the batch hydrolysis and CSTMR products made with Amano Protease A.

5.6 Conclusions

The choice of ultrafiltration membrane feed pump proved to be critical to the successful operation of the CSTMR in the experiments described in the present study. When a peristaltic pump was used the hydrolysate tended to form large precipitate particles which blocked the membrane channels. A positive displacement pump performed better in the CSTMR system.

Using Amano Protease A and a short HRT, as in CSTMR 4, it is possible to prepare a hydrolysate with a low proportion of free amino acids and large proportion of material in the molecular weight range 3000-5000 daltons. The same result may also be possible using Alcalase 2.4L if a low enough HRT is used. In general, it appears that to substantially lower the free amino acid peak in the product, the HRT or enzyme activity must be adjusted so that the enzyme reaction does not go to completion. The HRT will have no effect on the product unless it is low enough to prevent complete hydrolysis of the feed material.

The % conversion increases with increasing concentration of peptides in the reaction vessel due to the equilibrium partitioning effect (described by Cheryan, 1986) until fouling becomes dominant.

CHAPTER 6
FINAL DISCUSSION AND CONCLUSIONS

The "ideal" protein hydrolysate for use in hypoallergenic infant formulae can be defined from the literature. The hydrolysate should have a low proportion of free amino acids (< 10%) in order to minimize the negative flavour contribution of these free amino acids and to prevent side effects such as osmotic diarrhoea in infants consuming a formula containing the hydrolysate. In order to reduce the allergenicity of the hydrolysate, it should not contain any peptides greater than 5,000 daltons molecular weight. The hydrolysate should also contain the greatest proportion possible of peptides in the molecular weight range 1500-3000 daltons to maximise the possibility of retaining the physiological function of the peptides.

Traditional batch hydrolysis processes alone cannot be used to produce milk protein hydrolysates with these properties. Hydrolysates from these processes contain either a high proportion of free amino acids or a large proportion of material greater than 5,000 daltons. Hydrolysates manufactured using traditional batch processes must be treated further in order to reduce their allergenicity.

CSTMRs have been described in the literature. These systems consist of a CSTR connected to an ultrafiltration module. The reaction mixture containing unhydrolysed protein, peptides of various sizes and enzyme, is pumped through the membrane module. The permeate from the ultrafiltration module is collected and forms the product. The retentate is recycled back to the CSTR for further reaction. The CSTR is fed substrate continuously at a rate equivalent to the permeate flowrate in order to maintain constant volume in the CSTR.

The aim of the present study was to use the CSTMR system to produce a hydrolysate that was low in free amino acids and had a maximum molecular weight of 5000 daltons. Two enzymes were selected, on the basis of time series MWPs, to achieve this aim (Chapter 3). Alcalase

2.4L produces casein hydrolysates that are low in free amino acids. Amano Protease A produces casein hydrolysates with MWPs similar to Alcalase but forms a significant proportion of free amino acids late in the hydrolysis reaction. Because the formation of free amino acids occurs late in the Amano Protease A hydrolysis reaction it should be possible to select a HRT that would allow the peptides to pass through the ultrafiltration membrane before they can be hydrolysed further to free amino acids. Both enzymes have a high activity for casein, a necessary requirement to prevent the build-up of unhydrolysed protein and subsequent fouling of the membrane in the CSTMR.

The type of ultrafiltration membrane used in the CSTMR is important to the long-term stability of the system. Four different ultrafiltration membranes were evaluated for their effect on the separation of a casein hydrolysate. A spiral wound membrane with a nominal MWCO of 30,000 daltons was selected for the bioreactor experiments because it had a high flux, gave the highest recovery of peptides, was not susceptible to particulate fouling and, most importantly gave the most desirable MWP relative to the target product.

Four CSTMR experiments were conducted: two experiments per enzyme with two different HRTs each. In addition two experiments were carried out, one for each enzyme, using batchwise hydrolysis followed by ultrafiltration, to assess the products and the efficiency of their production compared to CSTMR-type hydrolyses.

The choice of ultrafiltration membrane feed pump proved to be critical to the successful operation of the CSTMR in the experiments described in the present study. When a peristaltic pump was used the hydrolysate tended to form large precipitate particles which blocked the membrane channels. A positive displacement pump performed well in the CSTMR system used in the present study.

