CHARACTERIZATION AND FUNCTIONAL PROPERTIES OF

CANOLA PROTEIN HYDROLYSATES

A Thesis

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Angela Hooi Leng Han

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ANGELA HOOI LENG HAN

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ABSTRACT

Functional properties of proteins can be modified by the use of hydrolysis. Canola proteins (*Brassica napus*) prepared by the protein micellar mass (PMM) method were subsequently hydrolysed by acid, trypsin, chymotrypsin, bacterial and fungal proteases at varying time intervals. Different hydrolysis procedures showed different impacts on the molecular weight distribution, subunit analysis, isoelectric points and protein conformation as well as the surface hydrophobicity. Enzyme treatments cleaved PMM into smaller fragments compared to both acid methods. Some protein aggregation was observed in alternate acid hydrolysates. After hydrolysis with four different enzymes, the resultant hydrolysates showed isoelectric points in the range of 6.2-6.7 whereas all acid hydrolysates from both acid methods had isoelectric points in the range of 4.4-5.7. Fungal and bacterial hydrolysis methods provided the mildest conditions in terms of the enthalpy of denaturation and the thermal denaturation temperature. Overall, T5 had a greater aromatic surface hydrophobicity whereas C20 showed an increased aliphatic surface hydrophobicity. Nitrogen solubility, water holding capacity, fat absorption capacity, emulsion stability, foaming capacity as well as foaming stability of all the hydrolysates were investigated. Hydrolysates treated with chymotrypsin for 20 min (C20) showed the highest nitrogen solubility at pH 4.5 whereas acid hydrolysates of 4 h exhibited the highest solubility at pH 7.0. Hydrolysates prepared by acid hydrolysis for 7 h showed the best water holding capacity whereas chymotrypsin hydrolysates (10 min) exhibited the best fat absorption capacity. Emulsion stability was slightly decreased

from the original PMM in most enzyme hydrolysates and mild acid hydrolysates whereas this stability was significantly improved using an alternate acid hydrolysis method. An acid hydrolysate (A5) possessed the best foaming capacity whereas the A7 acid hydrolysate showed the best foaming stability. Relationships between molecular characteristics and functional parameters were established.

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I wouldn't achieved that far without both my parents who sacrifice too much all their lives. Ma and Ba, I love you two dearly ! My grandmom is a role model whom I love so much, and also my great grandmon who raised me, I wish she rest in peace forever ! My brother Koon Juan, Lee Juan and sis, Michelle are always there for me and I love them all ! Last but not least, I would like to thank my husband, Mickey Ma, for his patience, moral support, guidance, encouragement, friendship and companionship. Without him, I wouldn't have the determination to achieve my goal in life.

DEDICATION

This thesis is specially dedicated to my beloved parents -
Tan Joo Kian and Han Jin Fong,
my brothers and sister -
Koon Juan, Lee Juan, and Michelle,
and my dearest husband,
Mickey Ma.

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I. INTRODUCTION

Tailoring the functional properties of proteins for meeting the complex needs of the manufactured food products can determine the effective utilization of protein in food systems. The use of plant proteins in food products is limited mainly due to the lack of desirable functional performance of these proteins in foods. Therefore, proteins usually require modification using enzymatic or chemical methods to improve such functional properties as solubility, whippability and emulsification activity. Generally, protein modifications for food utilization can be classified into three areas: (1) nutrition - introduction of deficient nutritional components into the protein or improvement in its digestibility by the biological agents; (2) functionality - alternations in the nonnutritional, but still useful, properties of the proteins (solubility, water or fat uptake, viscosity, etc.); (3) organoleptic properties - modifications in the taste and flavour of the protein material for improved palatability.

The main objective of this study is to obtain a canola protein hydrolysate which has optimal functional properties. Canola proteins were isolated using the protein micellar mass (PMM) procedure. The resultant PMM was subsequently modified using various enzymatic and chemical methods in order to obtain different hydrolysates. Following this, several molecular characteristics (including molecular weight, subunit analysis, isoelectric point, thermal stability, hydrophobicity) of the PMM and all hydrolysates were studied. Several functional properties (such as nitrogen solubility, water and fat binding, emulsion and foaming properties) were examined with the PMM

and all the hydrolysates in order to evaluate the possible use of canola protein hydrolysates in food systems. Then, the relationships of molecular characteristics of the proteins and their functional properties were considered so that some understanding of the relationships between the structure and function of proteins could be established.

II. LITERATURE REVIEW

A. Canola Protein - General Considerations

1. Protein Content and Protein in Canola Seed

Canola is a major oilseed crop in Canada. Knowledge of the composition and properties of the components of canola is important for achieving an optimal production of protein flours, concentrates and isolates (Mieth *et al.*, 1983). In terms of content, canola ranges from 11-42% protein; this is influenced by genetic and environmental factors (Mieth *et al.*, 1983).

Canola meal contains three protein fractions : salt-soluble globulins or storage proteins, water-soluble albumins and alkali soluble proteins (Norton, 1989). These fractions can be separated not only by ultracentrifugal, chromatographical and electrophoretical behaviour, but also by differences in isoelectric points as well as solubilities (Mieth *et al.*, 1983). According to Norton (1989), the albumins represent the majority of the metabolically active proteins which are responsible for the biosynthesis and degradation of globulins. Albumins are located in the cellular cytoplasm in the seeds. Globulins, which serve as nitrogen reserves for the embryonic axis during germination, constitute the majority of the storage proteins. Storage globulins are situated in the protein bodies in the parenchyma cells of the seeds (Norton, 1989).

Various protein fractions are commonly designated by their sedimentation coefficients. In general, *Brassica* spp. possess four protein fractions, namely 1.7S, 7S, 12S and 15S or 17S (Bhatty *et al.*, 1968; MacKenzie and Blakely, 1972). According to

Norton (1989), the 12S globulin (cruciferin) and the 1.7S albumin (napin), which account for about 60% and 20% of the total seed proteins respectively, are two of the major seed proteins. The 12S globulin is a high molecular weight, neutral complex, composed of several polypeptide chains. In contrast, the 1.7S albumin is a low molecular weight, basic protein, composed of two disulfide-linked polypeptide chains (Ericson *et al.*, 1986). Norton (1989) stated that the 7S protein is less widely distributed in *Brassica* spp. Prakash and Rao (1986) concluded that the 15 S or 17 S fraction is a polymer resulting from possible aggregation of the 1.7S, 7S, or 12S proteins rather than being inherently present in the seed.

2.   Physico-chemical Properties of the Canola 12S Globulins

   a. Molecular Weight and Subunit Profile. The 12S globulin represents an oligomeric protein with a molecular weight of 300,000 (Schwenke *et al.*, 1983). As mentioned, the 12S globulin is the major storage protein in the seeds of *Brassica* spp. According to Bhatty *et al.* (1968), 21-33% of the nitrogen in NaCl extracts of defatted rapeseed varieties or 18-28% of the total seed nitrogen correspond to this protein.

   The 12S globulin was first isolated by Bhatty *et al.* (1968) from oil-free rapeseed meal by extraction with 10% NaCl, precipitation by dialysis against water, and chromatographic separation on Sephadex G-100. Other techniques such as the application of a combined gel and ion-exchange chromatographic purification method have been used by Schwenke and co-workers (1981) to isolate the 12S fraction. The physico-chemical properties and structure of the 12S globulin are summarized in Table

TABLE 1.   Physico-chemical properties and structures of the 12S globulin from rapeseed[1]

PROPERTY	VALUE
Molar Mass (g/mol)	300,000
Isoelectric Point	7.2
Sedimentation Coefficient $S^{o}_{20,\,w}$ [10^{-13} sec]	12.7
Diffusion Coefficient $D^{o}_{20,\,w}$ [10^{-7} m^2/sec]	3.8
Stokes Radius (Rs, nm)	
Quasielastic light scattering	5.7
Gel chromatography	5.5
Partial Specific Volume (ml/g)	0.729
Frictional Ratio (f/fo)	1.28
Molecular Weight	
Sedimentation velocity and diffusion	300,000
Sedimentation velocity and gel chromatography	294,000
Dimension (nm)	
Electron microscopy	11.3 x 11.3 x 9.2
Small angle scattering	10.5 x 10.5 x 9.2

TABLE 1. (Cont'd)

PROPERTY	VALUE
Secondary Structure	
α-helix	11%
β-sheet	31%
aperiodic	58%
Quarternary Structure	trigonal antiprism
number of subunits	6
number of polypeptide chains	12
Molar Mass of Polypeptide Chains (g/mol)	
PPC1	18500 ± 800
PPC2	21100 ± 500
PPC3	26800 ± 900
PPC4	31200 ± 1600

[1] adapted from Mieth *et al.* (1983)
         Schwenke *et al.* (1983)
         Prakash and Rao (1986)

1.

Reichelt *et al.* (1980) has used electron microscopy to study the quaternary structure of the 12S globulin and they have proposed that the subunits are arranged in the form of a trigonal antiprism with a point symmetry of 32. Schwenke *et al.* (1983) also confirmed that the quaternary structure was composed of six ordered subunits arranged as a trigonal antiprism with each subunit made up of two polypeptide chains. These polypeptide chains have molecular weights in the range of 18,500 to 31,000 (Table 1).

b. Dissociation Profile. The 12S globulin has been shown to dissociate in the presence of urea and under varying pH conditions plus ionic strengths (MacKenzie, 1975). Fig. 1 illustrates the dissociation profile of the 12S globulin.

Bhatty *et al.* (1968) showed that the rapeseed protein exists as an hexameric native 12S globulin in high ionic strength solution. Schwenke *et al.* (1983) concluded that the 12S globulin dissociates as a trimeric 7S unit in a low ionic strength solution. However, this 7S unit will re-associate nearly completely when placed in high ionic strength solution. Contrary to this reversible dissociation, Goding *et al.* (1970) proved that the 12S globulin undergoes an irreversible dissociation into 2-3S monomeric subunits in the presence of strong dissociating agents (4-6M urea), especially in an acidic condition of pH $\leq$ 3.6.

FIGURE 1. The association - dissociation profile of the 12S canola globulin. (Schwenke *et al.*, 1981; 1983)

12S

low ionic
strength

high ionic
strength

7S

extremely high
or low pH

urea

2-3S
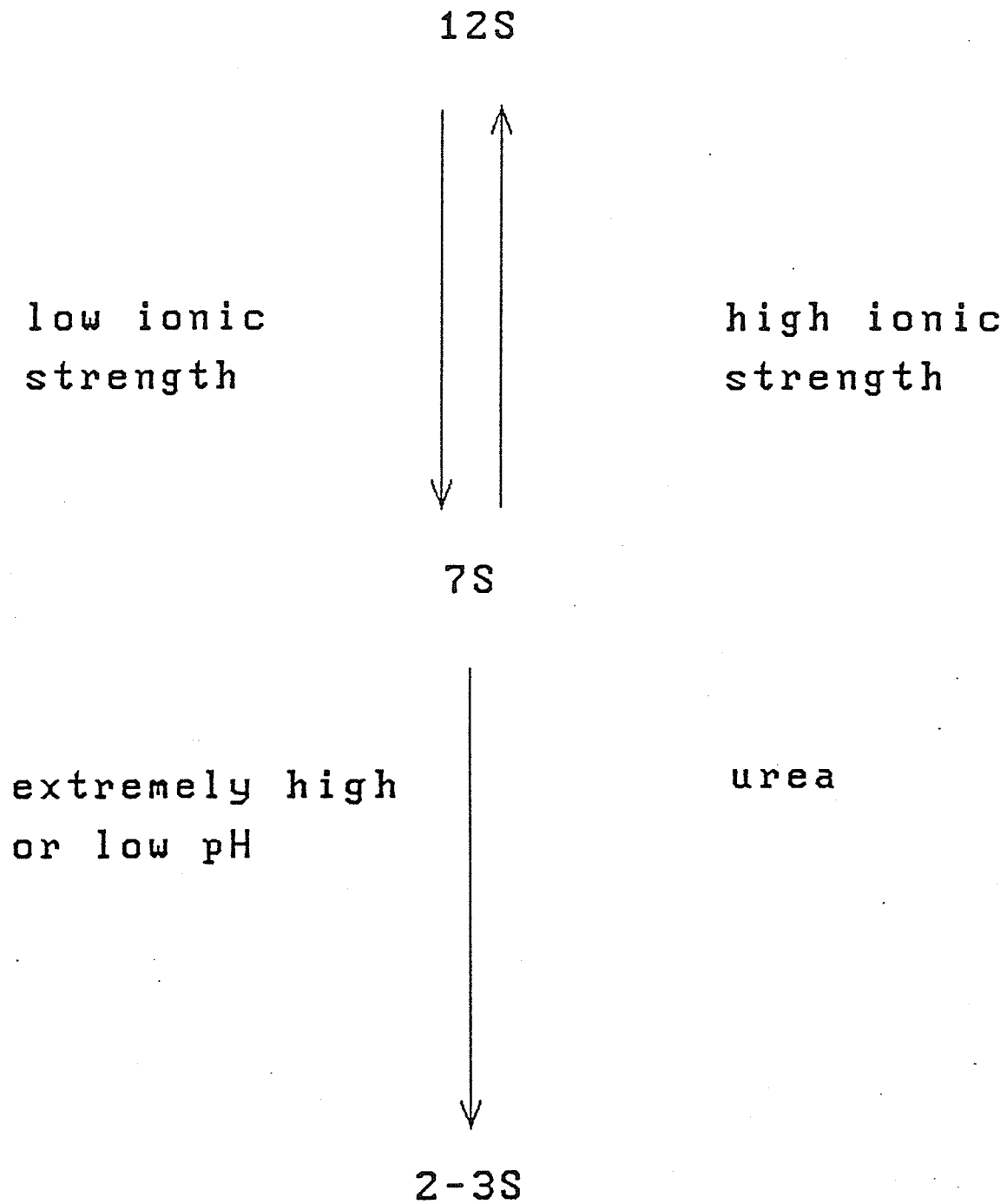
B. Micelle Phenomena

When amphiphilic molecules are dissolved in water they can achieve segregation of their hydrophobic portions from the solvent by self-aggregation (Tanford, 1973). The aggregated products are known as micelles. In 1981, Murray *et al.* used a noncovalent approach to processing and utilizing plant proteins such as fababean protein, by formation of a viscous gelatinous mass called a "protein micellar mass" (PMM). The process involved the solubilization of the protein in a high salt environment followed by a rapid reduction of ionic strength and hence the formation of insoluble protein micelles.

1.   Molecular Forces for Micelle Formation

Protein-protein interactions may occur as a result of either covalent or noncovalent interactions. According to Karp (1984), covalent interactions usually refer to high energy disulfide linkages. Murray *et al.* (1981) and Cheftel *et al.* (1985) emphasized the role of the disulfide bond as more of a stabilizing than a conformation directing force in micelle formation. Therefore, noncovalent forces may be of prime importance in micelle formation (Murray *et al.*, 1981). These noncovalent forces include van der Waal forces, hydrogen bonds, electrostatic interactions as well as hydrophobic interactions.

However, Burgess (1991) concluded that van der Waals forces, hydrogen bonds and electrostatic interactions play no or little role in micelle formation and association. Hence, hydrophobic interactions are thought to be the entropic driving force for micelle formation (Burgess, 1991). According to Nakai and Li-Chan (1988), the hydrophobic effect arises when water interacts with the nonpolar residues of a protein, and this

interaction frequently decreases its entropy. In order to minimize the unfavourable entropy changes, Nakai and Li-Chan (1988) suggested that the contact surface of protein with water should be reduced by forcing the nonpolar portion to coalesce together into droplets or globules. Therefore, the protein chain is forced to fold into a micellar structure with the hydrocarbon moiety on the inside of the globule and the polar groups on the outside. Thus, hydrophobic interactions are important in the formation of protein micelles.

2.   Criteria for Micelle Formation

Several criteria are important for micelle formation. Reynolds (1979) proposed that protein molecules will self-associate and form micelles at a critical micelle concentration (cmc). Furthermore, the amount and type (aliphatic or aromatic) of hydrophobic residues are critical for micelle formation (Tanford, 1973). Bigelow (1967) concluded that the knowledge of amino acid composition and hydrophobicity of the protein is important to identify proteins with good micelle forming capacity.

In 1984, Ismond proposed that proteins with high numbers of hydrophobic amino acid residues generally have a potential for micelle formation. Furthermore, the protein molecule must possess sufficient polar residues on the surface to bury the hydrophobic portion inside the moeity. Nakai and Li-Chan (1988) stated that some hydrophobic residues were able to be positioned on the outside as well as inside the protein; therefore, the importance of this flexibility and balance of internal and external hydrophobic groups will become more apparent when considering the effects of environmental manipulation

on micelle formation.

The amount of aqueous solvent used to bury the hydrophobic groups is also important. The type of micelle response is strongly dependent upon the dilution factor (Burgess, 1991).

3. Isolation of Protein Using Micelles

As mentioned before, Murray *et al.* (1981) have isolated seed globular proteins using a protein micellar mass (PMM) procedure. This involved stirring defatted meal in a high ionic strength salt solution and then diluting the high salt protein extract (HSPE) containing the solubilized protein, with cold distilled water. The low water solubility of the globular protein plus the decrease in ionic strength resulted in protein aggregation, micelle formation and interaction, and precipitation of the protein. In 1985, Arntfield *et al.* recovered 42.5% of the protein from fababean whereas Welsh (1988) recovered 4% protein from canola. Burgess (1991) modified the procedure used by Welsh and was able to recover approximately 31% protein from canola. The two important criteria in this procedure are the extraction step and the dilution of the high salt protein extract (Burgess, 1991).

Burgess (1991) found that extraction of canola proteins in 0.5M NaCl at pH 6.0 was optimal. Micelle formation did not occur in extreme pH environments (pH 4 or 9) due to the strong repulsive forces. However, the dilution factor was strongly dependent on the pH and ionic strength of the extracting environment. In this case, a dilution factor of one to six was used to obtain the optimal micelle response.

In 1992, Ismond and Welsh also used the PMM procedure to isolate the canola globular protein. They proposed that 0.1M NaCl/0.1M NaH$_2$PO$_4$ buffer at pH 5.5 was the best condition to remove both phytic acid and phenolic compounds whereas 0.01M NaCl/0.01M NaH$_2$PO$_4$ buffer at pH 5.5 was the optimal condition to remove glucosinolates.

4.   Effects of Environmental Manipulation on Micelle Formation

Research by Murray *et al.* (1981), Ismond (1984), Welsh (1988) and Burgess (1991) suggested that the formation and association of micelles are strongly affected by the initial protein concentration, pH and ionic strength of the solubilizing environment as well as the dilution factor.

In general, the micelle response at any protein concentration depended on the surrounding milieu. The surface properties of the protein can be altered by changing the solubilizing environment. As previously mentioned, hydrophobic interactions with a good hydrophobic-hydrophilic balance as well as slight repulsive electrostatic forces were critical for micelle formation and interaction (Burgess, 1991).

Ismond *et al.* (1986a) identified a strong micelle response from fababean vicilin occurring at pH 6.0 to 6.8. Further micellization did not occur above the optimal pH range due to the changes in protein conformation as a result of the increase in net negative surface charge. Furthermore, a decrease in surface hydrophobicity, indicating that a reduction of exposed nonpolar residues occurred at higher pH values, resulted in fewer micelle interactions.

Ismond *et al.* (1986b) selected various salts to study the influence of hydrophobic interactions on micelle formation. Ismond *et al.* (1986b) concluded that nonchaotropic salts were inadequate to promote extensive hydrophobic associations between micelles as the nonpolar residues were buried within the protein molecule. Moderately stabilizing salts (NaC$_3$H$_6$O$_7$, NaBr and NaCl) were shown by Ismond *et al.* (1986b) and Georgiou (1987) to be the best environments to produce highly interactive networks from fababean micelles ($\mu \leq 1.0$). In these situations, the hydrophobic-hydrophilic forces were balanced.

C. Hydrolysis of Proteins

1. Hydrolysis as a Tool to Modify Proteins

Many food proteins, particularly those from plant sources, require modification to improve such functional properties as solubility, emulsification and others (Shih, 1992). Modification of protein functionality can also make food products better suited for human nutritional utilization, therefore increasing the world's food supply (Hamada, 1992). As a result, Hamada (1992) concluded that the purpose of protein modification was to create new and unique products that would possess better functional properties in food systems than the unmodified protein.

Chemical and enzymatic hydrolysis are the two most popular forms of protein modification. The final product, referred to as a protein hydrolysate, is defined as a mixture containing amino acids and other substances such as salt and peptides, obtained by the hydrolysis of plant or animal proteins (Olsman, 1979).

According to Olsman (1979), industrial interest in protein hydrolysates grew sharply after Ikeda's discovery in 1908 of monosodium glutamate (MSG) as the major flavouring compound in protein hydrolysates. These hydrolysates were found to be relatively rich and cheap sources for the isolation of MSG.

Industrially, two basic hydrolysis methods are used : acid and enzymatic hydrolysis.

2. Mild Acid Hydrolysis

Acid hydrolysis can be employed as a tool to improve protein functionality as well as to produce flavouring materials. According to Shih (1992), peptide bonds on either side of aspartic acid may be cleaved at a rate 100 times greater than other peptide bonds. Fig. 2 illustrates the possible pathways for the release of aspartic acid from proteins. Cleavage of the N-peptide bond proceeds via an intermediate containing a six-membered ring, whereas fission of the C-peptide bond proceeds via a five-membered ring (Inglis, 1983). Therefore, this easy release of aspartic acid under mild acidic conditions provides control of the peptide bond hydrolysis and hence benefits the development of protein functionality.