Using Amano Protease A and a short HRT, as in CSTMR 4, it is possible to prepare a hydrolysate with a low proportion of free amino acids and large proportion of material in the molecular weight range 3000-5000

daltons. The same result may also be possible using Alcalase 2.4L if a low enough HRT is used. In general, it appears that to substantially lower the free amino acid peak in the product, the HRT or enzyme activity must be adjusted so that the enzyme reaction does not go to completion. This adjustment cannot be predicted from batch experiments of the kind used for the enzyme selection phase (Chapter 3). The HRT will have no effect on the product unless it is low enough to prevent complete hydrolysis of the feed material.

The efficiency of the membrane separation increases with increasing concentration of peptides in the reaction vessel due to the equilibrium partitioning effect (described by Cheryan, 1986).

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APPENDIX I
COMPOSITION OF CASEIN SUBSTRATE

Table A1 Composition of Alacid Acid Casein (Factory 1032, Batch J4176)

Component	Units	Value
Protein	% w/w	89
Moisture	% w/w	9.4
Ash	% w/w	2.0
Lactose	% w/w	0.06
Sodium	mg/100 g powder	6.7
Potassium	mg/100 g powder	23

APPENDIX II

RAW DATA FROM ENZYME SCREENING EXPERIMENTS

Table A2 Base consumption and DH for enzyme screening experiments

	Alcalase 2.4L		Neutrase 0.5L		Amano Protease A		Amano Protease B	
Hydrolysis time	4N NaOH added	DH	4N NaOH added	DH	4N NaOH added	DH	4N NaOH added	DH
(min)	(ml)	(%)	(ml)	(%)	(ml)	(%)	(ml)	(%)
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	5.75	3.03	0.56	0.72	3.61	4.00	0.80	1.95
10	9.35	4.93	1.07	1.37	4.89	5.41	1.09	2.66
15	11.49	6.05	1.19	1.53	5.73	6.34	1.35	3.29
30	15.82	8.33	1.82	2.33	7.26	8.04	1.76	4.29
45	18.64	9.82	-	-	8.29	9.18	1.98	4.83
60	20.35	10.72	2.85	3.66	9.54	10.56	2.12	5.17
90	22.92	12.07	3.24	4.16	10.67	11.82	2.27	5.54
120	24.52	12.92	3.66	4.70	11.26	12.47	2.40	5.85
150	25.79	13.59	3.98	5.11	11.65	12.90	2.53	6.17
180	26.56	13.99	4.24	5.44	11.87 *	13.14	2.63	6.41
210	-	-	4.45	5.71			-	-
240	28.01	14.76	4.70	6.03			-	-
270	-	-	4.79	6.15			-	-
300	28.86	15.20	4.93 *	6.32			2.87	7.00
330	-	-					-	-
360	29.51	15.55					3.02	7.37
420	30.13 *	15.97					3.07	7.49
540							3.43	8.37
1260							5.04 *	12.29

* = hydrolysis stopped

Table A2 (continued)

	Papain W40		Amano Bromelain		Rohm Bromelain		Corolase N	
Hydrolysis time	4N NaOH added	DH	4N NaOH added	DH	4N NaOH added	DH	4N NaOH added	DH
(min)	(ml)	(%)	(ml)	(%)	(ml)	(%)	(ml)	(%)
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	0.22	0.54	0.80	0.89	2.84	3.14	0.58	0.64
10	0.74	1.80	1.61	1.78	4.18	4.63	1.26	1.40
15	0.99	2.41	2.20	2.44	4.77	5.28	1.78	1.97
30	1.34	3.27	3.11	3.44	5.60	6.20	2.74	3.03
45	1.48	3.61	3.48	3.85	6.07	6.72	3.35	3.71
60	1.60	3.90	3.70	4.10	6.44	7.13	3.76	4.16
90	1.70	4.15	4.00	4.43	6.66	7.37	4.41	4.88
120	1.75	4.27	4.12	4.56	6.81	7.54	4.83	5.35
150	1.83	4.46	4.21	4.66	6.88	7.62	5.15	5.70
180	1.87 *	4.56	4.28 *	4.74	6.92 *	7.66	5.39	5.97
210							-	-
240							5.83 *	6.46
270								
300								
330								
360								
420								
540								
1260								

* = hydrolysis stopped

Table A2 (continued)

	Corolase 7089		Biocon Fungal Protease		Corolase S50		Rhozyme P41	
Hydrolysis time	4N NaOH added	DH	4N NaOH added	DH	4N NaOH added	DH	4N NaOH added	DH
(min)	(ml)	(%)	(ml)	(%)	(ml)	(%)	(ml)	(%)
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	2.08	2.30	1.78	1.97	2.57	2.24	0.79	0.87
10	3.14	3.48	2.80	3.10	3.94	3.44	1.50	1.66
15	3.82	4.23	3.64	4.03	4.39	3.83	2.03	2.25
30	-	-	5.01	5.55	5.27	4.60	3.47	3.84
45	5.83	6.46	5.86	6.49	5.69	4.97	4.13	4.57
60	6.34	7.02	6.44	7.13	5.78	5.05	4.64	5.14
90	7.08	7.84	7.31	8.09	5.94	5.19	5.38	5.96
120	7.66	8.48	7.83	8.67	6.05	5.28	5.91	6.54
150	7.95	8.80	8.22	9.10	-	-	-	-
180	8.32 *	9.21	8.56	9.48	6.19 *	5.40	6.36 *	7.04
210			8.75	9.69				
240			8.97	9.93				
270			9.11	10.09				
300			9.26	10.25				
330			9.33 *	10.33				
360								
420								
540								
1260								

* = hydrolysis stopped

Table A2 (continued)

	Rhozyme P64	
Hydrolysis time (min)	4N NaOH added (ml)	DH (%)
0	0.00	0.00
5	1.36	1.19
10	2.42	2.11
15	3.21	2.80
30	4.66	4.07
45	5.63	4.92
60	6.30	5.50
90	7.44	6.50
120	8.19	7.15
150	-	-
180	9.26 *	8.09
210		
240		
270		
300		
330		
360		
420		
540		
1260		

* = hydrolysis stopped

APPENDIX III

RAW DATA FROM pH-DROP EXPERIMENTS

Table A3 Raw data from pH-drop experiments

Hydrolysis Time (min)	Amano Protease A i pH	Amano Protease A ii pH	Corolase N pH	Rhozyme P64 pH	Rhozyme P41 pH	Rohm Bromelain pH	Corolase S50 pH
0	7.96	7.95	8.10	8.08	7.79	7.97	7.95
15	7.64	7.64	7.94	8.00	7.72	7.70	7.77
30	7.45	7.45	7.78	7.90	7.63	7.54	7.66
45	7.30	7.31	7.67	7.81	7.56	7.42	7.56
60	7.18	7.20	7.57	7.76	7.49	7.33	7.47
90	7.01	7.05	7.41	7.64	7.39	7.19	7.34
120	6.91	6.94	7.29	7.54	7.32	7.10	7.24
150	6.84	6.87	7.19	7.46	7.26	7.03	7.16
180	6.78	6.81	7.12	7.38	7.21	6.97	7.10
210	6.74	6.77	7.06	7.32	7.18	6.93	7.06
240	6.71	6.73	7.00	7.26	7.15	6.90	7.02
270	6.69	6.69	6.96	7.22	7.12	6.87	6.98
300	6.67	6.67	6.93	7.18	7.10	6.85	6.96
330	6.66	6.64	6.90	7.14	7.09	6.83	6.93
360	6.65	6.62	6.87	7.11	7.08	6.81	6.91
390	6.64	6.60	6.86	7.08	7.07	6.80	6.89
420	6.64	6.58	6.84	7.06	7.06	6.79	6.87
450	6.63	6.56	6.82	7.04	7.05	6.78	6.86
480	6.63	6.55	6.81	7.03	7.05	6.77	6.85
510	6.63	6.53	6.80	7.02	7.05	6.76	6.84
540	6.63	6.52	6.80	7.01	7.04	6.76	6.83
570	6.63	6.51	6.79	7.00	7.04	6.75	6.82
600	6.63	6.50	6.78	6.99	7.03	6.75	6.81
Initial slope (pH units/min)	0.0130	0.0125	0.0088	0.0053	0.0050	0.0107	0.0080
Total pH drop (pH units/10 min)	1.3300	1.4500	1.3200	1.0900	0.7600	1.2200	1.1400