From a negative perspective, Shih (1992) stated excessive peptide bond hydrolysis is undesirable because it could release bitter and off-flavour peptide components. Moreover, retaining macromolecular characteristics is of prime importance for the protein ingredient to function effectively in food systems. Too great a decrease in molecular size could result in reduced functionality. According to Shih (1992), reaction

FIGURE 2. Possible pathways for the release of aspartic acid from proteins. (Inglis, 1983)

$$-HCONHCHCONH- \\ \quad\quad CH_2C_ONH_2$$

$$-CONHCHCONH-$$

Left bracket structure:

$$\left[\begin{array}{c} H \\ O \quad N \\ -C \quad\quad CHCONH^- \\ O \quad CH_2 \\ C \\ O \end{array}\right]$$

Right bracket structure:

$$\left[\begin{array}{c} OH \\ -CONHCHC-NH- \\ | \quad\quad O \\ CH_2CO \end{array}\right]$$

Fission | N-peptide bond

C-peptide | Fission
bond

Bottom left bracket:

$$\left[\begin{array}{c} H \; H \\ N \\ -CO \quad CHCONH \\ O \quad CH_2 \\ C \end{array}\right] \xrightarrow{H_2O}$$

Middle bottom:

$$-CO + NH_2CHCOOH + NH_2^- \\ OH \quad\quad CH_2COOH$$

Bottom right:

$$\xleftarrow{H_2O} \left[\begin{array}{c} -CONHCHCO \\ | \quad\quad O \\ CH_2CO \end{array} + NH_2^- \right]$$

17
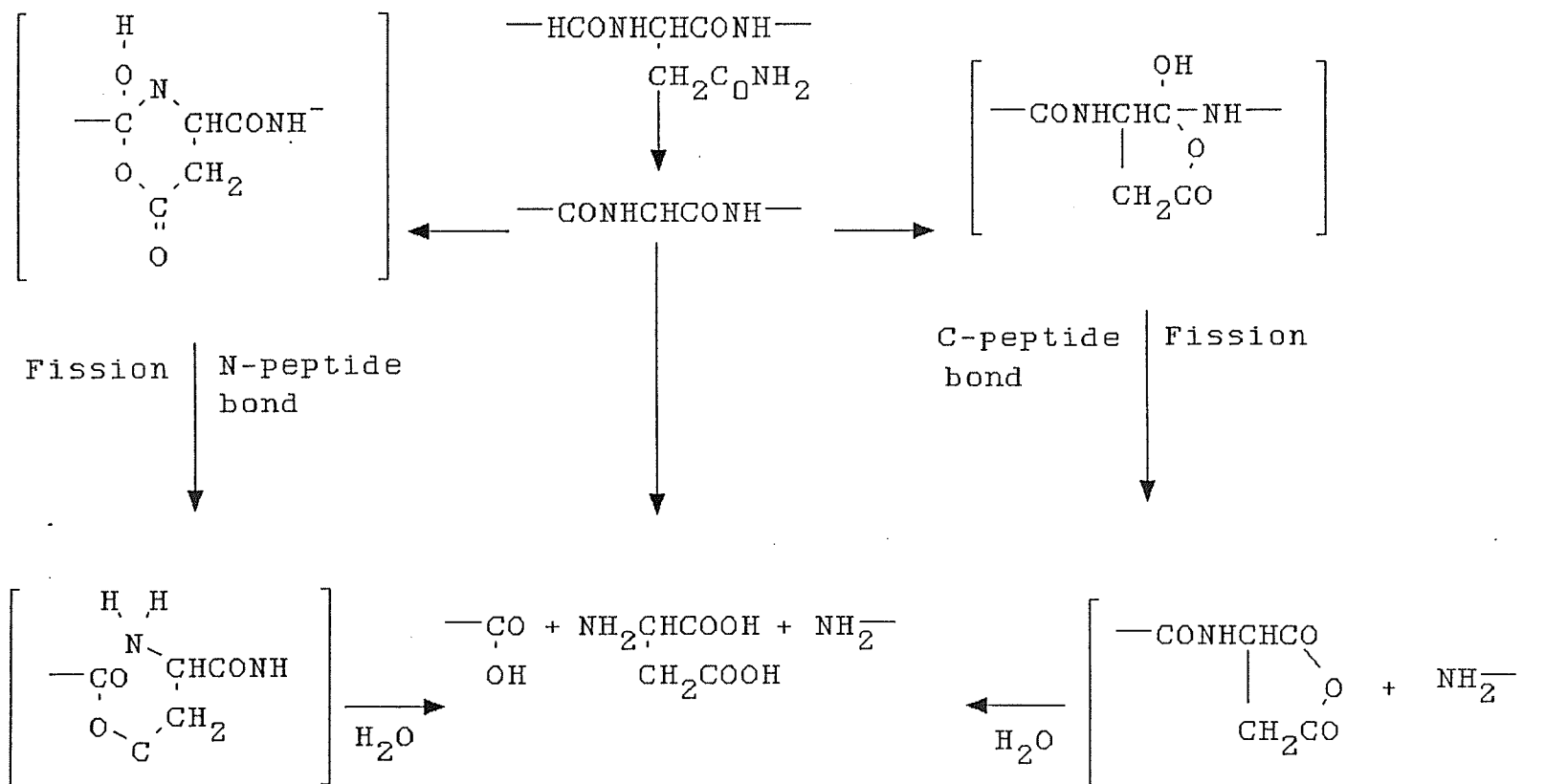
conditions have to be carefully controlled to prevent excess hydrolysis. The hydrolysis is preferentially limited to less than 4% of the peptide bonds in the protein. Under mild conditions for limited hydrolysis and with very low level peptide bond hydrolysis, both amide bond hydrolysis and its effect on the functional properties of the protein could be significant (Shih, 1992).

During mild acid hydrolysis of the protein, peptide bond hydrolysis seldom occurs without deamidation and vice versa. The mechanisms for the release of aspartic acid and ammonia and sequence and size of amino acids in the deamidated substrate have an influence on the conversion products for deamidation. Of all factors, pH seems to have the major control over the deamidation. The mechanism of deamidation is illustrated in Fig. 3. The reaction proceeds via hydrolysis of asparagine residues to aspartate residues.

According to Matsudomi *et al.* (1985), treatment of the protein under mild acid conditions normally results in significant deamidation (10-20%) but low peptide bond hydrolysis (<7%). Therefore, many researchers consider mild acid hydrolysis an effective method to achieve deamidation. This is mainly responsible for the ensuing changes in physical and functional properties of the protein.

3. **Enzymatic Hydrolysis**

The enzyme-catalysed process of hydrolysis as applied to protein containing raw materials used by the food industry has been the object of intense study (Petersen, 1991). Many of the uses for proteolytic enzymes in foods are involved in either changing the functional properties of the protein, or for nutritional purposes. In some cases, enzymes

FIGURE 3. Mechanism of deamidation. (Inglis, 1983)

20

$$\sim\!\!\sim\!\!\sim\!\!\sim-NH-CH-\underset{\|}{\overset{O}{C}}-NH-\underset{\overset{|}{R}}{CH}-\underset{\|}{\overset{O}{C}}-\sim\!\!\sim\!\!\sim\!\!\sim$$

$$CH_2-\underset{\|}{\overset{}{C}}-NH_2$$
$$O$$

↓

$$\sim\!\!\sim\!\!\sim\!\!\sim-NH-CH-\underset{\|}{\overset{O}{C}}-NH-\underset{\overset{|}{R}}{CH}-\underset{\|}{\overset{O}{C}}-\sim\!\!\sim\!\!\sim\!\!\sim$$

$$CH_2-\underset{\|}{\overset{}{C}}-OH$$
$$O$$
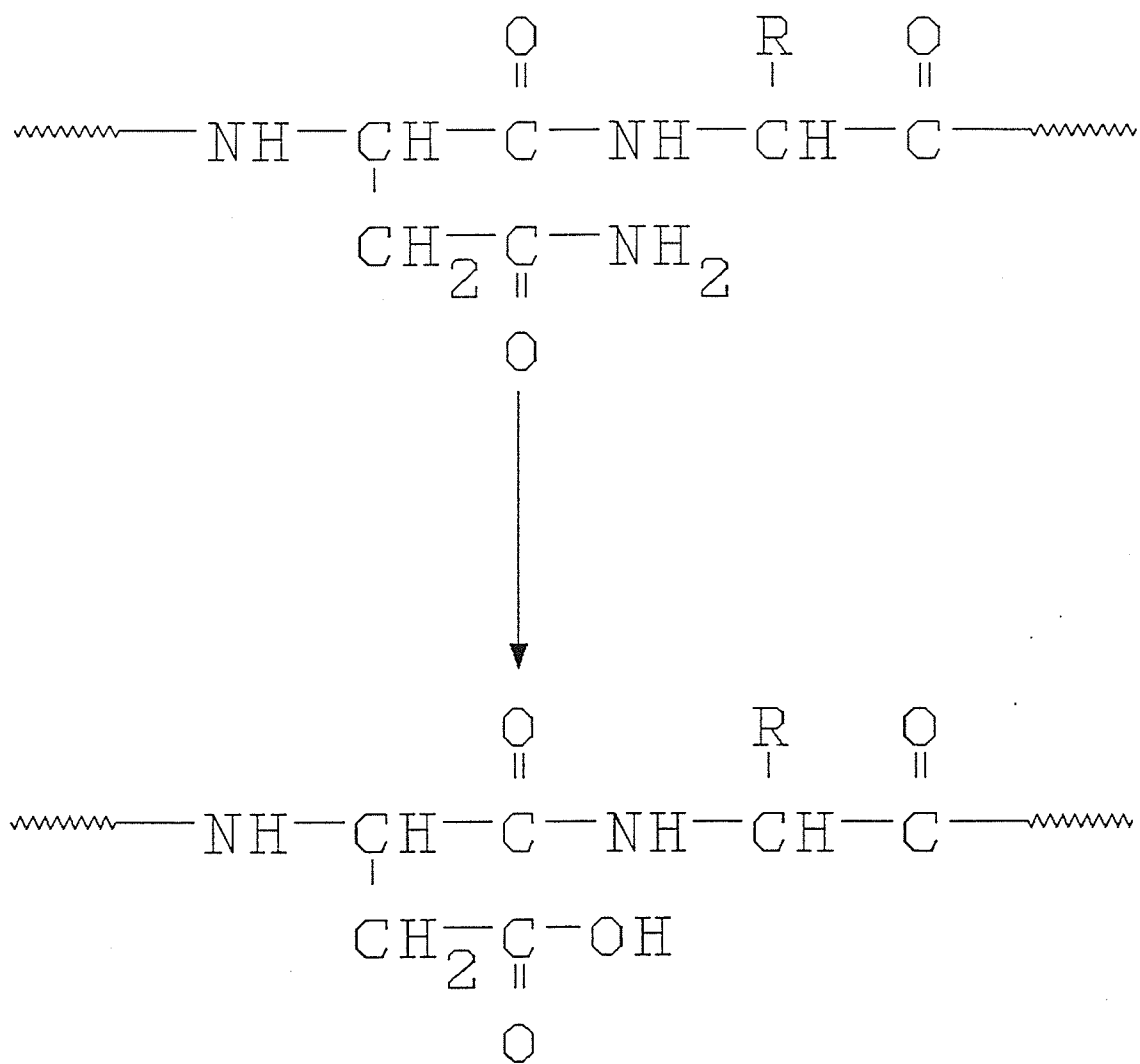
are also used to change the flavour of the products (Petersen, 1991). According to Hamada (1992), chemical modification of food proteins (such as acid hydrolysis) is not very desirable for food applications because of the harsh reaction conditions, non-specific chemical reagents, and the difficulty of removing residual reagents from the final product. On the other hand, enzymes provide several advantages including rapid reaction rates, mild conditions and, most importantly, high specificity.

a. **Degree of Hydrolysis (DH).** According to Adler-Nissen (1986), it is important to have a measure for the extent of the hydrolytic degradation. Moreover, the number of peptide bonds cleaved during the reaction is the entity which most closely reflects the catalytic action of proteases. In general, DH is defined as follows :

$$DH = ((\text{number of peptide bonds cleaved/total number of peptide bonds}) * 100\ \%$$

b. **Enzymes.** Enzymes used in food processing have at least one common characteristic : they have to be food grade and, if they are of microbial origin, the producing organism has to be non-pathogenic. Proteases are classified according to their source (animal, plant, microbial), their catalytic action (endopeptidases or exopeptidases), and the nature of the catalytic site. According to Adler-Nissen (1986), endopeptidases are always used in food protein hydrolysis. There are four major classes of endopeptidases of interest in food protein hydrolysis : serine proteases, cysteine proteases, metalloproteases, and aspartic proteases.

4.   The Impact of Protein Hydrolysis on Functional Properties

As mentioned earlier, acid and enzymatic hydrolysis methods can be used to modify the functional properties of food proteins. This area has been the subject of intense research with soy bean protein as the most studied example.

Unmodified soy protein has a low solubility in the neighbourhood of the isoelectric pH of the protein (pH 3-5). Petersen (1991) treated soy protein with Alcalase at a enzyme-substrate ratio of 2% at 50°C with the optimum pH controlled at 8.0. From this experiment, he concluded that the solubility of soy protein increased with a DH of 8% or above. Matsudomi et al. (1985) treated 2% soy protein solution with 0.05 N HCl at 95°C for 30 min and proposed there were significant changes in conformation and improved solubility. Bernardi Don et al. (1991) treated soy protein concentrate from toasted flour with fungal and bacterial proteases and found the solubility was improved.

The ability of a protein to form stable oil-in-water emulsions is one of the most useful functional properties of food proteins. Petersen (1991) found that emulsifying capacity was maximum at a DH of 5%. Matsudomi et al. (1985) concluded from their research that emulsifying activity was improved by mild acid treatment. Bernardi Don et al. (1991) using hydrolysed soybean protein found that the emulsifying capacity was unchanged and emulsion stability was decreased. Improved emulsifying capacity was confirmed by Kim et al. (1990) after treating soy protein isolate with trypsin (enzyme-substrate ratio of 2%, pH 8.0 and 37°C) for 30 min.

Petersen (1991) stated that foaming capacity was increased at least by 10 times with a DH of 3%. Bernardi Don et al. (1991) also concluded the foaming capacity and

the foaming stability were improved. Puski (1975) found that the foaming capacity of soy protein isolate treated with fungal protease was greatly improved; however, the foaming stability was very low.

The ability to bind oil is another important functional property of food proteins. Bernardi Don *et al.* (1991) concluded that treatment of soy protein with a bacterial protease improved oil absorption greatly. In addition, water binding capacity is essential for food protein applications. Puski (1975) concluded that fungal enzyme treated soybean proteins had slightly increased water absorption. However, Bernardi Don *et al.* (1991) noted a decreased water absorption capacity with the fungal protease treatment.

III. MATERIALS AND METHODS

A. Isolation of Canola Protein by the Protein Micellar Mass Procedure

Defatted canola meal (*Brassica napus*) was obtained from Northern Lite Canola in SexSmith, Alberta. A canola protein fraction was isolated based on the methodology of Burgess (1991) with some modifications. Canola meal was ground in a Micro-mill (Chemical Rubber Co., Cleveland, Ohio) for 30 sec and the ground meal samples were stirred in 0.1 M NaCl (1:10 ratio) for 4 h, and then centrifuged in 1 L centrifuge bottles for 30 min at 2,300 g (4°C) using a Sorvall Refrigerated Centrifuge, Model RC-3 (DuPont Co., Wilmington, Delaware). The precipitated pellets were discarded and the resultant supernatant containing the solubilized protein was filtered using Whatman No. 1 filter paper under vacuum in order to remove any debris. The supernatant was then concentrated using a 10^4 molecular weight cut-off Spiral Ultrafiltration (UF) cartridge in an Amicon UF unit (model RA2000, Oakville, Ontario) operating under a pressure of 30 psi. Using this method, the volume of supernatant was reduced at least eight times.

The concentrated protein solution was diluted by six times its volume with cold distilled water. The diluted protein solution was stored at 4°C for 12-16 h, to allow the insoluble protein micelles to precipitate. The protein micelles were recovered by centrifugation at 2,300 g for 30 min (4°C). The supernatant was discarded and the precipitated protein was frozen and freeze-dried for 72 h. The resultant protein micellar mass (PMM) was stored at 4°C.

B. Proximate Analysis

1.   Moisture Content Determination

The moisture content was determined for canola meal and PMM using a vacuum oven as described in the AOAC Official Methods (AOAC, 1975).

2.   Fat Content Determination

Fat content was determined for canola meal and PMM as described in the AOAC Official Methods (AOAC, 1975).

3.   Total Ash Determination

Total ash content was analyzed for canola meal and PMM as described in the AOAC Official Methods (AOAC, 1975).

4.   Protein Determination

The protein content of canola meal and PMM was determined by a micro-Kjeldahl method as described in the AOAC Official Methods (AOAC, 1975).

C. Preparation of Protein Hydrolysates

1.   Enzymatic Hydrolysis

a. Trypsin. The methodology of Kim *et al.* (1990) for trypsin hydrolysis was used with some modifications. A suspension of 20% (w/v) PMM was adjusted to pH 8.0 using 1 N NaOH. The suspension was subsequently heated in a water bath. When the suspension reached the optimum temperature (37°C), the trypsin solution (2% of PMM, w/w) was added. Prior to addition, the trypsin was dissolved in distilled water at a ratio

of 1:200 (w/w). The suspensions were incubated for 5, 10, 15 and 20 min, respectively, and then were heated at 87°C for 5 min to inactivate the trypsin. The hydrolysates were adjusted to pH 5.5, frozen,freeze-dried, ground, and stored at 4°C. The trypsin hydrolysates were designated as T5, T10, T15 and T20.

b. α-Chymotrypsin. The same procedure for chymotrypsin hydrolysis was used as for trypsin. The chymotrypsin hydrolysates were referred to as C5, C10, C15 and C20.

c. Fungal Protease. An *Aspergillus oryzae* protease was used to hydrolyse the PMM. The same hydrolysis procedure was used as for trypsin with some variation. Prior to hydrolysis, the PMM suspension was adjusted to pH 7.0. The optimum temperature for enzyme incubation was 45°C (Bernardi Don *et al.*, 1991). After incubation for 5, 10, 15 and 20 min, respectively, the suspensions were heated at 75°C for 10 min to inactivate the protease. The fungal protease hydrolysates were designated as F5, F10, F15 and F20.

d. Bacterial Protease. A *Bacillus subtilis* protease was used to prepare the bacterial hydrolysates. The same hydrolysis procedure was used as for trypsin with some modification. The hydrolysis was carried out at an optimum pH of 7.0 and with an optimum temperature of 50°C (Bernardi Don *et al.*, 1991). The suspensions were incubated for 5, 10, 15 and 20 min, respectively, and then heated at 75°C for 10 min to inactivate the protease. The bacterial protease hydrolysates were referred to as B5, B10,

B15 and B20.

2. Acid Hydrolysis

a. Mild Acid Treatment. Acid hydrolysates were prepared according to the method used by Matsudomi *et al.* (1985) with some modification. A 2% PMM solution in 0.05 N HCl was heated in a water bath at 95°C for 10, 20, 30 and 40 min (hence the designations A10, A20, A30 and A40). The acid-modified protein solution was adjusted to pH 5.5 with 1 N NaOH or HCl. The suspension was centrifuged for 30 min at 2,300 g (4°C). The precipitated protein was frozen, freeze-dried and stored at 4°C.

b. Alternate Acid Treatment. An alternate method was used to prepare the acid hydrolysates. The hydrolysates were prepared as in the mild acid treatment except the PMM solution was hydrolysed by refluxing for extended hours (1 to 8 h). The designations for these alternate acid hydrolysates were A1 to A8.

D. Degree of Hydrolysis

The degree of hydrolysis (DH) of each hydrolysate was estimated by measuring the nitrogen content soluble in 10% trichloroacetic acid (TCA) as described by Kim *et al.* (1990). A 0.5 g sample was dissolved in 50 ml of distilled water and 1 ml of the suspension was assayed for nitrogen by the micro-Kjeldahl method (AOAC, 1975). A 10 ml aliquot of the aqueous suspension of each hydrolysate (1% (w/v)) was mixed with 10 ml of 20% TCA and then centrifuged at 10,200 g for 30 min at 4°C using a Sorvall Refrigerated Centrifuge, model RC2-B (DuPont Co., Wilmington, Delaware). The

soluble nitrogen in the supernatant was assayed by the micro-Kjeldahl method. The %DH was expressed as :

$$\frac{10\% \text{ TCA-soluble N } \times 100}{\text{Total N}} = \%DH$$

E. Molecular Characteristics of PMM and Hydrolysates

1.   Molecular Weight Determination

a. Gel Filtration/High Performance Liquid Chromatography (HPLC). The molecular weights of PMM and all hydrolysates were assessed using gel filtration. Protein samples were solubilized in 0.03 M sodium citrate in 0.01 M sodium phosphate buffer at pH 8.0. This solution was also used as the mobile phase. Equivalent amounts of protein for the PMM, enzyme hydrolysates and acid hydrolysates were applied to the column. The protein content of each solution was measured by the Lowry *et al.* (1951) method using bovine serum albumin (Sigma) as a standard. Gel filtration was carried out on a Waters HPLC system (Mississauga, Ontario) consisting of one M-45 pump, one Model 6000 pump, a U6K injector and a Model 441 absorbance detector set a 280 nm. The column used was a 250 x 4.6 mm Brownlee Aquapore OH300 SEC (size exclusion) gel filtration column. Retention times were determined using a Spectra-Physics 4270 integrator. Standards obtained from Bio-Rad Laboratories are given in Table 2. The molecular weight of each protein sample was estimated from the calibration curve (Appendix 1) established by plotting the log molecular weight of protein standards as a function of retention time.

TABLE 2. Bio-Rad protein standards used for gel filtration analysis

STANDARD	MOLECULAR WEIGHT (Dalton)
Thyroglobulin (bovine)	670,000
Gamma Globulin (bovine)	158,000
Ovalbumin (chicken)	44,000
Myoglobin (horse)	17,000
Vitamin B-12	1,350

b. Subunit Analysis with SDS-PAGE. The molecular weight of the major subunits for each protein sample were estimated according to the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) procedure described by Ng *et al.* (1988) with some modification. The stock solutions used for SDS-PAGE are outlined in Table 3 and the compositions of the 3.5% stacking gel and the 10% separating gel are given in Table 4. The Bio-Rad SDS protein standards are described in Table 5.

Protein samples were prepared in the extraction buffer at a concentration of 1 mg/ml. Clear extract (8μl) was loaded into each slot and electrophoresed at 50 mA/gel for 4 h. The gels were rinsed with rinsing solution twice in order to reduce opaqueness caused by precipitation of SDS in the gels. The gels were then stained for 12 to 18 h and destained for another 12 h. The gels were photographed using Kodak Tech pan film (ESTAR-AH Base). The molecular weight of the subunits of each sample was estimated from the calibration curve (Appendix 2) established by plotting the log molecular weight of protein SDS standards as a function of migration distance.

2. Isoelectric Point Determination by Isoelectric Focusing

The isoelectric point for each protein sample was determined by procedures described by Winter and Anderson (1977). The composition of stock solutions used for isoelectric focusing are outlined in Table 6 and the Pharmacia protein standards (with a pH range of 3.5-9.3) are given in Table 7.

Protein samples were solubilized in 0.03 M sodium citrate in 0.01 M sodium phosphate buffer at pH 8.0. Solutions of PMM, enzyme hydrolysates and acid

TABLE 3. Stock solutions used for SDS-PAGE

SOLUTION		SOLUTION COMPOSITION
Tris-HCl Buffer **(1.0 M, pH 8.8)**	30.28 g 200 ml	Tris distilled water adjusted the pH to 8.8 with 6 N HCl and adjusted the final volume to 250 ml with distilled water.
Tris-HCl Buffer **(1.0 M, pH 6.8)**	12.11 g 60 ml	Tris distilled water adjusted the pH to 6.8 with 6 N HCl and adjusted the final volume to 100 ml with distilled water.
Separating Gel **Buffer**	9.93 g 0.07 g 0.005 g 37.5 ml	acrylamide bisacrylamide SDS Tris-HCl Buffer pH 8.8 adjusted to 100 ml with distilled water.
Stacking Gel **Buffer**	3.45 g 0.049 g 0.1 g 12.5 ml	acrylamide bisacrylamide SDS Tris-HCl Buffer pH 6.8 adjusted to 100 ml with distilled water.
Ammonium Persulfate **Solution (1%)**	0.1 g	ammonium persulfate diluted to 10 ml with distilled water. Prepared fresh prior to use.

TABLE 3. (Cont'd)

SOLUTION	SOLUTION COMPOSITION	
Extracting Buffer	20 mg	pyronin y
	4 g	SDS
	12.5 ml	stacking gel buffer
	20 ml	glycerol
	24.1 ml	distilled water
Electrode Buffer	24.23 g	Tris
	115.31 g	glycine
	80 ml	10% SDS solution
		made up to 8 L with distilled water.
Rinsing Solution	100 ml	100% (w/v) TCA
	330 ml	methanol
	570 ml	distilled water
Staining Solution	1 g	Coomassie Brilliant Blue G-250 dissolved in 500 ml of distilled water.
	500 ml	2 N H_2SO_4
		Allowed to stand for 4 h and filtered
	100 ml	10 N KOH
	140 ml	100% (w/v) TCA
		The mixture was filtered and stored at room temperature.
Destaining Solution	300 ml	ethanol
	100 ml	acetic acid
	600 ml	distilled water

TABLE 4.   Composition of 3.5% stacking gel and 10% separating gel for SDS-PAGE

	VOLUME (ml)	SOLUTION
3.5 % Stacking Gel	7.5	stacking gel buffer
	0.16	1% Ammonium persulfate
	0.008	N,N,N,N Tetramethyl ethylene diamine (TEMED)
10 % Separating Gel	30.0	separating gel buffer
	0.62	1% Ammonium persulfate
	0.031	TEMED

TABLE 5. Molecular weights of Bio-Rad SDS-PAGE standards

STANDARD	MOLECULAR WEIGHT (Dalton)
Myosin	200,000
β-galactosidase	116,000
Phosphorylase B	97,000
Bovine serum albumin	66,200
Ovalbumin	45,000
Carbonic anhydrase	31,000
Trypsin inhibitor	21,500
Lysozyme	14,400
Aprotinin	6,500

TABLE 6. Solutions used for the staining of isoelectric focusing bands.

SOLUTION		SOLUTION COMPOSITION
Fixing Solution	57.5 g 17.25 g	trichloroacetic acid sulphosalicyclic acid adjusted to 500 ml with distilled water
Destaining Solution	500 ml 160 ml	ethanol acetic acid adjusted to 2000 ml with distilled water
Staining Solution	0.46 g 400 ml	Coomassie Brilliant Blue R-250 destaining solution mixed and filtered through Whatman No. 1 filter paper
Preserving Solution	300 ml 40 ml	destaining solution glycerol

TABLE 7.   Isoelectric points of the Pharmacia protein
standards used for isoelectric focusing

STANDARD	pI (24°C)
Amyloglucosidase	3.50
Soybean trypsin inhibitor	4.55
β-lactoglobulin A	5.20
Carbonic anhydrase B (bovine)	5.85
Carbonic anhydrase B (human)	6.55
Myoglobulin (acidic, horse)	6.85
Myoglobulin (basic, horse)	7.35
Lentil lectin (acidic)	8.15
Lentil lectin (middle)	8.45
Lentil lectin (basic)	8.65
Trypsinogen	9.30

hydrolysates were prepared at equivalent protein concentrations (1mg/ml). The protein content of each solution was assayed by the Lowry *et al.* (1951) method using bovine serum albumin (Sigma) as a standard. Isoelectric focusing of the protein samples was carried out on an LKB 2117 Multiphor apparatus (Sweden) with an LKB 2197 Constant Power Supply. A Haake circulating water bath (West Germany) was used to control the temperature at 10°C. An LKB Ampholine PAG plate polyacrylamide gel with a pH range of 3.5 to 9.5 was used. After the PAG plate was positioned on the Multiphor apparatus, one electrode strip (soaked in 1 M H_3PO_4) was situated at the anode while another electrode strip (soaked in 1 M NaOH) was placed at the cathode. A 10 μl aliquot of each of the protein samples and the protein standards were applied on the surface of the gel.

In order for the migration of the proteins to their isoelectric points to occur, the gel was focused at a constant power of 30 W but varying amperage (mA) and voltage (V). The gel was focused for 1.5 h and the electrode strips were removed immediately after the completion of focusing. The gel was fixed for 30 min, destained for 5 min, stained at 60°C in an air oven for 20 min, destained for 24 h, and preserved for 1 h. The gel was dried at room temperature for 30-45 min and a plastic sheet was then carefully placed over the sticky gel surface. The gels were photocopied using a Kodak 1550 Coloredge copier (Rochester, New York).

A calibration curve (Appendix 3) was established by plotting the isoelectric points of the standards as a function of the migration distance from the cathode. The isoelectric points of the protein samples were determined by estimating the distance each band

moved from the cathode.

3.   Protein Conformation Assessment

a. Differential Scanning Calorimetry (DSC) Analysis. Conformational changes of PMM and all hydrolysates were examined by measuring the thermal properties of the proteins using a DuPont 990 Thermal Analyzer with a 910 Differential Scanning Calorimeter cell base (Westtec Industrial Ltd., Mississauga, Ontario).

The DSC analysis procedure of Burgess (1991) was used with some variation. Protein samples were prepared at 20% (w/w) and samples weighing 10-15 mg were hermetically sealed in DuPont aluminum pans, coated on the interior with an inert polymer. A silicon heat-sink compound (Dow Corning Corp., Midland, Michigan) was used to improve contact between pans and thermocouple detectors. The reference (a sealed empty pan) and the sample pan were placed in the DSC cell. The heating rate of the sample was 10°C/min over a temperature range of 25°C to 120°C. The cell was cooled to room temperature with ice after each run. Each protein sample was analyzed in triplicate.

The DSC Standard Data Analysis Program (Version 2.2C) was used to collect and analyze the thermal data. The results were expressed as thermal curves with heat flow as a function of temperature. From the thermal curve, the temperature of maximum heat flow into the sample or thermal denaturation temperature (Td) and the area of the endothermic thermal curve or enthalpy of denaturation (ΔH) were determined. After analysing the thermal properties of the protein, the thermal curve was plotted by a

Hewlett Packard Model HP7470A graphics plotter.

b. Surface Hydrophobicity. The methodology of Georgiou (1987) was used to determine the surface hydrophobicity. Each protein sample was serially diluted with 0.01 M sodium acetate in 0.01 M phosphate buffer pH 6.0 to obtain a range of protein concentrations from 0.02 to 0.6 mg/ml.

Two fluorescence probes were used: 1-anilino-8-naphthalenesulfonate (ANS, Sigma) and cis-parinaric acid (CPA, Calbiochem.). The ANS solution was prepared according to the method of Hayakawa and Nakai (1985); specifically, 8 mM magnesium ANS was dissolved in 0.02 M phosphate buffer, pH 7.4. This ANS solution was used to determine the aromatic hydrophobicity of the protein samples. A solution of CPA was prepared by the procedure of Kato and Nakai (1980); this consisted of equimolar (3.6 mM) CPA and butylated hydroxytoluene in ethanol. This CPA solution was used to estimate the aliphatic hydrophobicity of the protein samples.

Relative fluorescence intensity (RFI) was measured with a Perkin-Elmer LS-5 fluorescence spectrophotometer (Coleman Instruments Division, Oak Brook, Illinois) using a slit width of 0.5 nm and a fixed scale of 1.0. Temperature was controlled at 20°C with a Haake-G water bath. Wavelengths of excitation and emission were 390 and 470 nm for ANS and 325 and 420 nm for CPA. A 10 μl aliquot of ANS or CPA was added to 2 ml of each sample. The net fluorescence intensity (FI) at each protein concentration was determined by subtracting the fluorescence intensity of each solution without probe from that with the probe. The initial slope of a plot of fluorescence

intensity as a function of protein concentration was used as an index of protein surface hydrophobicity (So).

F. Impact of Hydrolysis on the Functional Properties of Hydrolysates

The functional properties of PMM and all hydrolysates were determined so that the effects of protein hydrolysis on functional properties could be examined.

1. Nitrogen solubility

a. At pH 4.5. The nitrogen solubility at pH 4.5 of all protein samples was estimated by the method of Kim *et al.* (1990) with some modification. Protein samples were dissolved in 0.1 N NaOH at a concentration of 1% (w/v) and the pH was adjusted to 4.5 with 0.1 N HCl. After stirring for 1 h, the suspension was centrifuged at 10,200g using a Sorvall Refrigerated Centrifuge, Model RC2-B (DuPont Co., Wilmington, Delaware), for 20 min at 4°C. The protein in the supernatant was determined by the Lowry *et al.* (1951) method. The percent solubilities of all hydrolysates were expressed as :

$$\frac{\text{Protein content of the hydrolysate supernatant} \times 100}{\text{Protein content of the PMM supernatant}} = \% \text{ solubility}$$

b. At pH 7.0. The same procedure as the nitrogen solubility at pH 4.5 was used with the exception that all the protein solutions were adjusted to pH 7.0 with 0.1 N HCl.

2. Water Holding Capacity

The water holding capacity (WHC) of all protein samples was measured according

to the method of Thompson *et al.* (1982). A protein sample (0.5g) and distilled water (3 ml) were mixed in a pre-weighed 15 ml conical graduated centrifuge tube. The suspension was held for 30 min with occasional stirring, followed by centrifugation at 1,760g using a Sorvall GLC-1 centrifuge (Dupont Co., Wilmington, Delaware) for 20 min. The supernatant was discarded and the tube was re-weighed. The %WHC was defined as :

$$\frac{\text{Weight of water bound} \times 100}{\text{Weight of sample}} = \% \text{ WHC}$$

3. Fat Absorption Capacity

The procedure of Lin and Humbert (1974) was used to estimate the fat absorption capacity (FAC) of all protein samples. A 0.3 g sample was added to 3 ml of corn oil in a 15 ml conical graduated centrifuge tube. The contents were stirred for 1 min with a thin brass wire to disperse the sample in the oil. After a holding period of 30 min, the tube was centrifuged for 1,760g using a Sorvall GLC-1 centrifuge for 25 min and the sample was reweighed. The %FAC was defined as :

$$\frac{\text{Weight of oil bound} \times 100}{\text{Weight of sample}} = \% \text{ FAC}$$

4. Emulsion Stability

The method of Lee *et al.* (1982) was used with some modification to measure the emulsion stability (ES). Protein samples of 5% (w/v) were prepared in 0.1 M sodium phosphate buffer pH 7.0. These protein samples (30ml) were added into an Omnimixer

(Ivan Sorvall Inc., Norwalk, Connecticut) container with 10 ml of corn oil. The mixture was homogenized for 1 min at a setting of 7. Immediately after mixing, 10 ml of emulsion were placed into a 50 ml centrifuge tube and another 5 ml of emulsion were pipetted into a pre-weighed aluminum dish. The moisture content of this emulsion was analyzed and was designated as Mo. After holding the 10 ml emulsion for 30 min at room temperature, 5 ml of emulsion were carefully pipetted from the bottom into a pre-weighed aluminum dish. The moisture content of this emulsion was also analyzed and the moisture content was designated as M_{test}. Emulsion stability was defined as:

$$\frac{100 - M_{test}}{100 - Mo} \times 100 = \% \text{ Emulsion Stability}$$

5.   Foaming Capacity and Stability

The method of Puski (1975) was used to measure the foaming capacity (FC) and the foaming stability (FS). Protein solutions (5%, w/v) were prepared in 0.1 M sodium phosphate buffer at pH 7.0. The solutions (50ml) were homogenized with an Omnimixer for 1 min at a setting of 7. After the mixing, the contents were poured immediately into a graduated cylinder and the volume of foam was recorded. After standing for 1 h at room temperature, the volume of remaining foam was assessed. Foaming capacity was defined as follows :

$$\frac{\text{Foam volume immediately after mixing}}{\text{Starting volume of liquid phase}} \times 100 = \% \text{ Foaming Capacity}$$

Foaming stability was defined as :

$$\frac{\text{Foam volume after standing for 1 h} \times 100}{\text{Foam volume immediately after mixing}} = \% \text{ Foaming Stability}$$

G. Statistical Analysis

All statistical analysis were performed on a HP 9000/380 microcomputer using SAS statistical analysis software program package (SAS Institute, 1990). Significant differences among treatments were determined by Duncan's multiple range test ($p \leq 0.05$). Correlation analysis between all data was carried out using the procedure corr (correlation analysis).

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IV. RESULTS AND DISCUSSION

A. Proximate Analysis

Proximate analyses were performed on the starting canola meal from *B. napus* and the PMM. The purpose of this analysis was to evaluate how the moisture, fat, ash and protein contents were influenced by the PMM procedure. The proximate analysis results are given in Table 8. Canola meal contained 7.4% moisture, 4.2% fat, 6.4% ash and 32.5% protein (N x 5.67). As mentioned previously, the protein content of canola, ranging from 11-42%, is influenced by genetic and environmental factors (Mieth *et al.*, 1983). The protein content of canola meal reported by Welsh (1989) and Burgess (1991) was 34.5% and 35.6% (*B. napus* in both studies), respectively. Therefore, the experimental value of 32.5% protein was comparable to these two findings and was within the range of percentages observed in other literature (Mieth *et al.*, 1983; Appelqvist and Ohlson, 1972). The ash content of canola in the literature varied with the species or cultivar of the sample. Appelqvist and Ohlson (1972) observed the ash content ranged from 7.0% to 7.5% (in *B. napus* and *B. campestris*, respectively) and Burgess (1991) found an ash value of 9.94%. Bell (1989) reported a similar value of 6.3% (*B. napus*) to that of 6.4% ash observed in this study. Bell (1989) and Burgess (1991) reported values of 9.0% and 9.95% for moisture contents, respectively. A lower value of 7.4% was observed for moisture content for this study. The fat content of the meal was found to be 4.2% in this study and this was comparable to the findings of Bell (1989) and Burgess (1991) (3.7% and 4.4%, respectively).

TABLE 8. Proximate analysis of canola meal and PMM

COMPONENT	CANOLA MEAL (% dry weight basis)[1]		PMM
Moisture	7.4 ± 0.0		7.3 ± 0.1
Fat	4.2 ± 0.0		12.1 ± 0.4
Ash	6.4 ± 0.0		1.6 ± 0.2
Protein	32.5 ± 0.5		76.2 ± 1.5

[1] Each value represents a mean of two determinations.

The resultant PMM contained 7.3% moisture, 12.1% fat, 1.6% ash and 76.2% protein. Using the PMM protein isolation procedure, the fat content was concentrated by three times; this was probably due to the decrease in other components such as phenolics, phytic acid, glucosinolates and ash in the meal (ie. the possibilities of these components remaining soluble in the supernatant during the PMM procedure). The ash content of PMM was reduced by almost 75% and the protein content of PMM was concentrated by at least a factor of two.

B. Degree of Hydrolysis (DH)

Native globular proteins are generally resistant to hydrolysis and this can be explained by the compact tertiary structure of the protein which protects most of the peptide bonds (Adler-Nissen, 1976). In any quantitative work on protein hydrolysis it is necessary to have a measure for the extent of the hydrolytic degradation (Adler-Nissen, 1986). It should be kept in mind that the number of peptide bonds cleaved during the reaction is the entity which most closely reflects the catalytic action of proteases (in the case of enzyme hydrolysis) or the hydrolysis condition using acid or alkali.

The DH for all hydrolysates are given in Table 9 and the graphs of DH as a function of hydrolysis time for all hydrolysates are presented in Figs. 4, 5 and 6. The hydrolysis of PMM with trypsin, chymotrypsin, fungal protease and bacterial protease proceeded at a rapid rate during the initial 5 min and then decreased for the next 15 min Fig. 4). The DH values varied from 2.3% to 3.9% after 20 min of incubation, depending upon the enzymes involved.

TABLE 9.   The degree of hydrolysis (%) values for all hydrolysates.

SAMPLE	DEGREE OF HYDROLYSIS[1] (%)
T5	3.5 ± 0.1^a
T10	3.7 ± 0.1^a
T15	3.7 ± 0.1^a
T20	3.9 ± 0.1^a
C5	3.0 ± 0.1^a
C10	3.2 ± 0.2^{ab}
C15	3.5 ± 0.1^{ab}
C20	3.6 ± 0.1^b
F5	1.9 ± 0.1^a
F10	2.0 ± 0.1^a
F15	2.2 ± 0.1^a
F20	2.3 ± 0.1^a
B5	2.4 ± 0.1^a
B10	2.6 ± 0.1^a
B15	2.8 ± 0.1^a
B20	2.9 ± 0.1^a

[1]   Each value represents a mean of two determinations.
[2]   Values followed by the same letter are not significantly different $(p \le 0.05)$ within each hydrolysis group.

TABLE 9. (Cont'd)

SAMPLE	DEGREE OF HYDROLYSIS[1] (%)
A10	0.6 ± 0.0^a
A20	0.7 ± 0.1^{ab}
A30	0.8 ± 0.0^b
A40	0.8 ± 0.1^b
A1	0.2 ± 0.0^a
A2	0.2 ± 0.0^{ab}
A3	0.3 ± 0.1^{bc}
A4	0.3 ± 0.0^{bc}
A5	0.4 ± 0.0^{cd}
A6	0.5 ± 0.1^{de}
A7	0.6 ± 0.0^e
A8	0.6 ± 0.0^e

[1] Each value represents a mean of two determinations.

[2] Values followed by the same letter are not significantly different ($p \leq 0.05$) within each hydrolysis group.

FIGURE 4. The degree of hydrolysis (%) as a function of hydrolysis time (min) for all enzyme hydrolysates.
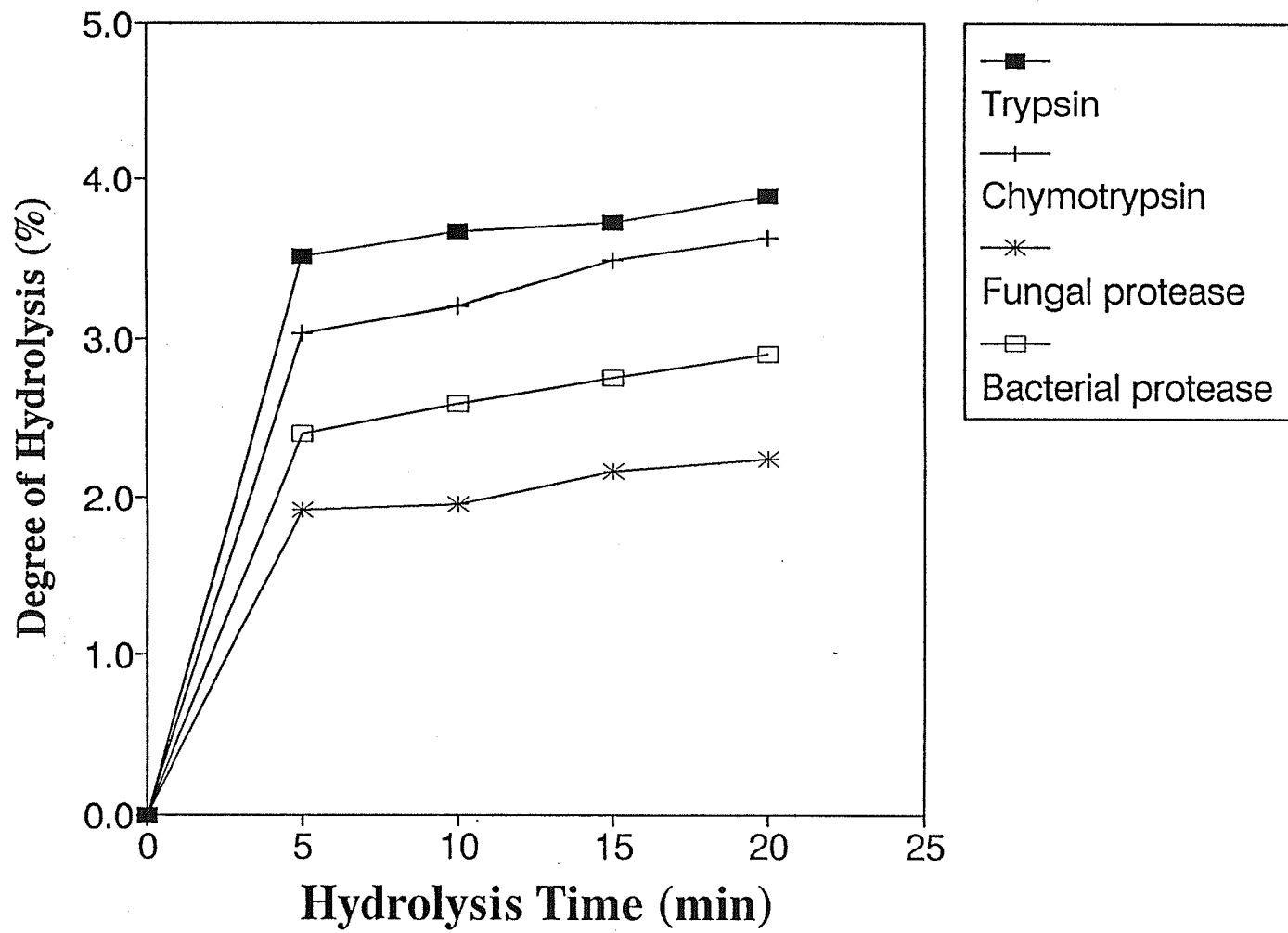
FIGURE 5. The degree of hydrolysis (%) as a function of hydrolysis time (min) for mild acid hydrolysates.
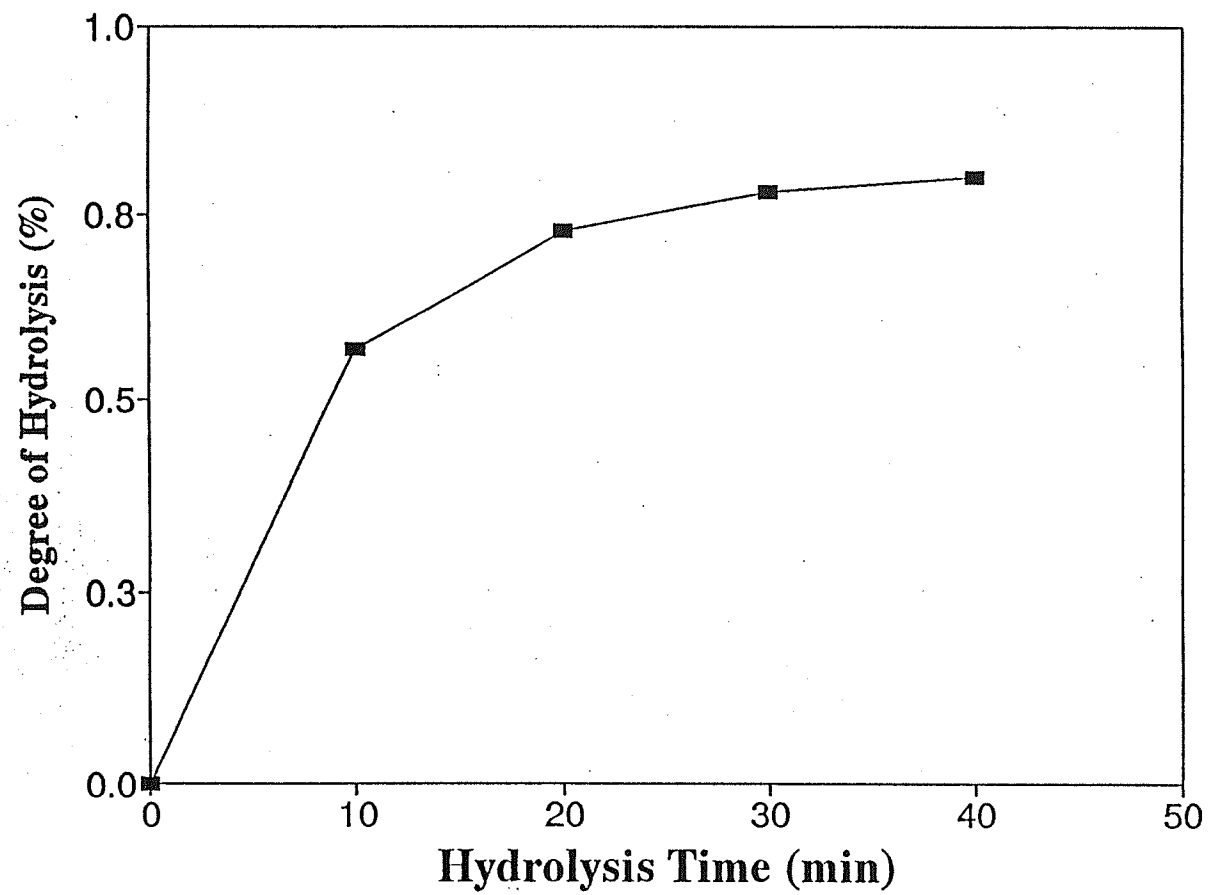
FIGURE 6. The degree of hydrolysis (%) as a function of hydrolysis time
(min) for alternate acid hydrolysates.
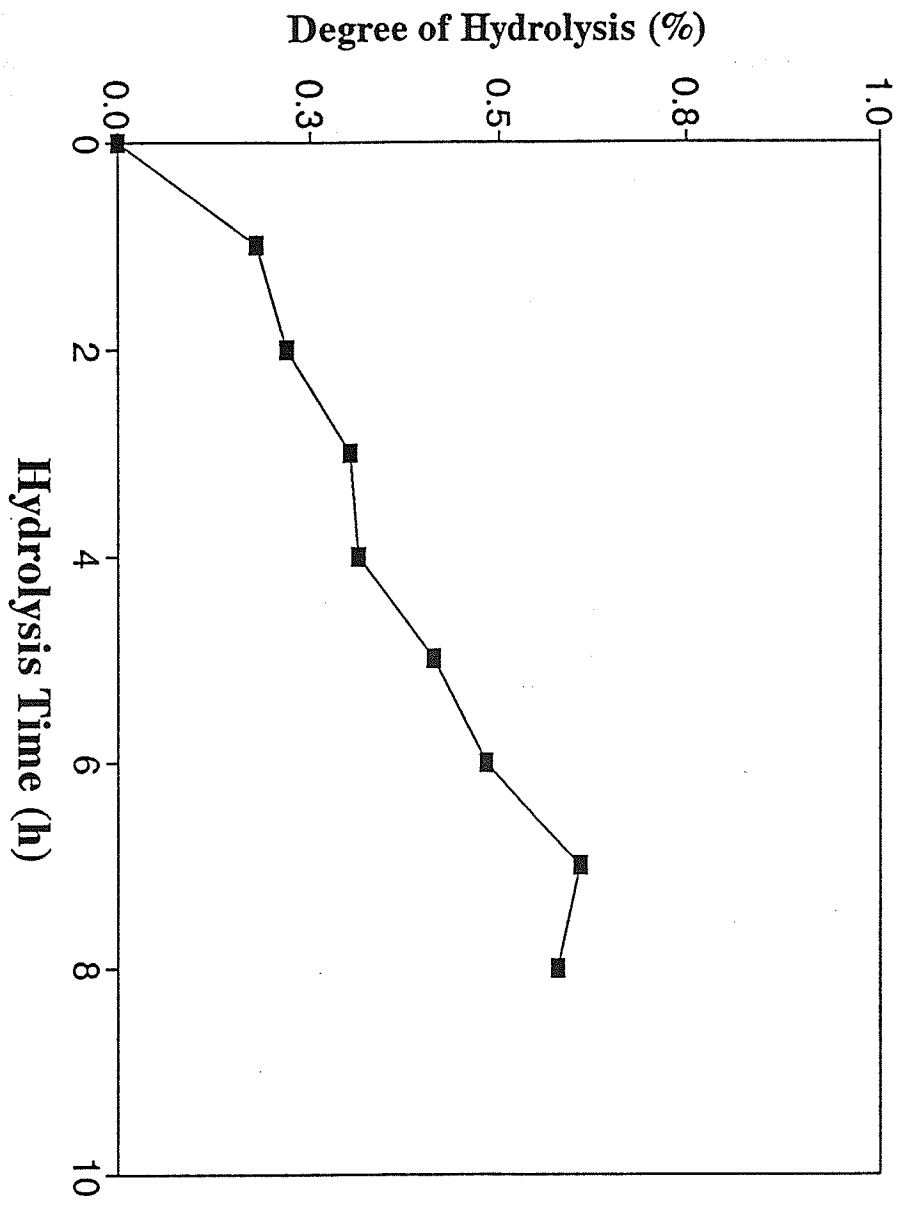
Trypsin showed the highest DH values among the enzymes used (Table 8). This agreed with Kim *et al.* (1990) although soy protein isolate was used as the substrate. However, much higher DH values of 20% were obtained by Kim *et al.* (1990) for trypsin hydrolysates (hydrolysis time of 30 min). The 12S globulins contain a number of basic amino acids and thus contain cleavage sites such as the carbonyl groups of lysine and arginine for trypsin to act on (Adler-Nissen, 1986). Burgess (1991) concluded that the 12S canola globulin was rich in glutamic acid, aspartic acid, valine, leucine and arginine but was low in cysteine, methionine, tryptophan and histidine. Chymotrypsin is known to preferentially cleave proteins at the carbonyl side of aromatic amino acids such as phenylalanine, tyrosine and tryptophan (Kimball *et al.*, 1981). Furthermore, it will also cleave after a number of other amino acids, including leucine, methionine, glutamine and asparagine. However, Kimball *et al.* (1981) concluded that many of the bonds in this second group are split more slowly and are not major sites of cleavage.