Table A3 (continued)

Hydrolysis Time (min)	Biocon Fungal Protease	Amano Bromelain		Alcalase 2.4L	Corlase 7089	Neutrase 0.5L
		i	ii			
	pH	pH	pH	pH	pH	pH
0	7.95	7.90	8.00	7.97	7.97	8.02
15	7.80	7.72	7.55	7.66	-	7.95
30	7.71	7.60	7.36	7.48	7.50	7.87
45	7.63	7.47	7.23	7.35	7.37	7.80
60	7.56	7.40	7.13	7.25	7.27	7.74
90	7.42	7.25	7.00	7.08	7.12	7.63
120	7.32	7.14	6.92	6.99	7.00	7.53
150	7.23	7.05	6.86	6.92	6.92	7.45
180	7.15	6.97	6.82	6.87	6.86	7.38
210	7.08	6.92	6.79	6.84	6.82	7.33
240	7.03	6.86	6.76	6.81	6.79	7.27
270	6.98	6.82	6.74	6.78	6.76	7.22
300	6.94	6.79	6.73	6.76	6.74	7.18
330	6.91	6.76	6.72	6.74	6.73	7.14
360	6.88	6.73	6.71	6.72	6.72	7.11
390	6.86	6.71	6.70	6.71	6.72	7.08
420	6.84	6.70	7.00	6.70	6.71	7.06
450	6.82	6.68	6.68	6.69	6.70	7.03
480	6.80	6.67	6.68	6.68	6.70	7.01
510	6.79	6.66	6.68	6.67	6.70	6.99
540	6.78	6.65	6.67	6.66	6.70	6.97
570	6.77	6.65	6.67	6.66	6.70	6.96
600	6.76	6.64	6.67	6.65	6.70	6.95
Initial slope (pH units/min)	0.0065	0.0083	0.0145	0.0120	0.0117	0.0047
Total pH drop (pH units/10 min)	1.1900	1.2600	1.3300	1.3200	1.2700	1.0700

Table A3 (continued)

Hydrolysis Time (min)	Amano Protease B pH	Amano Papain pH
0	8.05	7.99
15	8.02	7.83
30	7.99	7.74
45	7.96	7.65
60	7.94	7.58
90	7.89	7.45
120	7.83	7.35
150	7.78	7.27
180	7.73	7.21
210	7.68	7.15
240	7.63	7.10
270	7.59	7.06
300	7.54	7.02
330	7.49	6.98
360	7.45	6.95
390	7.40	6.92
420	7.36	6.89
450	7.32	6.86
480	7.28	6.84
510	7.24	6.81
540	7.21	6.78
570	7.18	6.77
600	7.14	6.75
Initial slope (pH units/min)	0.0018	0.0068
Total pH drop (pH units/10 min)	0.9100	1.2400

APPENDIX IV
RAW DATA AND FLUX CALCULATIONS FROM THE MEMBRANE SCREENING EXPERIMENTS

Table A4 Permeate flowrates and flux calculations for various ultrafiltration membranes tested with a casein hydrolysate

Time (min)	Permeate flowrate (ml/min) (Flux (L/m²/h))			
	S1Y30 Spiral wound, 30,000 daltons MWCO (area = 0.09 m²)	**S1Y10** Spiral wound, 10,000 daltons MWCO (area = 0.09 m²)	**H1P30-43** Hollow fibre, 30,000 daltons MWCO (area = 0.03 m²)	**H1P10-43** Hollow fibre, 10,000 daltons MWCO (area = 0.03 m²)
1	144 (96)	56 (37)	12 (24)	-
5	116 (77)	52 (35)	15 (30)	7 (14)
10	104 (69)	54 (36)	14 (28)	7 (14)
15	-	50 (33)	14 (28)	7 (14)
20	81 (54)	-	-	-
30	65 (43)	50 (33)	13 (26)	6 (12)
40	57 (38)	-	-	6 (12)
45	-	-	-	6 (12)
50	51 (34)	-	13 (26)	-
60	46 (31)	-	-	6 (12)
70	43 (29)	-	-	-
100	38 (25)	-	-	-
120	36 (24)	-	-	-

$$\text{Flux (L/m}^2\text{/h)} = \frac{\text{permeate flowrate (L/h)}}{\text{membrane area (m}^2\text{)}}$$