Bacterial protease (*B. subtilis*) is a mixture of metalloproteases and serine proteases with broad specificity and is known to release mainly terminal hydrophobic-COOH amino acids (Bernardi Don *et al.*, 1991). Fungal protease (*A. sojae*) is a mixture of aspartic, metallo, serine proteases and carboxypeptidases with a very broad specificity (Bernardi Don *et al.*, 1991).

From Appendix 4, the effect of hydrolysis time was found to be not significant on DH values for all hydrolysis methods except the alternate acid treatment (F=22.92, p=0.0111). When the PMM was hydrolysed for 1 h, the DH was found to be 0.2 and when the hydrolysis was proceeded for few hours further, the DH values increased

slightly. However, when the hydrolysis was carried out for 7 h or more, the DH values increased significantly (Table 8, Fig. 6).

Matsudomi *et al.* (1985) used a mild acid method to hydrolyse soy protein and they noticed that the electrophoretic properties of the protein did not show significant changes until heat treated for 30 min, but the high molecular fraction of the protein started decreasing to a lower molecular fraction at a heating time above 30 min. In this study, a mild acid method was used to hydrolyse the PMM for 10, 20, 30 and 40 min and the DH values for the four hydrolysates obtained were lower than 1%. The alternate acid hydrolysis using a reflux method for the hydrolysis of PMM also showed DH values below 1%. However, Shih (1992) concluded that hydrolysis is preferentially limited to less than 4% of the peptide bonds in the proteins.

Excessive peptide bond hydrolysis is undesirable due to the release of bitter and off-flavour peptide components. As mentioned earlier, retaining macromolecular characteristics is essential for the protein ingredient to function effectively in food systems, and too great a decrease in molecular size could lead to reduced functionality (Shih, 1992).

C. Molecular Characteristics

1. Molecular Weight Determination

a. Gel Filtration. Gel filtration profiles were examined to determine the effects of hydrolysis methods on the distribution of molecular weight. The molecular weights of PMM and all hydrolysates estimated by gel filtration are presented in Table 10.

TABLE 10. Molecular weights of PMM and all hydrolysates determined by gel filtration.

SAMPLE	MOLECULAR WEIGHT (Daltons)[1]	%
PMM	170800 ± 5000	92 ± 3
	7800 ± 100	8 ± 3
T5	170800 ± 5000	19 ± 2
	2900 ± 70	81 ± 2
T10	165400 ± 2500	26 ± 1
	2900 ± 40	74 ± 1
T15	165500 ± 2500	35 ± 2
	6400 ± 300	21 ± 2
	2900 ± 70	44 ± 1
T20	167200 ± 2500	32 ± 1
	5900 ± 200	22 ± 2
	2800 ± 80	46 ± 2
C5	172600 ± 6700	29 ± 4
	3200 ± 50	71 ± 4
C10	170800 ± 6700	22 ± 2
	2800 ± 180	78 ± 2
C15	172500 ± 2500	25 ± 1
	2800 ± 70	75 ± 1
C20	172500 ± 2500	20 ± 2
	2900 ± 40	80 ± 2

[1] Each value represents a mean of three determinations.

TABLE 10. (Cont'd)

SAMPLE	MOLECULAR WEIGHT (Daltons)[1]		%
F5	157100	$\pm$ 2300	63 $\pm$ 3
	5780	$\pm$ 150	13 $\pm$ 2
	3000	$\pm$ 40	24 $\pm$ 1
F10	160400	$\pm$ 2400	67 $\pm$ 3
	5900	$\pm$ 500	13 $\pm$ 1
	3100	$\pm$ 50	20 $\pm$ 2
F15	160400	$\pm$ 2400	58 $\pm$ 3
	6400	$\pm$ 400	14 $\pm$ 1
	3100	$\pm$ 100	28 $\pm$ 4
F20	157100	$\pm$ 2300	56 $\pm$ 2
	6600	$\pm$ 500	15 $\pm$ 0
	2900	$\pm$ 50	29 $\pm$ 2
B5	157100	$\pm$ 2300	58 $\pm$ 0
	6500	$\pm$ 200	15 $\pm$ 0
	2800	$\pm$ 70	27 $\pm$ 1
B10	158700	$\pm$ 0	56 $\pm$ 2
	6600	$\pm$ 200	15 $\pm$ 1
	2900	$\pm$ 40	29 $\pm$ 2
B15	149100	$\pm$ 0	52 $\pm$ 2
	6300	$\pm$ 400	13 $\pm$ 1
	2800	$\pm$ 40	35 $\pm$ 2
B20	157100	$\pm$ 2300	54 $\pm$ 2
	6600	$\pm$ 0	14 $\pm$ 1
	3000	$\pm$ 40	32 $\pm$ 2

[1] Each value represents a mean of three determinations.

TABLE 10. (Cont'd)

SAMPLE	MOLECULAR WEIGHT (Daltons)[1]	%
A10	34100 $\pm$ 2700	25 $\pm$ 4
	8200 $\pm$ 0	75 $\pm$ 4
A20	34400 $\pm$ 2400	40 $\pm$ 2
	8100 $\pm$ 400	60 $\pm$ 2
A30	34800 $\pm$ 2900	26 $\pm$ 5
	7600 $\pm$ 200	74 $\pm$ 5
A40	35800 $\pm$ 1400	28 $\pm$ 1
	6800 $\pm$ 500	72 $\pm$ 1
A1	1091400 $\pm$ 42200	3 $\pm$ 1
	32600 $\pm$ 1700	23 $\pm$ 2
	7900 $\pm$ 200	67 $\pm$ 3
	90 $\pm$ 10	8 $\pm$ 4
A2	1254000 $\pm$ 117700	14 $\pm$ 6
	7800 $\pm$ 300	85 $\pm$ 3
	90 $\pm$ 10	3 $\pm$ 3
A3	1249100 $\pm$ 31900	24 $\pm$ 1
	8200 $\pm$ 200	70 $\pm$ 1
	90 $\pm$ 10	7 $\pm$ 2
A4	1185800 $\pm$ 34600	17 $\pm$ 1
	8200 $\pm$ 500	75 $\pm$ 1
	100 $\pm$ 10	7 $\pm$ 1

[1] Each value represents a mean of three determinations.

TABLE 10. (Cont'd)

SAMPLE	MOLECULAR WEIGHT (Daltons)[1]	%
A5	1235900 ± 18100 7200 ± 200 100 ± 10	25 ± 1 65 ± 1 9 ± 0
A6	1316500 ± 50900 7400 ± 200 100 ± 10	25 ± 1 65 ± 1 10 ± 1
A7	1329700 ± 33900 7300 ± 200 100 ± 10	17 ± 0 73 ± 1 10 ± 2
A8	2170000 ± 64600 6800 ± 400 100 ± 10	42 ± 1 55 ± 2 3 ± 1

[1] Each value represents a mean of three determinations.

According to Mieth *et al.* (1983), the theoretical weight of the 12S protein was 294,000 to 300,000 D (Table 1). The molecular weights for PMM were found to be 170,800 D and 7,800 D (designated as P1 and P2, respectively); these values were much lower than the theoretical values. This was possibly due to the dissociation of the proteins into their subunit components (refer to Table 1 for the molecular weights of subunit components) which was caused by the low ionic strength of buffer used to solubilize the protein for gel filtration analysis.

After hydrolysis with trypsin, the subunit components of PMM were dissociated into smaller fractions. With an exposure time of 5 min, the hydrolysate contained about 81% of 2,900 and 19% of 170,800; this demonstrated that P1 and P2 fractions from PMM were dissociated into smaller units with a residual amount of P1. With an increase of the hydrolysis time above 10 min, the P1 and P2 in PMM were reduced into three components. For chymotrypsin hydrolysates, the trend of molecular weight distribution for all four samples were quite consistent with 2,800 to 3,000 D as the major subunit component and with 170,800 to 172,600 D as the minor subunit component.

The molecular weight distribution patterns are quite similar for all fungal and bacterial hydrolysates. For fungal hydrolysates, the major component was 157,000 to 160,400 D whereas for bacterial hydrolysates, the predominant component was 149,100 to 159,000 D. Trypsin and chymotrypsin enzymes dissociated the P1 component to a greater extent than fungal and bacterial proteases and this was supported by the slightly higher DH values for all trypsin and chymotrypsin hydrolysates (Table 9).

For mild acid hydrolysis samples, a consistent molecular weight distribution

pattern was observed; that is, the low molecular weight fraction appeared with an increase in hydrolysis time. The P1 from PMM was cleaved to a greater extent by acid compared with enzymic hydrolysis. The major components in these hydrolysates ranged from 6,800 to 8,200 D.

A similar trend was noted for all alternate acid hydrolysates. Each hydrolysate contained three components with the major one in the range of 5,900 to 8,200 D. There were four components in the A1 sample and three components in the other acid hydrolysates. All hydrolysates from the alternate acid treatment contained a component which had a relatively high molecular weight (ranged from 1,091,400 D in the A1 sample to as high as 2,170,000 D in the A8 sample) but occurred at a low percentage (3%). This is perhaps due to protein aggregation during prolonged hydrolysis.

b. Subunit Analysis. The electrophoregrams for PMM and all hydrolysates are presented in Fig. 7, 8 and 9. The molecular weights of subunits for PMM and all hydrolysates estimated by SDS-PAGE are given in Table 11. For SDS-PAGE, sodium dodecyl sulphate (SDS) was used to denature the protein into its subunits by disrupting noncovalent linkages. The SDS binds to the protein to obtain a protein with an excessive negative charge which is constant per subunit. Thus, the electrophoretic mobility of the protein-SDS complex is based solely on the molecular weight of the subunit. The SDS-PAGE was carried out under reducing conditions using mercaptoethanol to break the covalent disulphide bonds between the acidic and basic subunits.

As discussed by Schwenke *et al.* (1983), the 12S canola protein consists of

FIGURE 7. SDS-PAGE electrophoregrams of standards, PMM plus trypsin and chymotrypsin hydrolysates.

Lane	a.	SDS-PAGE standards
	b.	SDS-PAGE standards
	c.	PMM
	d.	T5
	e.	T10
	f.	T15
	g.	T20
	h.	C5
	i.	C10
	j.	C15
	k.	C20
	l.	PMM
	m.	SDS-PAGE standards
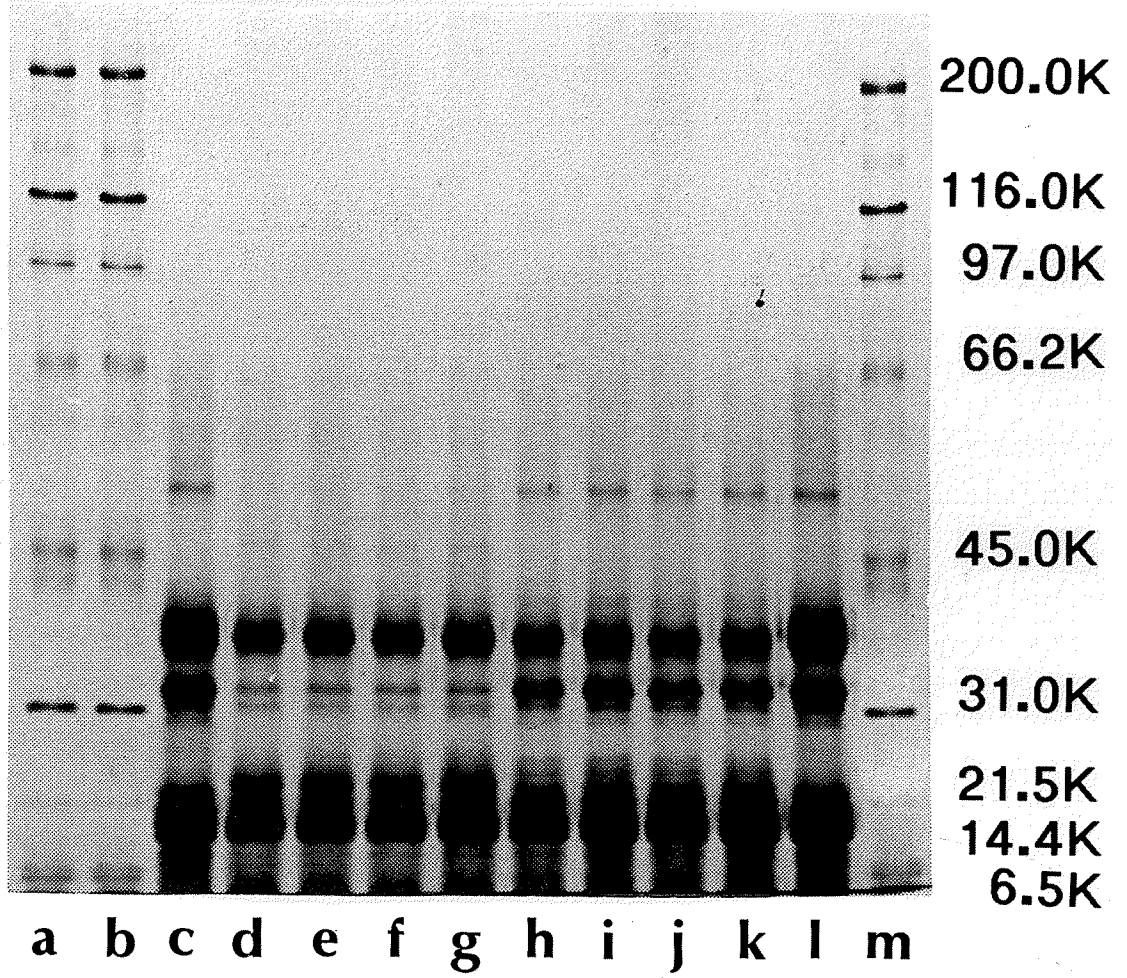
FIGURE 9. SDS-PAGE electrophoregrams of standards and acid hydrolysates.

Lane	a.	SDS-PAGE standards
	b.	A10
	c.	A20
	d.	A30
	e.	A40
	f.	A1
	g.	A2
	h.	A3
	i.	A4
	j.	A5
	k.	A6
	l.	A7
	m.	A8
	n.	OV

	200.0K
	116.0K
	97.0K
	66.2K
	45.0K
	31.0K
	21.5K
	14.4K
	6.5K

a b c d e f g h i j k l m n o
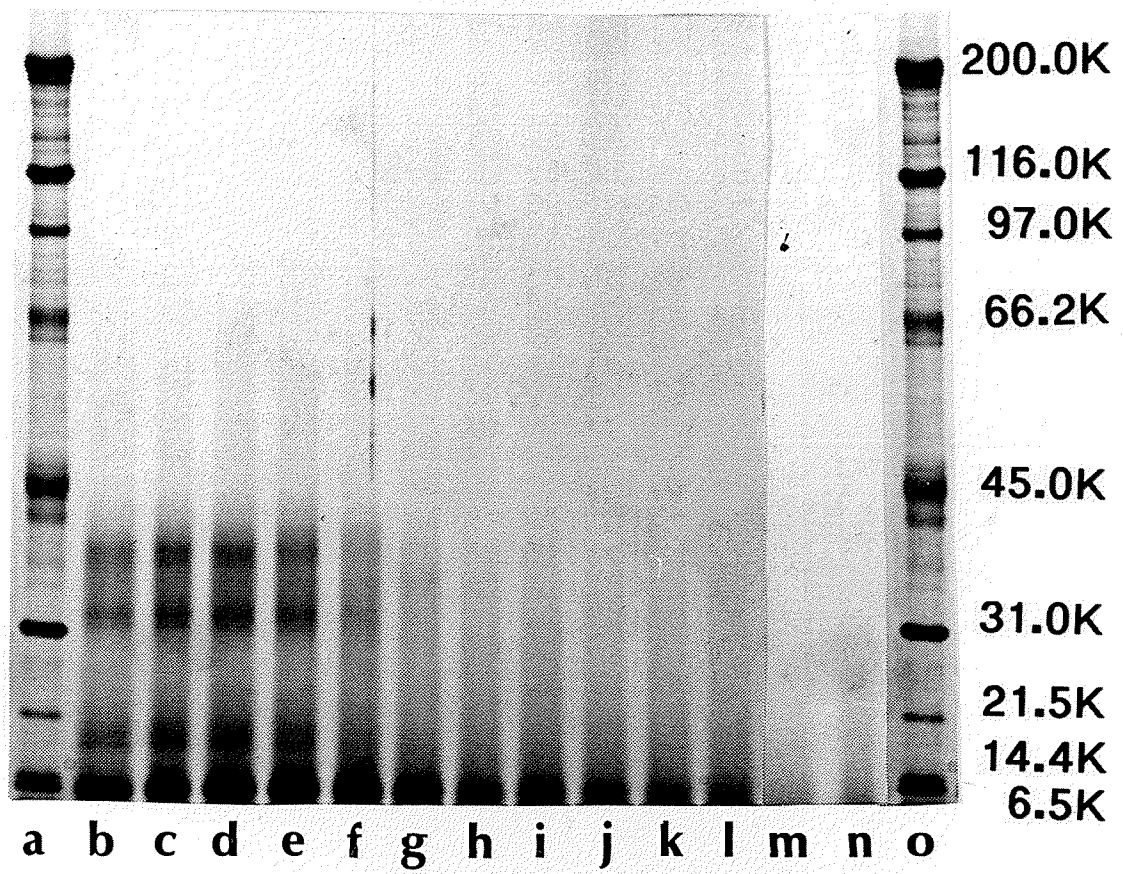
TABLE 11. Molecular weights of PMM and all hydrolysates estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

SAMPLE	MOLECULAR WEIGHT (Daltons)
PMM[1]	16500
	19900
	27000
	29000
	33400
	52200
T5 - T20	16100
	20400
	27000
	29000
	33400
C5 - C20	16100
	19900
	22400
	27000
	29000
	33400
	52200
B5 - B20	16400
	19500
	27600
	29000
	31300
	32900

[1] The 52200, 33400, 29000, 27000, 19900 and 16500 molecular fractions are designated as R1, R2, R3, R4, R5 and R6 respectively.

TABLE 11. (Cont'd)

SAMPLE	MOLECULAR WEIGHT (Daltons)
F5 - F20	16400
	19000
	27600
	32900
	51400
A10 - A40	15500
	18700
	27100
	28300
	34100
A1	15500
	27100
A2 - A8	15500

polypeptide chains with molecular weights in the range of 18,500 to 31,000 (Table 1). There are six subunits and each subunit is made up of two polypeptide chains. In this study, there were six bands in the PMM. The 52,200 fraction (designated as R1, Table 11) as observed in the PMM represented the 2-3S protein which should dissociate in the presence of mercaptoethanol. However, Burgess (1991) also observed this fraction in her study. In addition, a set of three bands at approximately 27,000, 29,000 and 33,400 (acidic polypeptides, designated as R4, R3 and R2, respectively) and another set of two bands at about 16,500 and 19,900 (basic polypeptides, designated as R6 and R5, respectively) were also observed. Schwenke *et al.* (1983) also noticed bands at 50,000-53,000 D in addition to the four bands of acidic and basic polypeptides found.

From Fig. 7, all trypsin hydrolysates appeared to have five bands. The R1 fraction from PMM was definitely cleaved by the trypsin enzyme into smaller subunits during hydrolysis and there was no evidence of the R1 band present in the electrophoregram. Trypsin seemed to hydrolyse the R3 fraction from PMM into a much smaller unit and hence the lighter intensity of the band. Furthermore, the R2 fraction in PMM was hydrolysed by trypsin and hence the lighter intensity of the band compared to PMM.

For chymotrypsin hydrolysates, the R1 fraction from PMM was not completely hydrolysed by the enzyme and hence the appearance of this band with a lighter intensity. The R2 and R3 fractions were hydrolysed by chymotrypsin and bands with lighter intensities at these positions were observed. For trypsin and chymotrypsin hydrolysates, the electrophoretic properties of the protein did not show significant changes with an

increase in hydrolysis time.

For bacterial hydrolysates, there were six bands; in contrast, all fungal hydrolysates had five bands (Fig. 8). Unlike the bacterial protease, the fungal protease did not cleave the R1 fraction from PMM. For bacterial hydrolysates, the R2 fraction of PMM was cleaved into two smaller subunits. These two bands appeared to be lighter and sharper with an increase in hydrolysis time and this pattern seemed to hold true for the hydrolysis of R3 and R4 fractions in PMM. For fungal hydrolysates, there were no significant changes in the electrophoretic properties of the protein with an increase in the hydrolysis time.

During acid hydrolysis, all the bands in the PMM were cleaved into several smaller subunits (Fig. 9). It was shown clearly that the R1 molecular fraction was completely cleaved during the acid hydrolysis. The R5 and R6 fractions in PMM were cleaved to a great extent by acid hydrolysis. However, the electrophoretic properties of the protein did not show significant changes with increasing the hydrolysis time. By using the alternate acid hydrolysis, the PMM was hydrolysed extensively after 1 h of treatment. Only two bands existed and, of these, the 27,100 fraction in the A1 sample decreased with an increase in hydrolysis time.

2. Isoelectric Point Determination

The isoelectric focusing pattern for PMM and all hydrolysates are given in Figs. 10 and 11 and the estimated isoelectric points for all protein samples are tabulated in Table 12.

FIGURE 10. Isoelectric focusing patterns for standards and all enzymic hydrolysates.

Lane a.   Isoelectric focusing standards
     b.   T5
     c.   T10
     d.   T15
     e.   T20
     f.   C5
     g.   C10
     h.   C15
     i.   C20
     j.   F5
     k.   F10
     l.   F15
     m.   F20
     n.   B5
     o.   B10
     p.   B15
     q.   B20
     r.   Isoelectric focusing standards
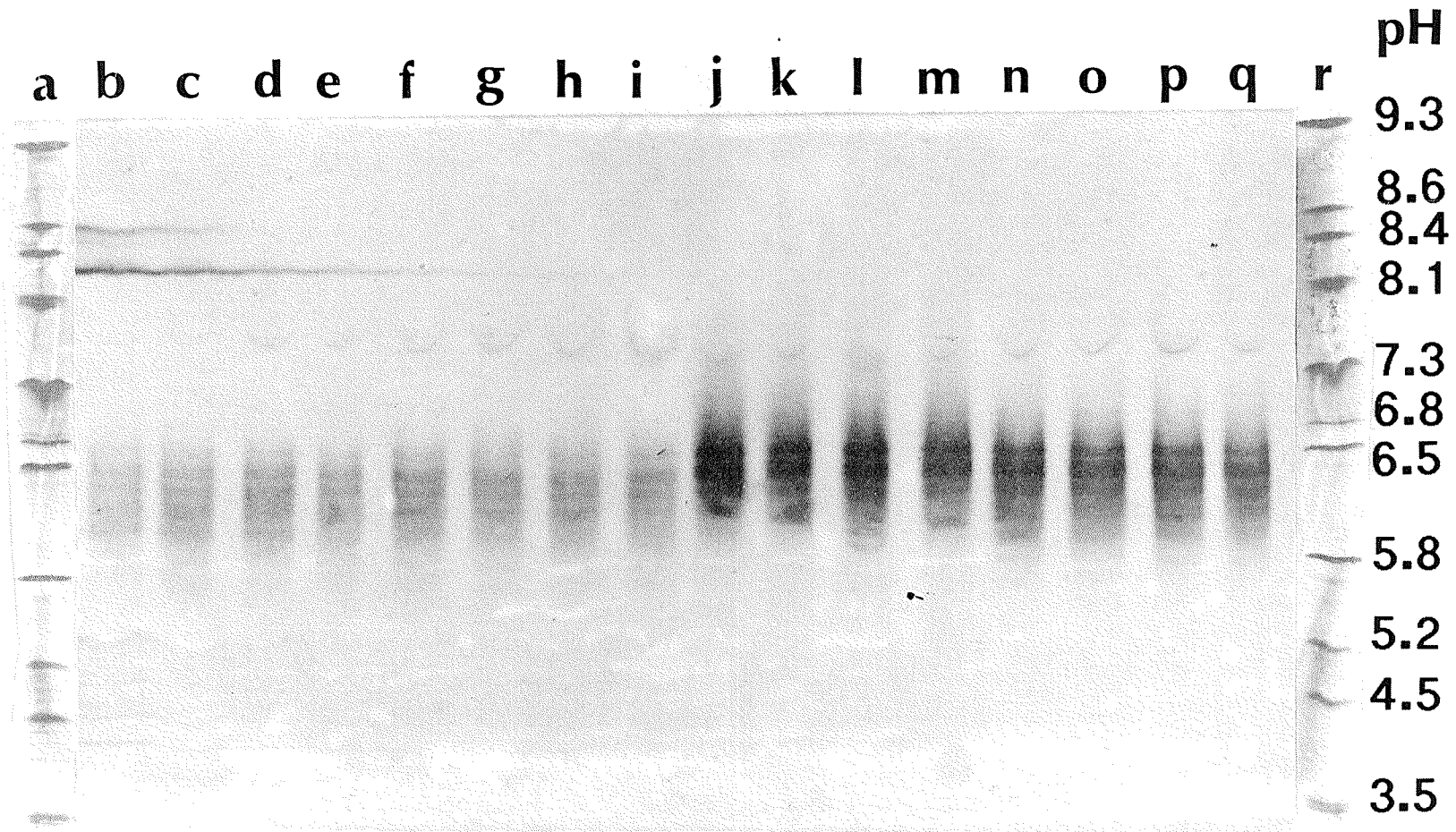
FIGURE 11. Isoelectric focusing patterns for standards, PMM and acid hydrolysates.

Lane		
	a.	Isoelectric focusing standards
	b.	A10
	c.	A20
	d.	A30
	e.	A40
	f.	A1
	g.	A2
	h.	A3
	i.	A4
	j.	A5
	k.	A6
	l.	A7
	m.	A8
	n.	OV
	o.	PMM
	p.	Isoelectric focusing standards

a b c d e f g h i j k l m n o p pH

9.3

8.6
8.4
8.1

7.3

6.8

6.5

5.8

5.2

4.5

3.5
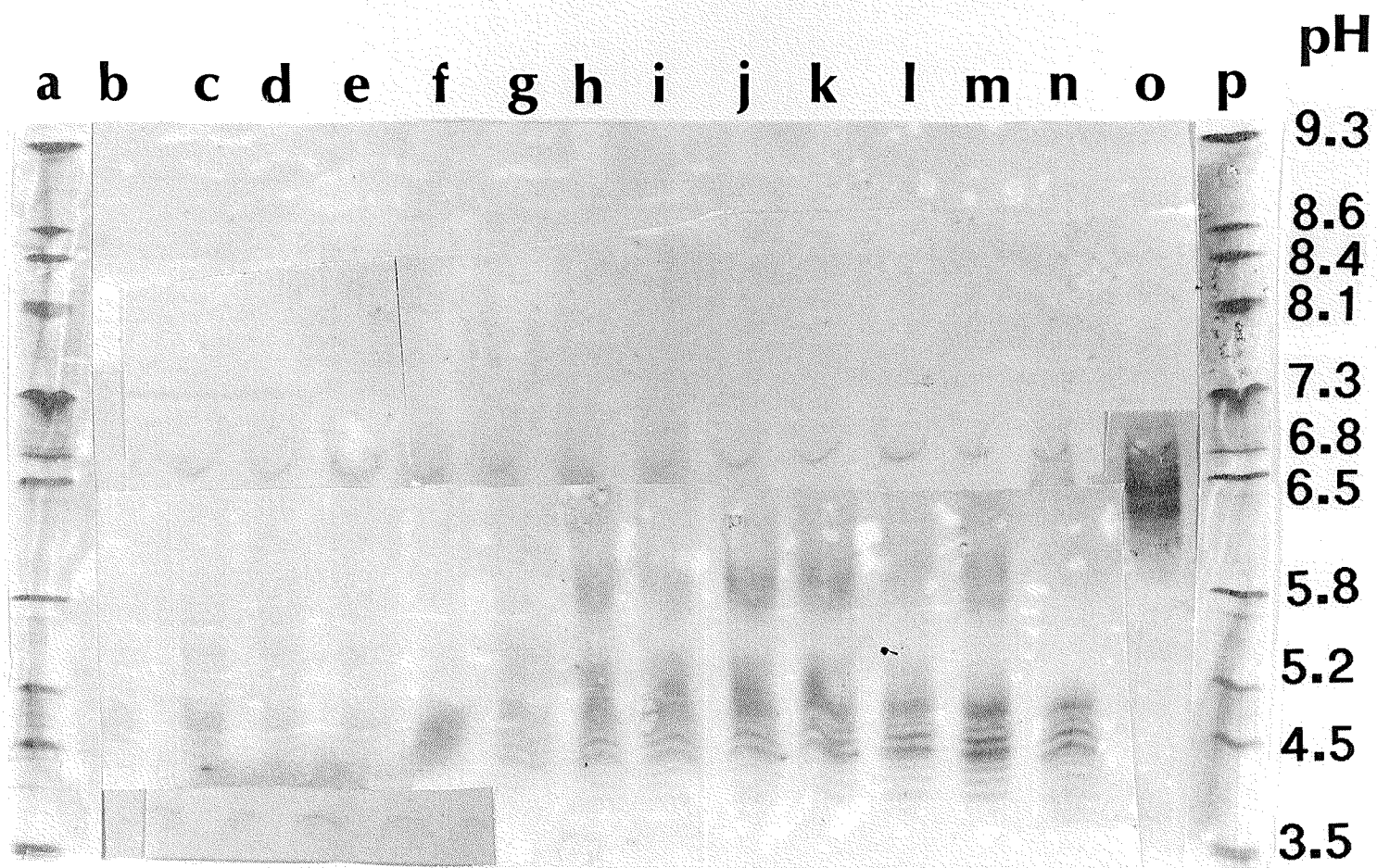
TABLE 12. Isoelectric points for PMM and all hydrolysates determined by isoelectric focusing.

SAMPLE	ISOELECTRIC POINT (pH)
PMM	6.6
T5 - T20	6.3
	6.4
	6.6
C5 - C20	6.2
	6.4
	6.6
B5 - B20	6.6
	6.7
F5 - F20	6.6
	6.7
A10 - A40	4.4
	4.6
	4.8
A1 - A2	4.5
	4.6
	4.7
	4.8

TABLE 12. (Cont'd)

SAMPLE	ISOELECTRIC POINT (pH)
A3 - A5	4.4
	4.6
	4.8
	5.1
	5.7
A6 - A8	4.5
	4.6
	4.7
	4.9
	5.7

A number of bands were observed in all situations except for the PMM. The isoelectric point for the 12S canola protein according to Schwenke *et al.* (1983) was 7.2 (Table 1). The experimental isoelectric point for PMM in this study was found to be 6.6.

For trypsin hydrolysates, the isoelectric points were found to be in the 6.3-6.6 range whereas chymotrypsin hydrolysates have isoelectric points in the 6.2-6.6 range (Table 12). As observed with the gel filtration data, trypsin and chymotrypsin convert the PMM into smaller units. These units have lower isoelectric points. Bacterial and fungal hydrolysates all have isoelectric points in the 6.6-6.7 range (Table 12). Therefore, it seems that both trypsin and chymotrypsin have more impact in terms of hydrolytic action compared to both bacterial and fungal proteases. This was proved by the slightly higher DH values (Table 9) for the trypsin and chymotrypsin hydrolysates at the same hydrolysis time.

As for the samples of mild acid hydrolysis, the hydrolysates had isoelectric points in the range of 4.4-4.8 whereas the alternate acid hydrolysis yielded samples that had isoelectric points in the 4.4-5.7 range.

3. Protein Conformational Assessment

a. Thermal parameters using DSC analysis. Thermal parameters determined by DSC are excellent tools to provide an insight towards the degree of conformational change of proteins when subjected to a manipulation of the environment (Arntfield and Murray, 1981). Two important parameters are used to assess these structural changes

in DSC analysis, namely the enthalpy of denaturation (ΔH) and the thermal denaturation temperature (Td). The results for these assays in this study are given in Table 13.

In food proteins, protein denaturation will have an adverse effect on the functionality of proteins in food systems (Arntfield and Murray, 1981). According to Arntfield and Murray (1981), some of the factors that may be responsible for denaturation of the protein are heat, organic solvents, acid, alkali, detergents, urea and guanidine hydrochloride.

The ΔH measures the enthalpy required for thermal denaturation. Lower ΔH values are an indication of any protein unfolding before the heat treatment. PMM has a ΔH of 11.0 Joules/g. This relatively large ΔH value demonstrates the mildness of the PMM process on the native state of the extracted protein. This is very similar to the results of Arntfield and Murray (1981) where proteins were isolated from soybeans and fababeans using the PMM process.

However, the PMM was subjected to different hydrolysis procedures involving the adjustment of temperature and pH for the purpose of optimum hydrolysis conditions. Therefore, all hydrolysates exhibited different thermal characteristics. Although the incubation temperature for both trypsin and chymotrypsin (37°C) hydrolysis was much lower than for fungal protease (45°C) and bacterial protease (50°C), a higher inactivation temperature of 87°C for a duration of 5 min was used to inactivate both trypsin and chymotrypsin enzymes compared to the 75°C for 10 min inactivation treatment for fungal and bacterial proteases. A decrease in ΔH values in all trypsin and chymotrypsin hydrolysates is observed and the decrease of ΔH is as high as 80% in the T20 sample

TABLE 13. The enthlphy of denaturation (ΔH in Joules/g) and the thermal denaturation temperature (Td in °C) for PMM and all hydrolysates

SAMPLE	ΔH (Joules/g)[1]	Td (°C)[1]
PMM	11.0 ± 1.2	90.4 ± 0.2
T5	1.8 ± 0.2[a]	80.5 ± 0.1[a]
T10	3.0 ± 0.3[a]	81.1 ± 0.6[a]
T15	2.8 ± 0.1[a]	79.4 ± 0.1[b]
T20	2.3 ± 0.6[a]	79.7 ± 0.1[b]
C5	3.6 ± 0.3[a]	81.6 ± 0.2[a]
C10	3.0 ± 0.1[a]	82.2 ± 0.0[b]
C15	3.3 ± 0.5[a]	83.0 ± 0.2[c]
C20	3.0 ± 0.2[a]	81.8 ± 0.0[a]
F5	10.9 ± 0.2[a]	80.1 ± 0.1[a]
F10	10.4 ± 0.7[a]	81.0 ± 0.3[a]
F15	11.3 ± 0.4[a]	81.6 ± 0.3[a]
F20	10.9 ± 0.5[a]	81.0 ± 0.4[a]
B5	10.7 ± 0.2[a]	88.2 ± 0.2[a]
B10	10.9 ± 0.9[a]	88.8 ± 0.5[ab]
B15	11.3 ± 0.4[a]	89.1 ± 0.1[b]
B20	11.1 ± 0.2[a]	88.7 ± 0.1[ab]

[1] Each value represents a mean of three determinations.
[2] Values followed by the same letter are not significantly different ($p \leq 0.05$) within each hydrolysis group

TABLE 13. (Cont'd)

SAMPLE	ΔH (Joules/g)[1]	Td (°C)[1]
A10	0.9 ± 0.0^a	67.5 ± 2.7^a
A20	1.0 ± 0.1^a	68.4 ± 3.5^a
A30	0.6 ± 0.1^b	67.6 ± 0.6^a
A40	0.6 ± 0.1^b	68.1 ± 0.8^a
A1	1.4 ± 0.1^a	63.6 ± 0.4^{ab}
A2	1.4 ± 0.2^a	68.6 ± 0.2^c
A3	2.0 ± 0.1^{bc}	68.6 ± 1.1^c
A4	1.6 ± 0.2^{ab}	62.2 ± 2.8^a
A5	1.2 ± 0.1^a	65.7 ± 1.8^{ab}
A6	1.3 ± 0.2^a	62.8 ± 1.0^{ab}
A7	1.5 ± 0.2^a	63.8 ± 0.6^{ab}
A8	2.1 ± 0.5^c	65.7 ± 1.7^{bc}

[1] Each value represents a mean of three determinations.

[2] Values followed by the same letter are not significantly different ($p \leq 0.05$) within each hydrolysis group

(Table 13). Decreased ΔH values denote protein denaturation which leads to possible reduced functionality of the protein in a food system.

Unexpectedly, only a slight decrease in ΔH values was observed in all fungal and bacterial hydrolysates. Therefore, fungal and bacterial hydrolysis methods provided milder treatments and hence the less denatured samples.

For both acid treatments, the hydrolysis is carried out in 0.05 N HCl at 95°C. Relatively low ΔH values are observed for all the acid hydrolysates. The acidic hydrolysis conditions and the high temperature used are responsible for the denaturation of the protein. In general, enzymic hydrolysis (especially the use of fungal and bacterial proteases) provides a milder treatment which leads to less protein denaturation compared to both acid hydrolysis methods.

The analysis of variance of ΔH is presented in Appendix 5. For all the enzyme hydrolysates tested, the effects of hydrolysis time on ΔH were not significantly different. However, the effects of hydrolysis time on the ΔH were significantly different for both acid treatments ($F = 32.88$, $p = 0.0004$ for mild acid; $F = 6.32$, $p = 0.0009$ for alternate acid).

The temperature required to denature a protein is indicated by the temperature of denaturation (Td). The PMM had a Td of 90.4°C and for all enzyme hydrolysates, the Td values were decreased by about 10°C with the exception of the bacterial hydrolysates (Table 13). From Appendix 6, the analysis of variance of Td shows that hydrolysis time had significant effects on the Td for trypsin, chymotrypsin ($F = 13.84$, $p = 0.0042$; $F = 52.02$, $p = 0.0001$ respectively) whereas the effect of hydrolysis time on Td is not

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significant for bacterial proteases and fungal proteases (F=2.91, p=0.1233; F=0.59, p=0.6457 respectively).

All acid hydrolysates showed lower Td values than other hydrolysates and this can be explained by the harsher conditions used. Hydrolysis time had no significant effect on Td for the mild acid treatment (F=0.05, p=0.9821) whereas significant effects from hydrolysis time on Td were observed in the alternate acid treatment (F=5.63, p=0.0017).

b. Surface Hydrophobicity. According to Kato and Nakai (1980), surface hydrophobicity has a greater significance than total molecular hydrophobicity in elucidating protein function. Although many hydrophobic residues are buried in the interior of most native proteins to avoid contact with the polar aqueous environment, some hydrophobic groups may remain exposed at the molecular surface. Surface properties of a protein will be indicative of its capacity for intermolecular interaction. Hayakawa and Nakai (1985) suggested that protein hydrophobicity may be classified into two types, aromatic and aliphatic, as influenced by aromatic and aliphatic amino acid residues, respectively. These may be related in different ways to protein functionality. In 1985, Li-Chan *et al.* concluded that both aromatic and aliphatic hydrophobicities were significant predictors of emulsifying and fat-binding properties of salt soluble muscle proteins. However, Hayakawa and Nakai (1985) found no difference between the two types of hydrophobicity measurements in relation to the thermal properties of ovalbumin. Nevertheless, Nakai and Li-Chan (1988) concluded that aromatic hydrophobicity showed

a significant relationship with protein solubility.

The surface hydrophobicity of proteins was assessed through the use of fluorescent probes which are not inherently fluorescent. However, they become fluorescent when bound to specific groups on proteins. Sklar *et al.* (1977) introduced cis-parinaric acid (CPA) to bind with aliphatic hydrocarbon side-chains of proteins. Hayakawa and Nakai (1985) used 1-anilino-naphthalene-8-sulphonate (ANS) to study the aromatic hydrophobicity of the proteins. The initial slope of a plot of fluorescence intensity as a function of protein concentration was an index of protein surface hydrophobicity (Hayakawa and Nakai, 1985).

The aromatic and aliphatic hydrophobicity results are shown in Table 14 and the analysis of variance for both aromatic and aliphatic hydrophobicity data are presented in Appendices 7 and 8. The aromatic hydrophobicity for PMM was 12.4 whereas the aliphatic hydrophobicity was 40.4. When PMM was subjected to trypsin, the resultant hydrolysates showed a dramatic increase in aromatic hydrophobicity whereas aliphatic hydrophobicity did not show any increase. From Appendices 7 and 8, the analysis of variance data showed that within the trypsin treatment, the hydrolysis time showed a significant effect on only aromatic hydrophobicity (F=32.02, p=0.0089). As for chymotrypsin treatment, the effect of hydrolysis time was significant only on aliphatic hydrophobicity (F=75.80, p=0.0025). The aliphatic hydrophobicity decreased slightly for the initial 10 min of chymotrypsin treatment and reached a minimum at 15 min. However, the greatest aliphatic hydrophobicity was observed with the C20 sample.

As for the fungal hydrolysates, hydrolysis time had significant effects (F=22.34,

TABLE 14. The surface hydrophobicity of PMM and all hydrolysates.