APPENDIX V
DATA AND CALCULATIONS FROM CSTMR EXPERIMENTS

Table A5 CSTMR Experiment 1 (Alcalase 2.4L, HRT = 160 minutes): Permeate flowrates, NaOH addition, total solids, total nitrogen and residual active protease results

Time (min)	Permeate Flowrate (ml/min)	Volume 4N NaOH added (ml)	Volume of permeate collected (ml)	Total solids (% w/w)	Total nitrogen (% w/w)	Residual active protease (+ positive; + + strong positive; - negative)	Conversion (% total nitrogen)
Substrate	-	-	-	5.15	0.755	-	-
15	8.0	4.11	120	-	-	+	-
30	8.0	4.99	240	1.86	0.271	+	36
45	7.0	6.28	345	-	0.379	+	50
60	6.5	6.76	443	-	-	+	-
90	7.0	9.03	653	2.69	0.377	+	50
120	6.0	10.28	833	2.12	0.304	+	40

Table A5 (continued)

Time (min)	Permeate Flowrate (ml/min)	Volume 4N NaOH added (ml)	Volume of permeate collected (ml)	Total solids (% w/w)	Total nitrogen (% w/w)	Residual active protease (+ positive; + + strong positive; - negative)	Conversion (% total nitrogen)
150	6.5	11.5	1028	2.93	0.396	+	52
180	6.5	13.75	1223	3.20	0.448	+	59
240	5.5	16.08	1553	3.72	0.526	+	70
300	6.5	19.41	1943	3.85	0.532	+	70
360	6.0	21.76	2303	3.11	0.446	+	59
420	6.0	24.31	2663	4.38	0.608	+	81
480	6.0	27.78	3023	4.34	0.609	+	81
540	5.8	29.65	3371	4.78	0.668	+	88
600	5.5	31.78	3701	4.96	0.705	+	93
Bulk Permeate	-	-	3701	3.77	0.519	-	69

Table A5 (continued)

Time (min)	Permeate Flowrate (ml/min)	Volume 4N NaOH added (ml)	Volume of permeate collected (ml)	Total solids (% w/w)	Total nitrogen (% w/w)	Residual active protease (+ positive; + + strong positive; - negative)	Conversion (% total nitrogen)
Reaction Vessel	-	-	-	13.85	1.934	+ +	-

Table A6 CSTMR Experiment 2 (Alcalase 2.4L, HRT = 100 minutes): Permeate flowrates, NaOH addition, total solids, total nitrogen and residual active protease results

Time (min)	Permeate Flowrate (ml/min)	Volume 4N NaOH added (ml)	Volume of permeate collected (ml)	Total solids (% w/w)	Total nitrogen (% w/w)	Residual active protease (+ positive; + + strong positive; - negative)	Conversion (% total nitrogen)
Substrate	-	-	-	7.04	1.08	-	-
15	7.5	2.04	113	0.92	0.141	+	13
30	7.5	3.33	225	1.85	0.275	+	26
45	7.0	4.70	330	1.98	0.294	+	27
60	8.0	5.55	450	2.19	0.320	+	30
120	5.3	8.57	1654	2.96	0.429	+	40
180	5.5	11.43	2434	3.74	0.541	+	50
300	-	15.02	3094	5.09	0.724	+	67
Bulk Permeate	-	-	3094	2.90	0.420	-	39
Reaction Vessel	-	-	-	10.97	0.786	+ +	-

Table A7 CSTMR Experiment 3 (Amano Protease A, HRT = 110 minutes): Permeate flowrates, NaOH addition, total solids, total nitrogen and residual active protease results