SAMPLE	AROMATIC HYDROPHOBICITY[1]	ALIPHATIC HYDROPHOBICITY[1]
PMM	12.4 ± 2.2	40.4 ± 2.0
T5	64.0 ± 3.0[a]	45.9 ± 2.9[ab]
T10	60.7 ± 1.7[a]	38.8 ± 3.0[b]
T15	47.1 ± 0.9[b]	51.7 ± 0.5[a]
T20	46.0 ± 1.0[b]	35.5 ± 2.2[b]
C5	11.5 ± 2.5[a]	30.2 ± 2.2[a]
C10	19.6 ± 2.4[a]	31.3 ± 0.5[a]
C15	13.5 ± 1.2[a]	21.5 ± 2.8[c]
C20	14.5 ± 1.2[a]	52.7 ± 2.6[b]
F5	15.4 ± 0.1[a]	30.9 ± 2.5[a]
F10	11.5 ± 0.1[b]	27.2 ± 1.5[a]
F15	13.8 ± 0.4[a]	27.0 ± 1.3[a]
F20	11.1 ± 0.9[b]	26.7 ± 0.5[a]
B5	14.2 ± 2.7[a]	31.8 ± 1.8[a]
B10	15.0 ± 0.3[a]	36.6 ± 0.8[a]
B15	14.3 ± 0.1[a]	40.2 ± 0.9[a]
B20	14.9 ± 0.0[a]	32.8 ± 2.5[a]

[1] Each value represents a mean of two determinations.
[2] Values followed by the same letter are not significantly different ($p \leq 0.05$) within each hydrolysis group   .

TABLE 14. (Cont'd)

SAMPLE	AROMATIC HYDROPHOBICITY[1]	ALIPHATIC HYDROPHOBICITY[1]
A10	13.8 ± 0.1[a]	32.2 ± 3.1[a]
A20	21.1 ± 0.4[b]	45.2 ± 1.1[b]
A30	28.9 ± 0.9[c]	51.3 ± 0.7[b]
A40	30.6 ± 0.6[d]	29.6 ± 0.3[a]
A1	3.0 ± 0.0[a]	33.2 ± 0.4[ab]
A2	8.2 ± 0.2[ab]	34.1 ± 2.2[ab]
A3	11.9 ± 0.5[b]	38.1 ± 1.6[a]
A4	12.6 ± 3.3[b]	43.1 ± 3.1[c]
A5	11.0 ± 4.6[b]	45.6 ± 0.3[c]
A6	6.8 ± 1.6[ab]	45.6 ± 0.9[c]
A7	7.5 ± 0.4[ab]	47.8 ± 1.1[c]
A8	9.6 ± 0.1[ab]	48.2 ± 0.6[c]

[1]   Each value represents a mean of two determinations.
[2]   Values followed by the same letter are not significantly different ($p \leq 0.05$) within each hydrolysis group

p=0.0149) on only aromatic hydrophobicity. With an increase in hydrolysis time, only aromatic hydrophobicity decreased and there was no significant difference for the aliphatic values. With all bacterial hydrolysates, the effect of hydrolysis time on both types of hydrophobicity was not significant. For mild acid hydrolysates, the effects of hydrolysis time were not significantly different in terms of the abilities to liberate either aromatic or aliphatic amino acid residues. For the alternate acid treatment, the effect of hydrolysis time was significantly different only on the aliphatic surface hydrophobicity (F=18.67, p=0.0002).

Overall, the hydrophobicity values obtained are relatively lower when compared to other literature (Nakai *et al.*, 1980; Nakai and Li-Chan, 1988; Georgiou, 1987). For example, Georgiou(1987) observed a value of 142 for the aliphatic hydrophobicity and a value of 466 for the aromatic hydrophobicity in *Vicia faba* legumin. The discrepancy is perhaps due to the type of protein Georgiou (1987) used; parameters such as buffer and pH were similar in the two studies. In general, the trypsin treatment is able to liberate more aromatic amino acid residues (and hence the higher aromatic hydrophobicity values) on the surface of the protein compared to other enzyme treatments. For both acid treatments, the mild acid treatment significantly increased the aromatic hydrophobicity values; in contrast, the extended acid treatment tended to reduce the aromatic hydrophobicity values. For aliphatic hydrophobicity values, it seems that aliphatic amino acid residues were reduced for hydrolysates from all treatments except samples from the alternate acid treatment (A4 to A8).

D. Impact of Hydrolysis on Functional Properties

1. Nitrogen Solubility (NS)

a. At pH 4.5. The solubility characteristics of proteins are among the most important functional properties since many functional performances of proteins depend upon their capacity to go initially into solution. Solubility at pH 4.5 was tested on all hydrolysates in order to evaluate the potential use of these proteins in food systems with an acidic pH such as soft drinks. The %NS as a function of hydrolysis time for all hydrolysates are shown in Figs. 12, 13 and 14. The numerical results for %NS at pH 4.5 are tabulated in Appendix 9 and the analysis of variance is presented in Appendix 10.

Among all the enzyme hydrolysates, chymotrypsin hydrolysates were the most soluble at pH 4.5. The effect of hydrolysis time on soluble nitrogen at pH 4.5 was found to be significant ($F=17.83$, $p=0.0198$). Chymotrypsin increased the solubility at pH 4.5 most effectively (approximately two times) during the initial 5 min of incubation, but showed decreased solubility when the hydrolysis was carried out for 10 min and 15 min. However, a solubility of 210.4% was obtained from the sample hydrolysed for 20 min Fig. 12). Its solubility was the highest among all the enzyme hydrolysates.

Bacterial hydrolysates showed the second highest solubility among all hydrolysates. The test of the effect of hydrolysis time on solubility at pH 4.5 was significant ($F=169.18$, $p=0.0008$). Solubility was increased by 1.5 times by bacterial protease during the first 10 min of hydrolysis and bacterial hydrolysates of 15 and 20 min had the highest solubility among this treatment group (Fig. 12).

Hydrolysates produced by the fungal protease exhibited the third highest nitrogen

FIGURE 12. The nitrogen solubility (%) at pH 4.5 as a function of hydrolysis
time (min) for all enzyme hydrolysates.
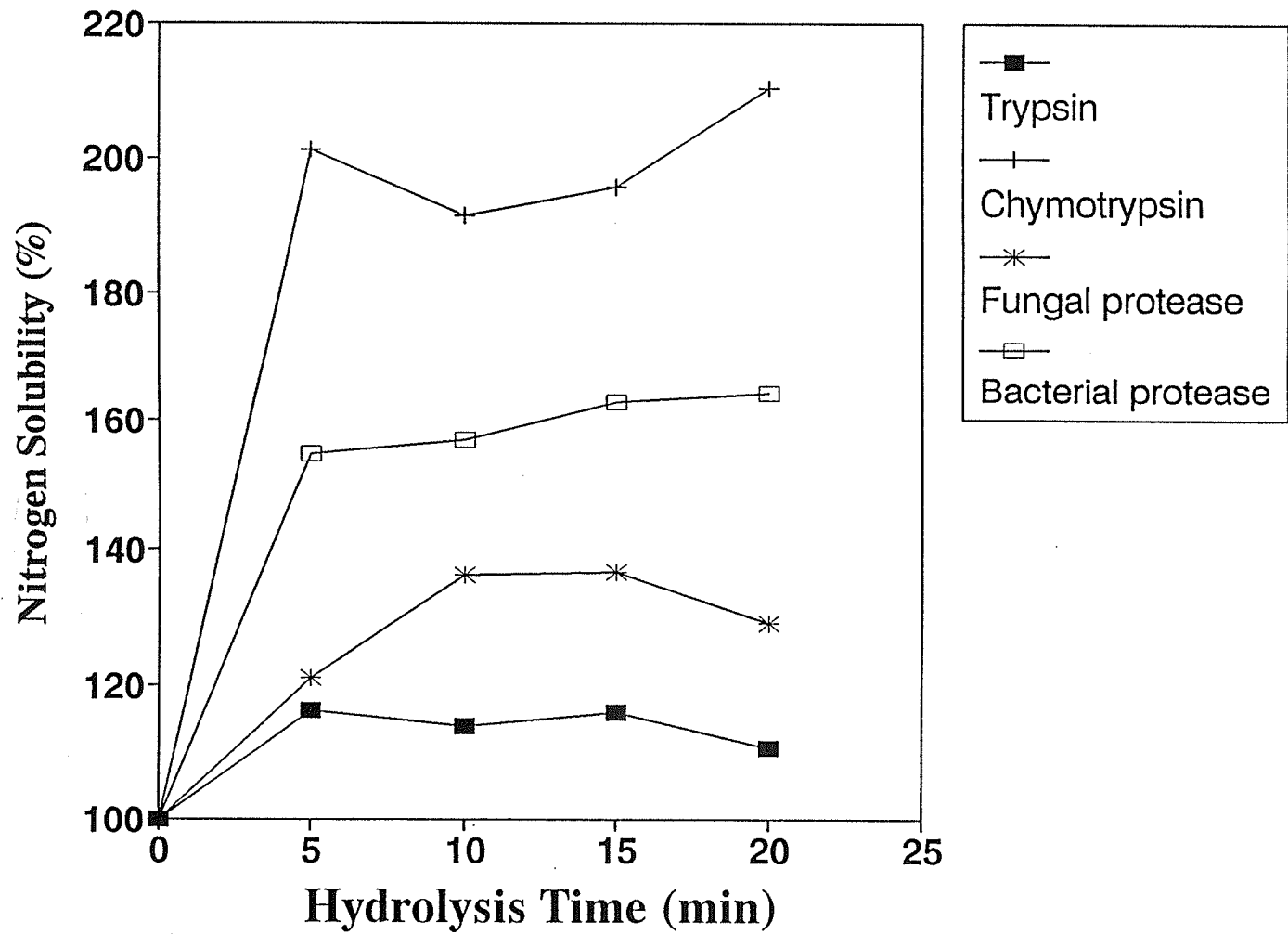
FIGURE 13. The nitrogen solubility (%) at pH 4.5 as a function of hydrolysis time (min) for mild acid hydrolysates.
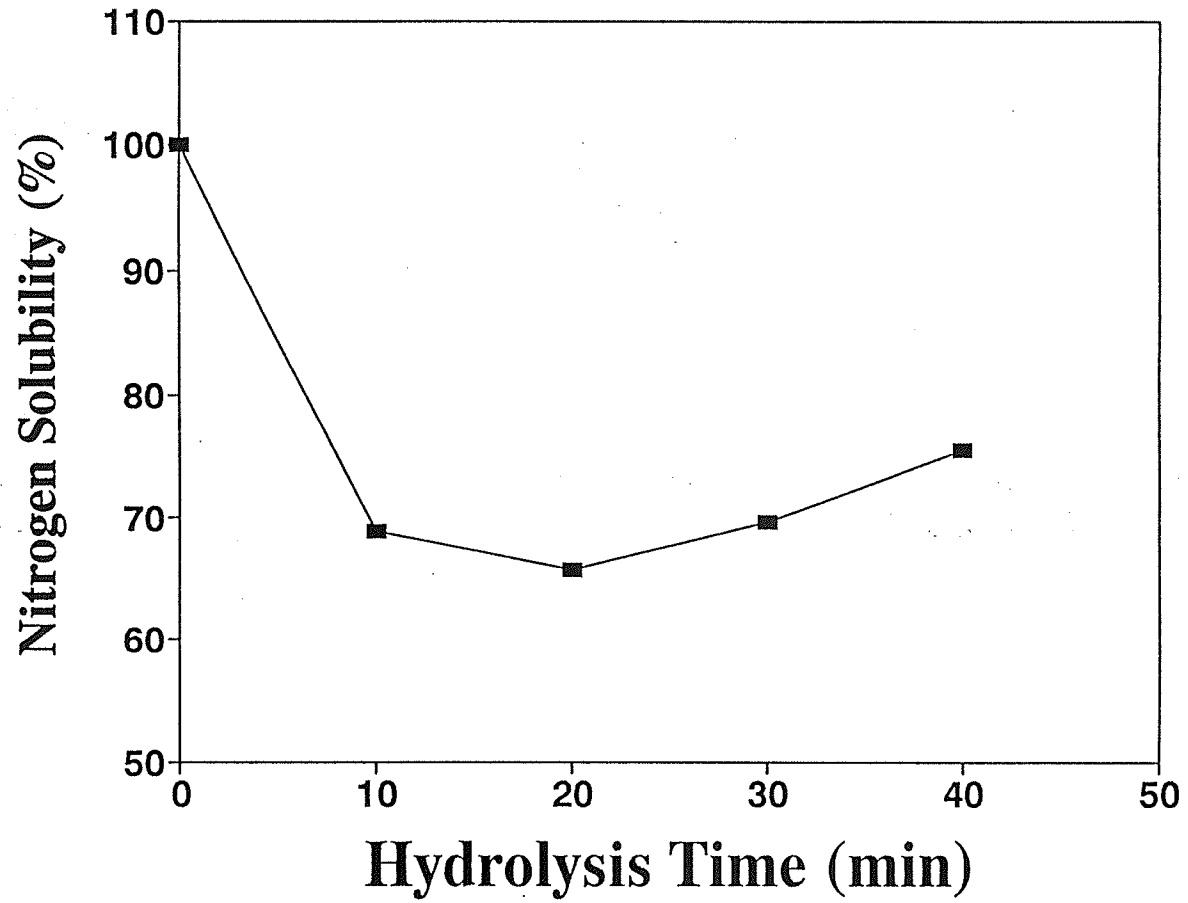
FIGURE 14. The nitrogen solubility (%) at pH 4.5 as a function of hydrolysis time (min) for alternate acid hydrolysates.
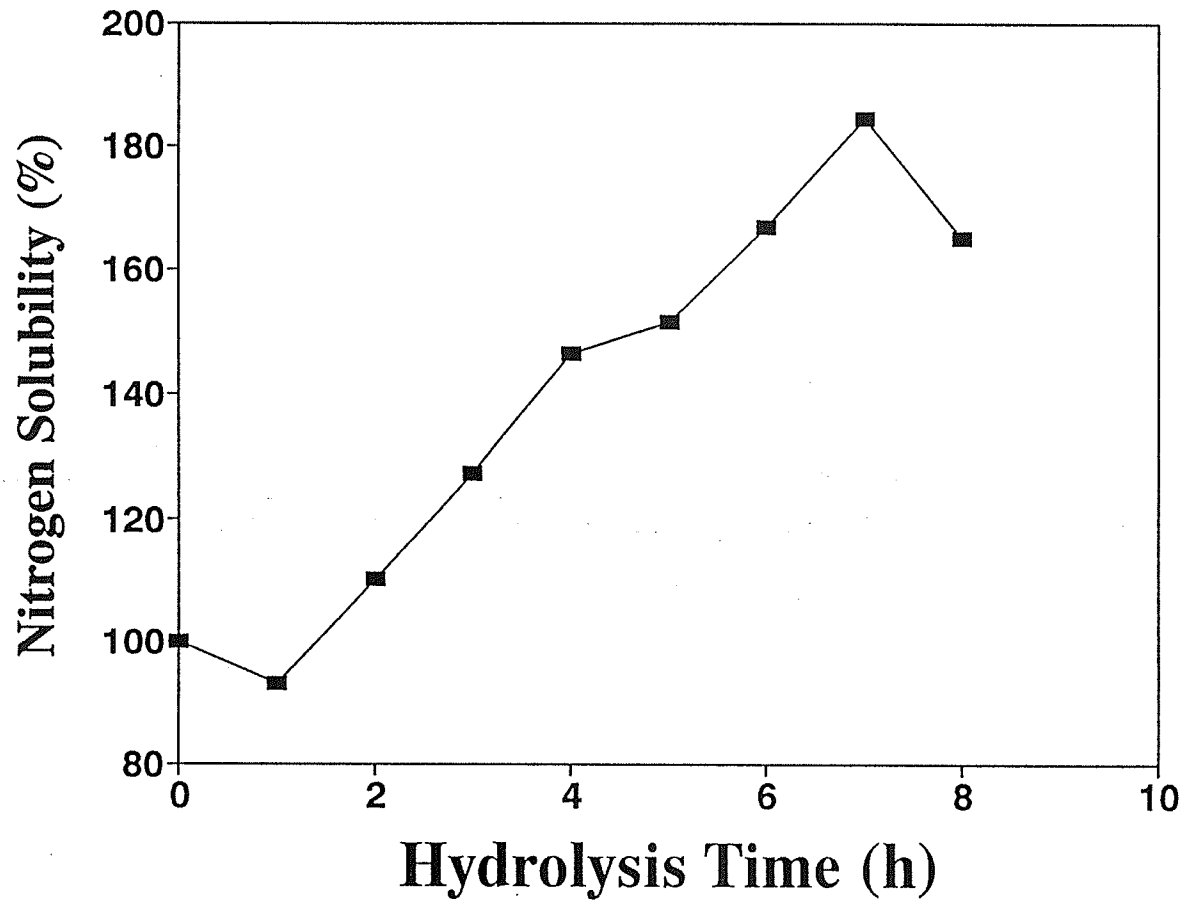
solubility among all the hydrolysates. The solubility increased during the initial 10 min of hydrolysis and the highest solubility was observed at 10 min with a value of 136.7% (Fig. 12). Hydrolysis time had a significant effect (F=22.37, p=0.0148) on nitrogen solubility at pH 4.5.

Trypsin hydrolysates showed the poorest solubility at pH 4.5 among all the enzyme hydrolysates. The T5 sample possessed the highest solubility of 116.3% and gradually decreased after 5 min hydrolysis time (Fig. 12). From the analysis of variance data, the effect of hydrolysis time on nitrogen solubility was significant (F=107.91, p=0.0015).

For mild acid hydrolysates, the solubility decreased dramatically during the initial 10 min of hydrolysis. The lowest solubility was shown at 20 min and the solubility at pH 4.5 improved slightly after that. The best solubility was observed at 40 min with a value of 75.5% (Fig. 13). Hydrolysis time had a significant effect (F=76.28, p=0.0025) on nitrogen solubility at pH 4.5.

With the alternate acid treatment, the lowest solubility was noted at 1 h. The solubility increased with an increase in hydrolysis time up to 7 h, then subsequently decreased. The A7 hydrolysate showed the highest %NS of 184.5% (Fig. 14). It was found that the effect of hydrolysis time on NS at pH 4.5 was statistically significant (F=375.85, p=0.0001).

b. At pH 7.0. The %NS as a function of hydrolysis time are presented in Fig. 15, 16 and 17 and the values of %NS for PMM and all hydrolysates are tabulated in

Appendix 11. Appendix 12 shows the analysis of variance data for this variable and Appendix 13 gives the correlation analysis of all variables.

For enzyme hydrolysates, the NS generally decreased after the different enzyme treatments. Chymotrypsin and bacterial hydrolysates gave the best solubilities among all enzyme hydrolysates and the C20 sample had the highest solubility of 101.7% (Fig. 15). The analysis of variance data shows that the effect of hydrolysis time on NS at pH 7.0 was significant (F=1976.52, p=0.0001) for chymotrypsin treatment. The solubility of bacterial hydrolysates showed a decrease during the initial 5 min of treatment and remained constant for the remainder of the hydrolysis treatment. Furthermore, it was found that the effect of hydrolysis time tested on NS at pH 7.0, was not statistically significant (F=6.32, p=0.0822).

Lower NS at pH 7.0 was also observed for the samples hydrolysed by fungal enzymes. The hydrolysis time had a significant effect (F=15.72, p=0.0244) on NS at pH 7.0. The lowest solubility (80.5%) was noted in the 20 min hydrolysate (Fig. 15).

Among all the enzyme treatments, trypsin hydrolysed samples showed the poorest solubilities. The trypsin enzyme reduced the solubility most significantly during the initial 5 min of hydrolysis compared to other enzyme treatments. However, the solubility increased significantly when the hydrolysis was carried out for a further 5 min. The solubility decreased significantly for the 15 and 20 min hydrolysates. The analysis of variance data showed the hydrolysis time exerted an effect on NS of trypsin hydrolysates at pH 7.0 (F=248.2, p=0.0004). Overall, all enzyme treatments had a negative impact on the NS at pH 7.0.