Time (min)	Permeate Flowrate (ml/min)	Volume 4N NaOH added (ml)	Volume of permeate collected (ml)	Total solids (% w/w)	Total nitrogen (% w/w)	Residual active protease (+ positive; + + strong positive; - negative)	Conversion (% total nitrogen)
Substrate	-	-	-	5.22	0.619	-	-
15	10.5	3.93	158	1.50	0.212	+	28
30	9.5	5.49	300	2.21	0.330	+	43
45	9.5	7.31	443	2.62	0.366	+	48
60	9.5	8.70	585	3.08	0.421	+	55
90	10.5	11.49	900	3.65	0.529	+	69
120	12.0	14.15	1260	3.80	0.542	+	71
150	11.5	16.98	1605	3.93	0.566	+	74

Table A7 (continued)

Time (min)	Permeate Flowrate (ml/min)	Volume 4N NaOH added (ml)	Volume of permeate collected (ml)	Total solids (% w/w)	Total nitrogen (% w/w)	Residual active protease (+ positive; + + strong positive; - negative)	Conversion (% total nitrogen)
180	11.0	19.53	1935	4.15	0.577	+	75
240	10.0	24.40	2535	4.41	0.639	+	84
300	9.0	29.26	3075	4.62	0.670	+	88
360	8.5	33.69	3585	5.06	0.728	+	95
420	8.5	37.86	4095	4.67	0.687	+	89
480	8.0	41.96	4575	4.75	0.673	+	88
540	8.5	45.84	5085	4.76	0.666	+	87
600	7.0	49.00	5505	5.16	0.781	+	-
Bulk Permeate	-	-	5505	4.21	0.641	-	84
Reaction Vessel	-	-	-	16.16	2.105	+ +	-

Table A8 CSTMR Experiment 4 (Amano Protease A, HRT = 50 minutes): Permeate flowrates, NaOH addition, total solids, total nitrogen and residual active protease results

Time (min)	Permeate Flowrate (ml/min)	Volume 4N NaOH added (ml)	Volume of permeate collected (ml)	Total solids (% w/w)	Total nitrogen (% w/w)	Residual active protease (+ positive; + + strong positive; - negative)	Conversion (% total nitrogen)
Substrate	-	-	-	5.74	0.843	-	-
15	10.0	1.59	150	2.03	0.294	+	35
30	7.0	2.59	255	2.50	0.361	+	43
45	3.8	3.13	312	3.33	0.479	+	57
60	3.5	3.73	365	3.46	0.491	+	58
90	5.0	4.63	515	3.91	0.580	+	69
120	5.0	5.50	665	4.38	0.625	+	74
150	4.0	6.39	785	4.33	0.626	+	74

Table A8 (continued)

Time (min)	Permeate Flowrate (ml/min)	Volume 4N NaOH added (ml)	Volume of permeate collected (ml)	Total solids (% w/w)	Total nitrogen (% w/w)	Residual active protease (+ positive; + + strong positive; - negative)	Conversion (% total nitrogen)
180	5.5	7.28	950	4.78	0.693	+	82
240	3.5	9.41	1160	5.52	0.771	+	91
300	3.0	12.78	1340	4.51	0.657	+	78
360	3.5	13.14	1550	5.19	0.752	+	89
420	3.5	16.96	1760	5.43	0.744	+	88
480	3.8	16.96	1988	3.82	0.580	+	69
540	2.5	16.96	2138	3.38	0.470	+	56
Bulk Permeate	-	-	2138	3.93	0.582	-	69
Reaction Vessel	-	-	-	17.34	2.146	+ +	-

APPENDIX VI

DATA FROM BATCH HYDROLYSIS EXPERIMENTS

Table A9 Batch hydrolysis experiments: base consumption, total solids, total nitrogen and residual active protease results

Experiment	Enzyme	Hydrolysis time (min)	4N NaOH added (ml)	Total solids (% w/w)	Total nitrogen (% w/w)	After Ultrafiltration	Residual Active Protease	Total solids (% w/w)	Total nitrogen (% w/w)
Batch 1	Alcalase 2.4L	150	32.14	-	-	Feed (Volume = 4.6 L)	-	5.52	0.786
						Permeate (3.0 L)	-	4.66	0.676
						Retentate (1.6 L)	-	7.25	0.951
Batch 2	Amano Protease A	120	28.91	5.22	0.773	Feed (4.7 L)	-	5.60	0.809
						Permeate (2.7 L)	-	5.00	0.718
						Retentate (2.0 L)	-	6.42	0.925