FIGURE 15. The nitrogen solubility (%) at pH 7.0 as a function of hydrolysis time (min) for all enzyme hydrolysates.
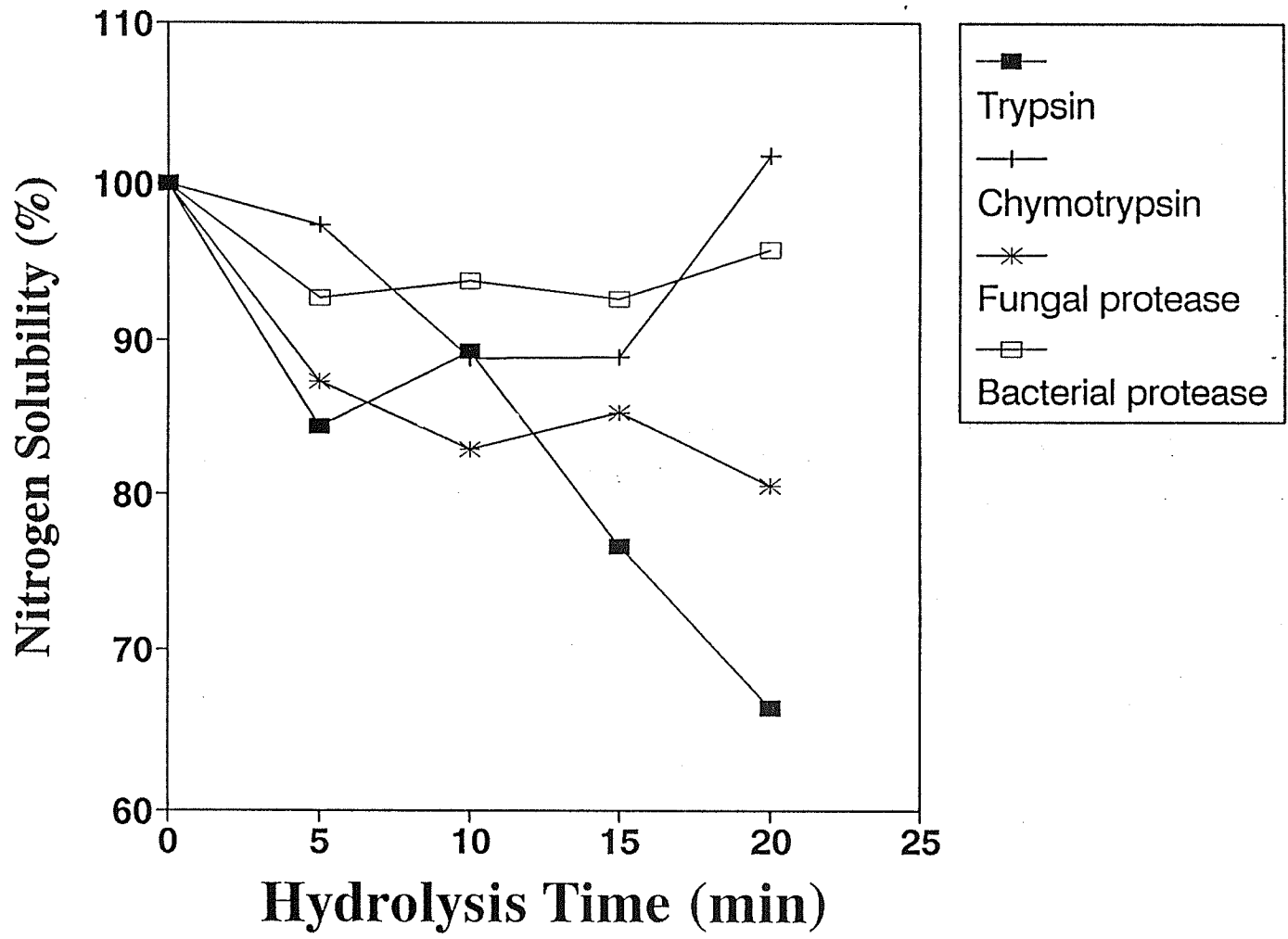
FIGURE 16. The nitrogen solubility (%) at pH 7.0 as a function of hydrolysis time (min) for mild acid hydrolysates.
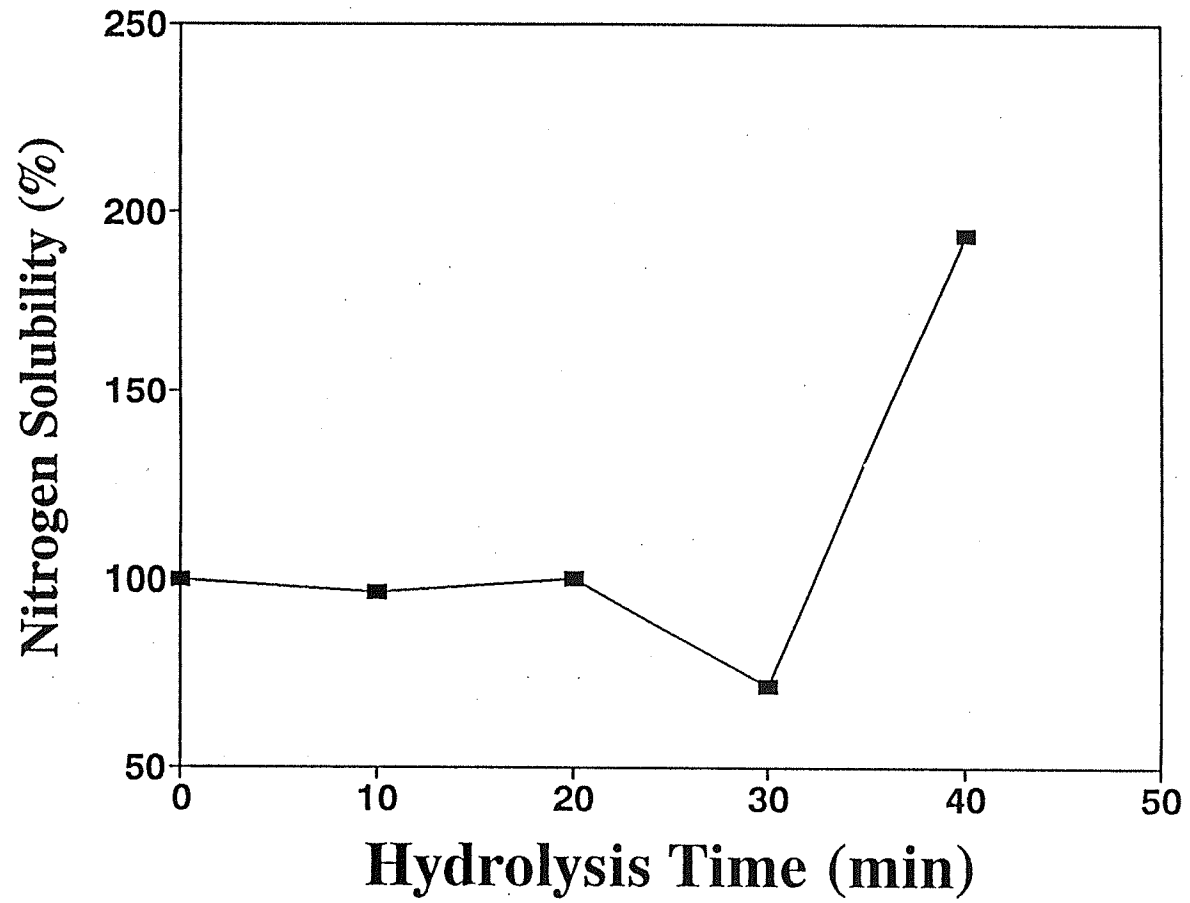
FIGURE 17. The nitrogen solubility (%) at pH 7.0 as a function of hydrolysis time (min) for alternate acid hydrolysates.
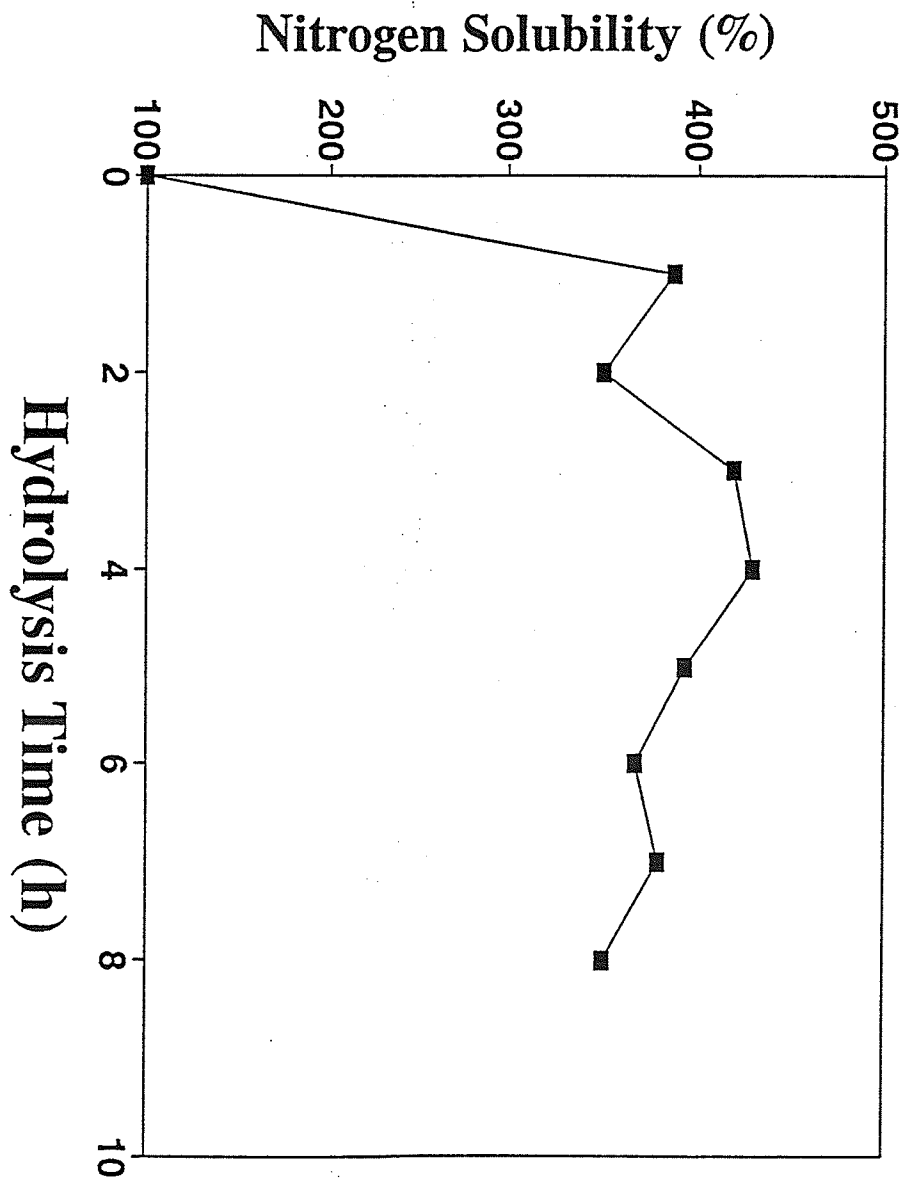
Using the mild acid treatment, the solubility remained constant during the first 20 min of hydrolysis and then decreased to about 71.7% at a hydrolysis time of 30 min (Fig. 16). The solubility then improved significantly when the hydrolysis was carried out for another 10 min (Appendix 11). The effect of hydrolysis time was found to be significant (F=48761.9, p=0.0001) on NS at pH 7.0.

After 1 h of alternate acid hydrolysis, the solubility was increased by a factor of 3.9 and then decreased significantly as hydrolysis was carried out for 2 h. However, the solubility was improved again as the hydrolysis proceeded for another 2 h. After this period of time, the solubility reduced significantly with an increase in hydrolysis time (Fig. 17). The NS at pH 7.0 was improved by the alternate acid treatment. The analysis of variance data showed that the effect of hydrolysis time was significant on the NS at pH 7.0 (F=90.13, p=0.001). Overall, the sample from the alternate acid treatment of 4 h showed the best solubility (427.8%) at pH 7.0.

According to Cheftel *et al.* (1985), from a thermodynamic standpoint, solubilization corresponds to separating the molecules of solvent, separating the molecules of proteins, and dispersing the latter in the solvent with maximum interaction between the protein and solvent. A protein should be able to interact as much as possible via hydrogen-bond, dipole-dipole, and ionic interactions with the solvent (Cheftel *et al.*, 1985). The solubility is dependent upon pH, ionic strength, the type of solvent, and temperature. In this study, only the pH was used to monitor the solubility profile.

Protein carries a negative or positive electric charge depending on the pH of the milieu and when water molecules interact with these charges, solubilization occurs

(Cheftel *et al.*, 1985). For pH values not far from the pI, protein molecules show minimal interactions with water and their net charges are small enough to allow polypeptide chains to approach each other which may lead to protein precipitation.

The starting material, PMM, has an isoelectric point at pH 6.6 (Table 11) and it is unexpectedly more soluble in neutral pH (2.047 mg/ml) than in the acid environment (0.951 mg/ml). All enzyme hydrolysates have isoelectric points in the range of pH 6.3-6.7 and therefore it is understood that all enzyme hydrolysates showed a higher solubility at pH 4.5 than at pH 7.0. With the mild and alternate acid treatments, the resultant hydrolysates have isoelectric points between the pH of 4.4-5.7 and therefore the solubility at pH 7.0 is expected to be greater due to the high net electric charges that resulted by the interaction of protein and water molecules. In this study, all acid hydrolysates exhibited higher solubilities at pH 7.0 than at pH 4.5.

Cheftel *et al.* (1985) concluded that the degree of insolubility is perhaps the most practical measure of protein denaturation plus aggregation. As seen from the correlation matrix in Appendix 13, only NS at pH 7.0 was correlated negatively to the ΔH and Td (r=-0.1854, p=0.0076; r=-0.7614, ==0.0001). It can be observed that hydrolysates that were less denatured exhibited lesser solubility at pH 7.0 than the hydrolysates that were denatured to a greater extent.

Kim *et al.* (1990) studied the NS at pH 4.5 and 7.0 using soy protein as a substrate. After exposure to trypsin and chymotrypsin, all hydrolysates from both treatments showed better NS at pH 7.0 than at pH 4.5. In general, all trypsin hydrolysates exhibited higher NS than chymotrypsin hydrolysates at these two different

pH values. Whereas in this study, all chymotrypsin hydrolysates showed a higher NS than trypsin hydrolysates at these two different pH levels (Figs. 12 and 15).

Matsudomi *et al.* (1985) used a mild acid treatment to hydrolyse soy protein. The solubility of the treated soy proteins increased markedly at the isoelectric region (around pH 4.5) due to the increased electrostatic repulsion and the decreased hydrogen bonding. However, the hydrolysates from the mild acid treatment in this study exhibited higher solubility at pH 7.0 than their isoelectric region. Bernardi Don *et al.* (1991) concluded that soy protein treated by bacterial protease (*B. subtilis*) was more soluble than those treated by fungal protease (*A. oryzae*) at the same DH values. This seems to hold true in this study. In addition, Petersen (1991) noticed an increase in solubility of soy protein hydrolysates especially in pH 3-5 if treated with Alcalase 0.6L.

The DH values were found to be negatively correlated (r=-0.7574, p=0.0001; Appendix 13) with NS at pH 7.0 and positively correlated with NS at pH 4.5 (r=0.3926, p=0.0001). As seen from Table 9 and Appendices 9 and 11, proteins that were cleaved to a lesser extent were more soluble at pH 7.0 and were less more soluble at pH 4.5. Nitrogen solubility at pH 4.5 was found to have positive correlation with DH values (r=0.3926, p=0.0351). Samples with higher DH values (ie. cleave to greater extent) will show better NS at pH 4.5.

2. Water Holding Capacity (WHC)

According to Cheftel *et al.* (1985), WHC is related to the progressive hydration of proteins starting from the dry state. The first step involves the adsorption of water

molecule to proteins via polar site binding and is followed by the adsorption of multilayer water. Then the condensation of liquid water occurs and hence the swelling of the protein molecules.

The relationships of %WHC as a function of hydrolysis time for all hydrolysates are presented in Figs. 18, 19 and 20. The values for WHC and the analysis of variance data for this functional parameter are tabulated in Appendix 14 and 15 respectively.

PMM exhibited a %WHC of 111.5% and after treatment with trypsin and chymotrypsin, the resultant hydrolysates showed the highest WHC among all enzyme hydrolysates (Appendix 12). After hydrolysis with trypsin for 5 min, the WHC improved and reached the best state at 10 min. The WHC decreased when the hydrolysis was continued for another 5 or 10 min (Fig. 18). Chymotrypsin treated samples also showed a high WHC. The WHC increased significantly during the initial 15 min and then showed a significant decrease at 20 min (Fig. 18). From the analysis of variance data, the effect of hydrolysis time on WHC for both trypsin and chymotrypsin hydrolysates were significant (F=335.11, p=0.0003 and F=193.75, p=0.0006, respectively).

From Fig. 18, it was noticed that after treatment with fungal protease, the resultant hydrolysates remain quite constant in WHC; however, WHC started to decrease after 15 min of hydrolysis treatment. It was shown that hydrolysis time had exerted an effect on WHC (F=336.03, p=0.0003). For 5, 10 and 15 min hydrolysates, bacterial protease treated samples exhibited the poorest WHC (Fig. 18). However, the hydrolysis time showed an effect on WHC (F=137.23, p=0.0010).

FIGURE 18. The water holding capacity (%) as a function of hydrolysis time (min) for all enzyme hydrolysates.
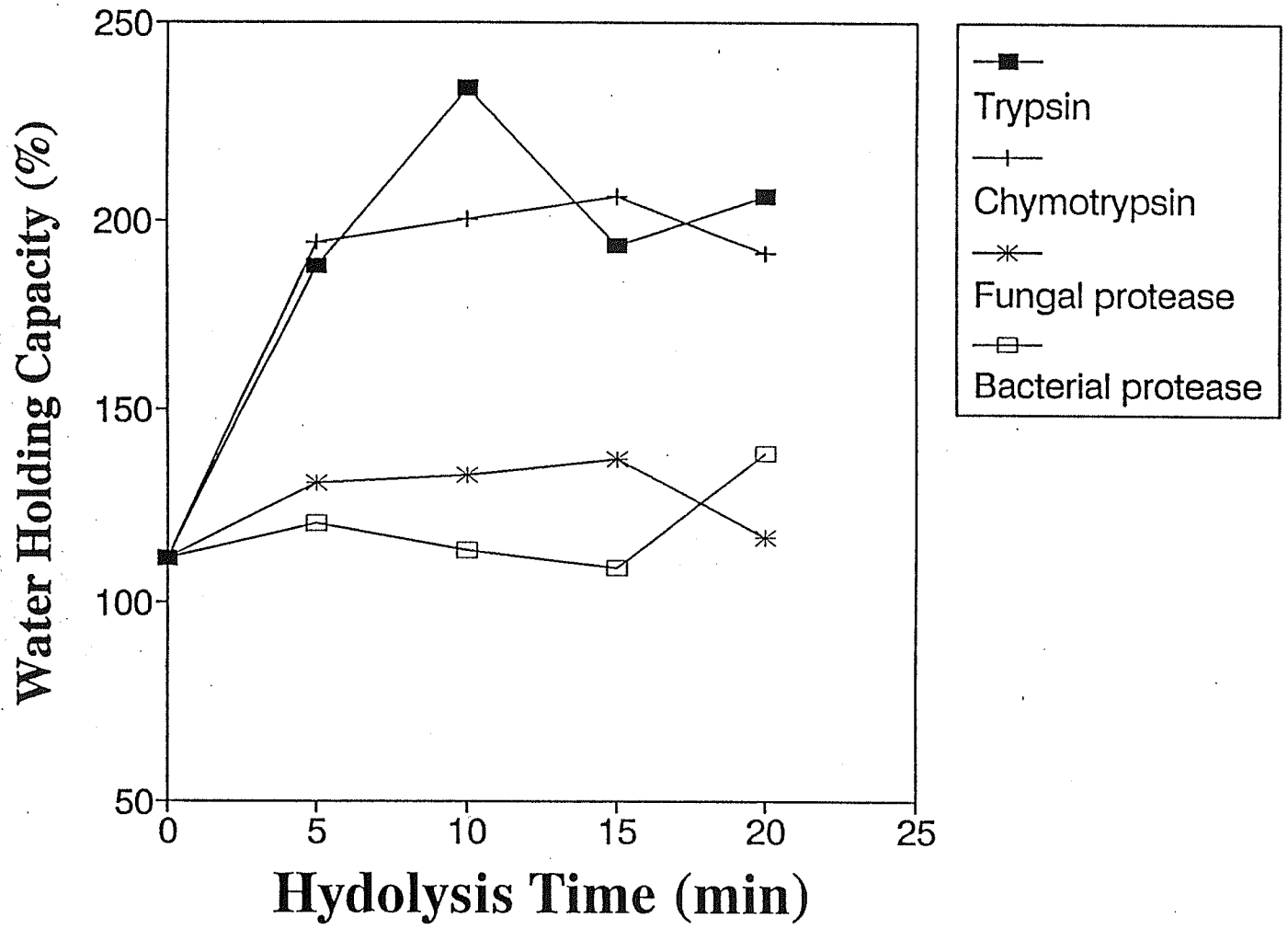
FIGURE 19. The water holding capacity (%) as a function of hydrolysis time (min) for mild acid hydrolysates.

III
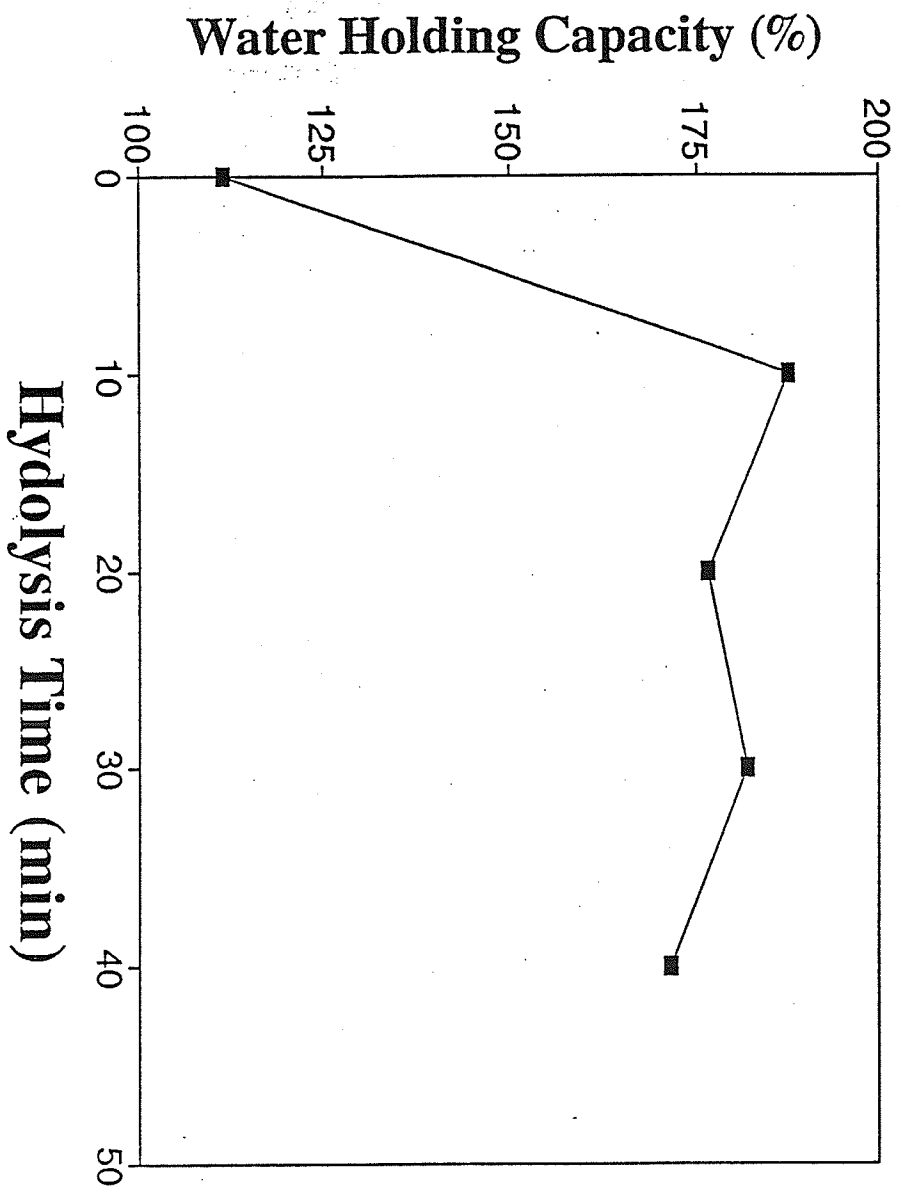
FIGURE 20. The water holding capacity (%) as a function of hydrolysis time (min) for alternate acid hydrolysates.
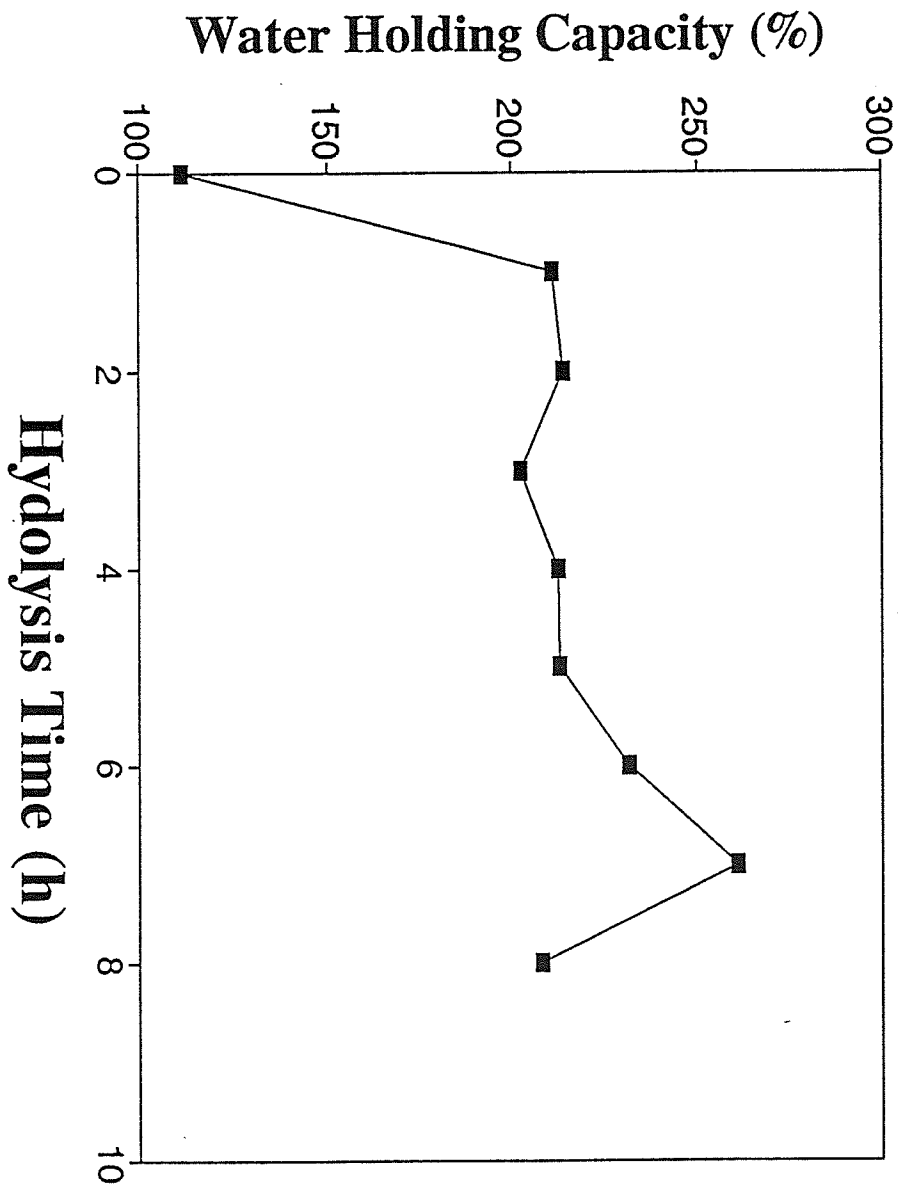
The WHC increased greatly during the initial 10 min of the mild acid treatment and reached the highest value of 187.7% (Fig. 19). Nevertheless, the WHC started to decrease when the hydrolysis was continued. The effect of hydrolysis time on WHC was found to be significant (F=48.32, p=0.0049), and this was also true for the alternate acid hydrolysis (F=8.95, p=0.0049). Both acid treatments improved the WHC of the protein samples. The WHC was increased by at least a factor of two for all the hydrolysates from the alternate acid treatment (Fig. 20). The A7 hydrolysate showed the highest WHC (260.8%, Appendix 14); this was also the sample with highest WHC among all the hydrolysates tested.

Bernardi Don *et al.* (1991) examined the WHC of soy proteins treated by fungal and bacterial proteases. Both hydrolysis treatments did not improve the WHC and a greater decrease in WHC was observed when fungal protease was used. However, both proteases improved the WHC of canola protein (except B15 sample) in this study and the effects on WHC from both proteases were similar.

Puski (1975) used *A. oryzae* to modify soy proteins and he observed a slight increase in water absorption. Mietsch *et al.* (1989) investigated the functional properties of partially hydrolysed soy and milk proteins (by Alcalase 0.6L and Neutrase 0.5L). They found that the WHC decreased in all enzyme hydrolysates tested.

In this study, it was found that WHC values for all hydrolysis treatments were correlated with both thermal parameters, ΔH and Td. The WHC values had a high negative correlation with the ΔH (r=-0.8797, p=0.0001; Appendix 13). As seen from Table 13 and Appendix 14, when the sample was more denatured (ie. lower ΔH values),

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the WHC values increased. In addition, WHC values are also correlated with the thermal denaturation temperature (r=-0.6203, p=0.0003; Appendix 13). When the protein sample was less denatured, a decrease in WHC value was observed (Table 13 and Appendix 14). Therefore, less denatured proteins (all enzyme hydrolysates) had better WHC than more denatured samples (all acid hydrolysates; Table 12 and Appendix 13). It was also concluded that WHC values were correlated with aliphatic hydrophobicity (r=0.3711, p=0.0475; Appendix 13) but had no significant correlation with aromatic hydrophobicity. When the hydrolysates possessed high aliphatic hydrophobicity, the WHC values tend to be higher (Table 14 and Appendix 14). Therefore, hydrolysates possessing higher numbers of surface aliphatic amino acid groups tend to have a higher capacity to absorb water.

3. Fat Absorption Capacity (FAC)

FAC of proteins is also important as it enhances flavour retention and improves mouth-feel in the food preparations. In terms of assessing this parameter, the amount of oil and protein sample, kind of oil, holding, centrifuging conditions, and units of expression have varied from one investigator to another (Nakai and Li-Chan, 1988).

The %FAC for PMM and all hydrolysates are tabulated in Appendix 16 and the analysis of variance data is presented in Appendix 17. The relationships of %FAC as a function of hydrolysis time are shown in Figs. 21, 22 and 23.

As observed from Fig. 21, chymotrypsin hydrolysates showed the highest %FAC among all the enzyme hydrolysates as well as all the hydrolysates tested (Appendix 16).

FIGURE 21. The fat absorption capacity (%) as a function of hydrolysis time (min) for all enzyme hydrolysates.
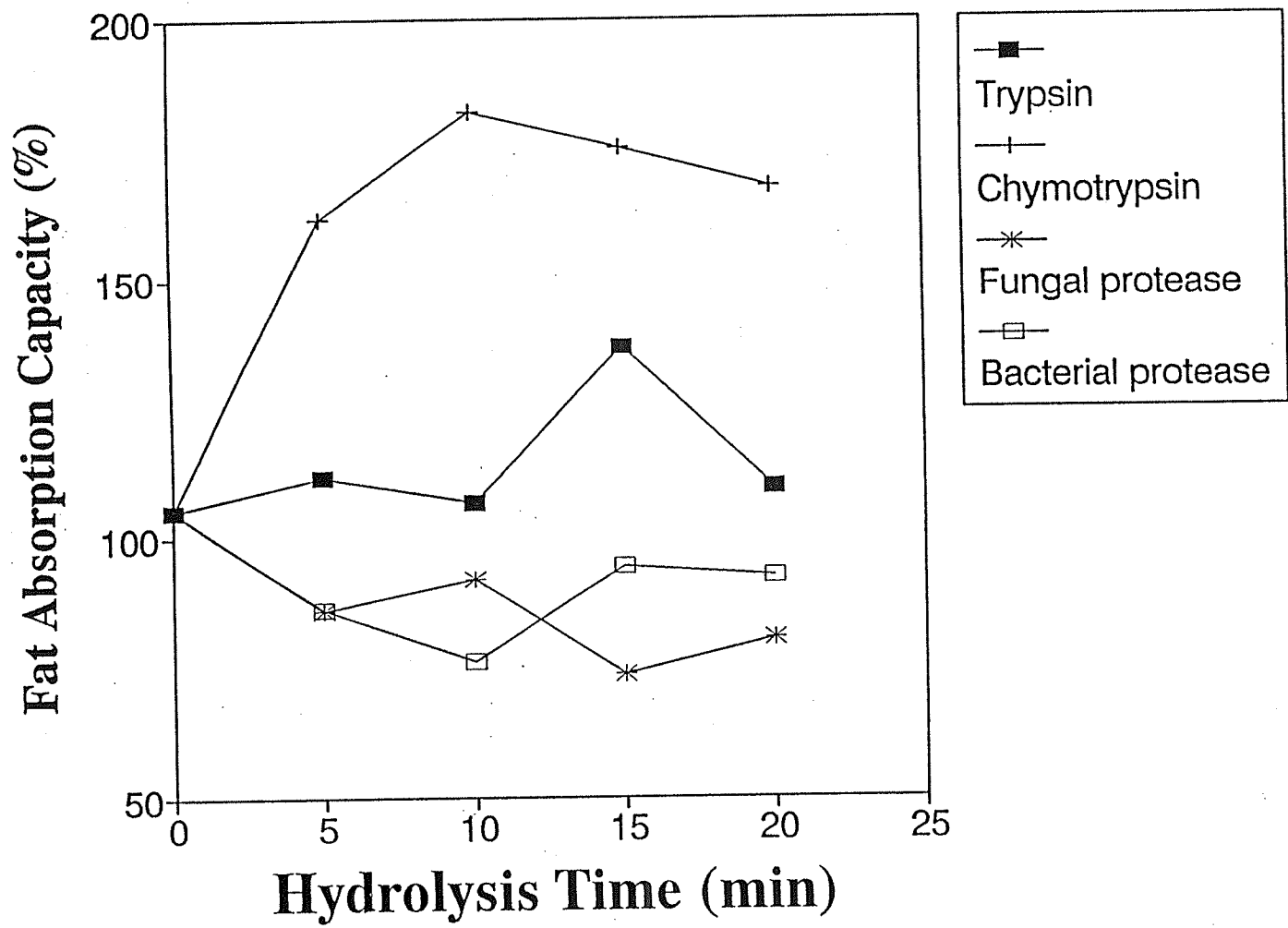
FIGURE 22. The fat absorption capacity (%) as a function of hydrolysis time
(min) for mild acid hydrolysates.
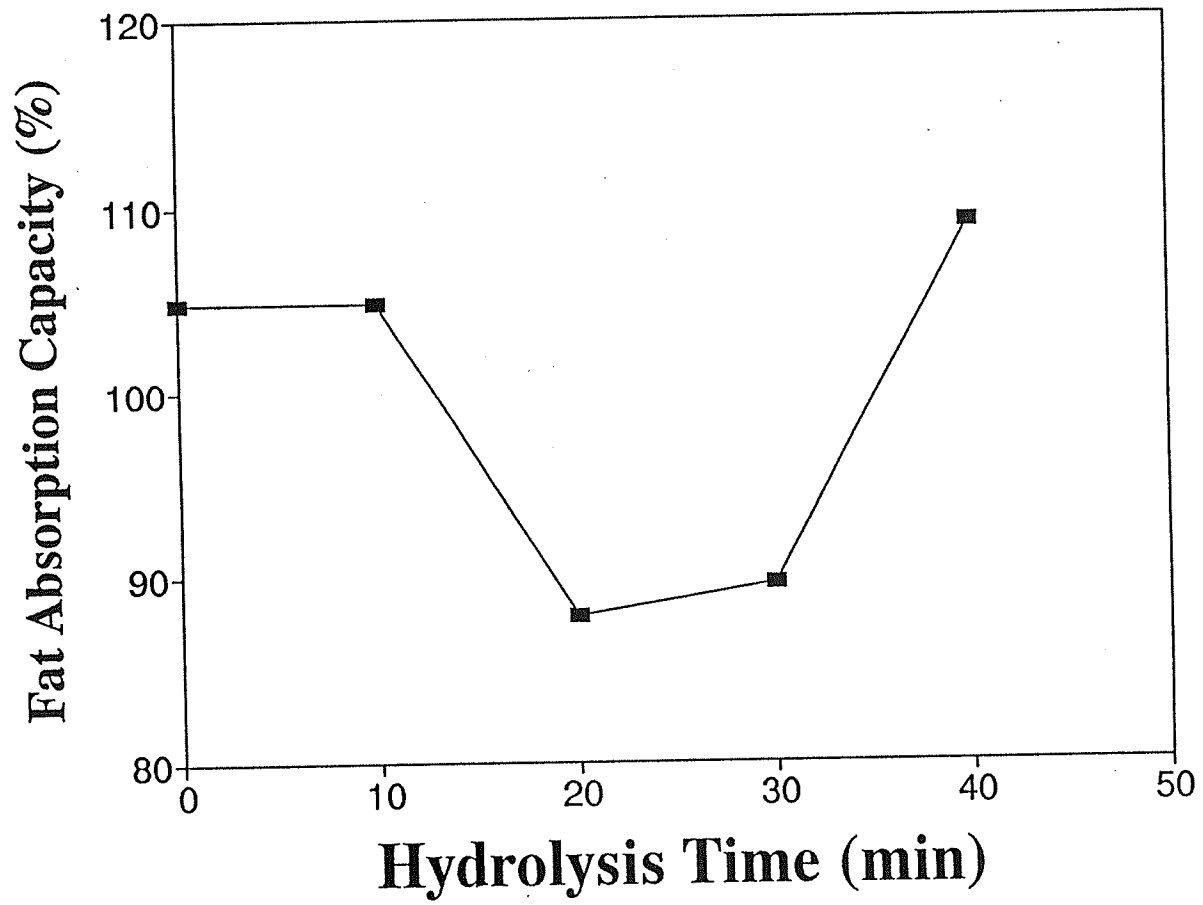
FIGURE 23. The fat absorption capacity (%) as a function of hydrolysis time (min) for alternate acid hydrolysates.
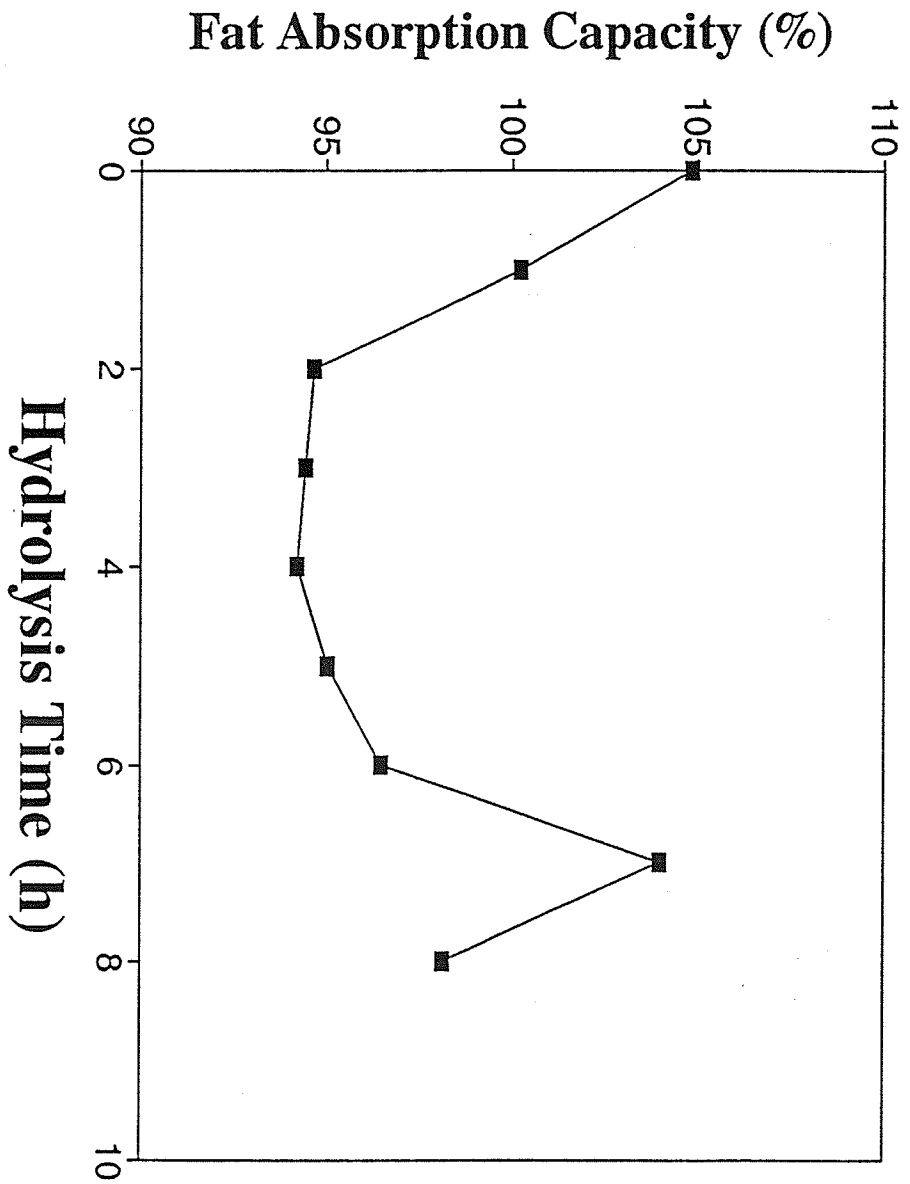
The PMM has only a FAC of 104.9%. After treatment with chymotrypsin, the resultant hydrolysates showed a significantly high %FAC and the effect of hydrolysis time on FAC was found to be significant $(F=119.86, p=0.0013)$.

Samples treated with trypsin remained quite constant in FAC values with the exception of C15, which exhibited the greatest %FAC of 136.6% (Fig. 21). It was found that hydrolysis time did not exert an effect on FAC $(F=8.44, p=0.0566)$ for trypsin hydrolysates.

Both fungal and bacterial protease treated samples showed much lower FAC values (range from 73.6-94.1%) (Fig. 21). Hydrolysis time exerted significant effect on FAC for both bacterial and fungal protease treated samples $(F=20.71, p=0.0166$ and $F=58.11, p=0.0037$, respectively). For the mild acid treatment, the FAC remained unchanged during the first 10 min of hydrolysis (Fig. 22). The FAC then decreased when the reaction proceeded for another 10 min and later remained constant when the hydrolysis time reached 30 min. After that, the FAC was improved significantly in A40 although the FAC was similar to that of the PMM. Hydrolysis time was found to have a significant effect on %FAC $(F=120.03, p=0.0013)$.

As shown in Fig. 30, the FAC for all hydrolysates from the alternate acid treatments stayed relatively constant. It was shown that the effect of hydrolysis time was not significant on FAC $(F=0.52, p=0.7941)$.

The mechanism of fat absorption is not clear. However, fat absorption has been attributed mostly to physical entrapment of the oil, and FAC could be correlated with increasing bulk density of the protein sample (Nakai and Li-Chan, 1985).

Protein-lipid interactions are affected by various factors including protein conformation, protein-protein interactions, and the spatial arrangement of the liquid phase resulting from the lipid-lipid interaction (Nakai and Li-Chan, 1988). Noncovalent interactions such as hydrophobic, electrostatic, and H-bonds are involved in the protein-lipid interactions. Voutsinas and Nakai (1983) found that there was correlation between FAC and aliphatic hydrophobicity and solubility. In this study, however, FAC was only correlated with NS at pH 4.5 ($r=0.5026$, $p=0.0064$). From Appendices 10 and 18, it can be found that samples with lower NS at this pH had lower FAC values. Fat absorption capacity was found to have positive correlation with DH values ($r=0.4767$, $p=0.0089$); therefore, hydrolysates that were cleaved to greater extent will exhibit better fat absorption properties.

Bernardi Don *et al.* (1991) examined the FAC of soy protein hydrolysed with fungal and bacterial proteases. It was found that only bacterial protease greatly improved the FAC; however, both proteases had similar effects on the FAC of canola protein in this study.

4. Emulsion Stability (ES)

Many food products are emulsions and protein constituents often play a major role in stabilizing these colloidal systems (Cheftel *et al.*, 1985). Proteins are generally poor stabilizers of water oil (W/O) emulsions due to the predominantly hydrophilic nature of most proteins, causing the bulk of an adsorbed protein molecule to reside on the water side of the interface (Cheftel *et al.*, 1985).

FIGURE 24. The emulsion stability (%) as a function of hydrolysis time (min) for all enzyme hydrolysates.
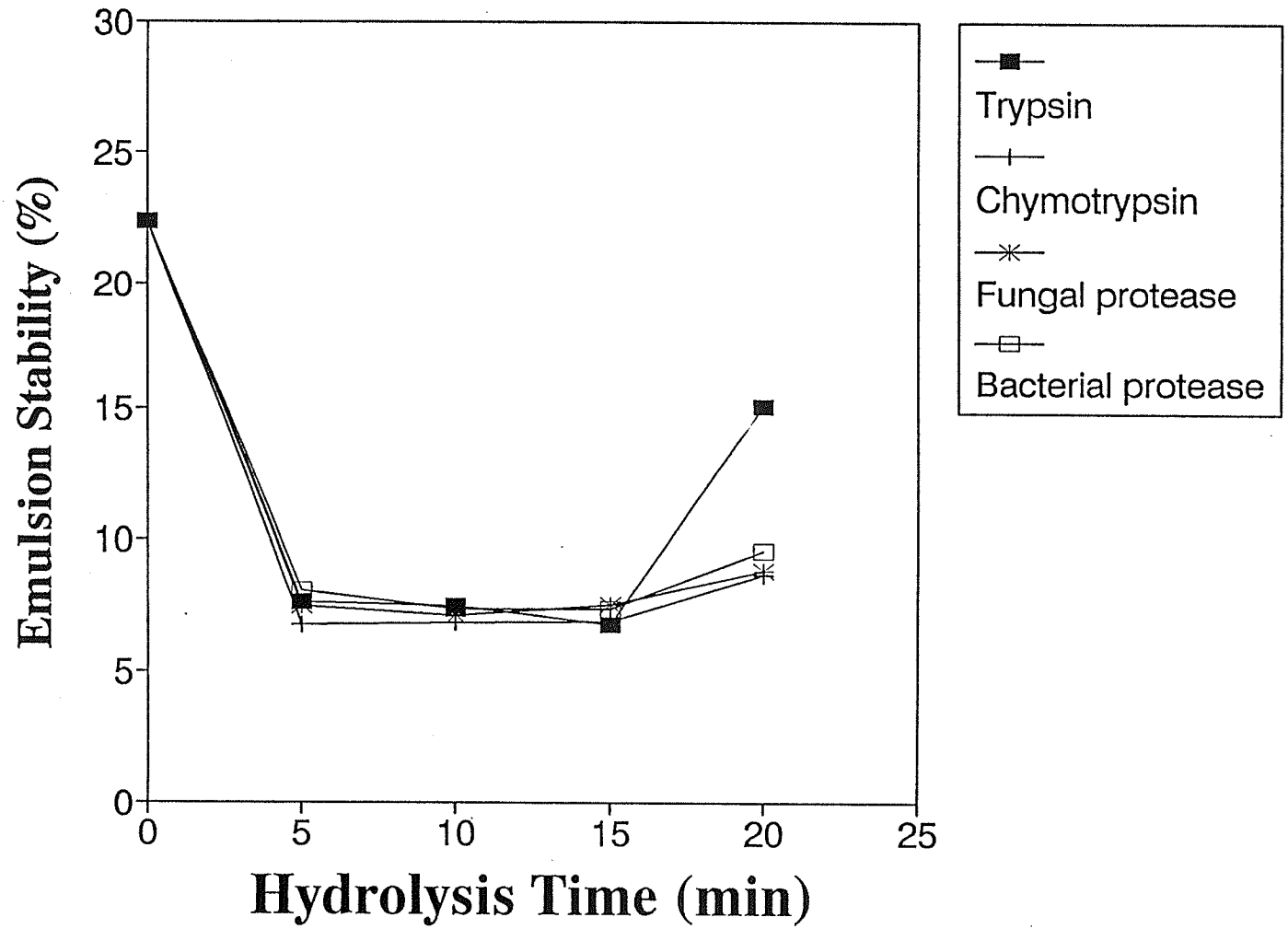
FIGURE 25. The emulsion stability (%) as a function of hydrolysis time (min) for mild acid hydrolysates.
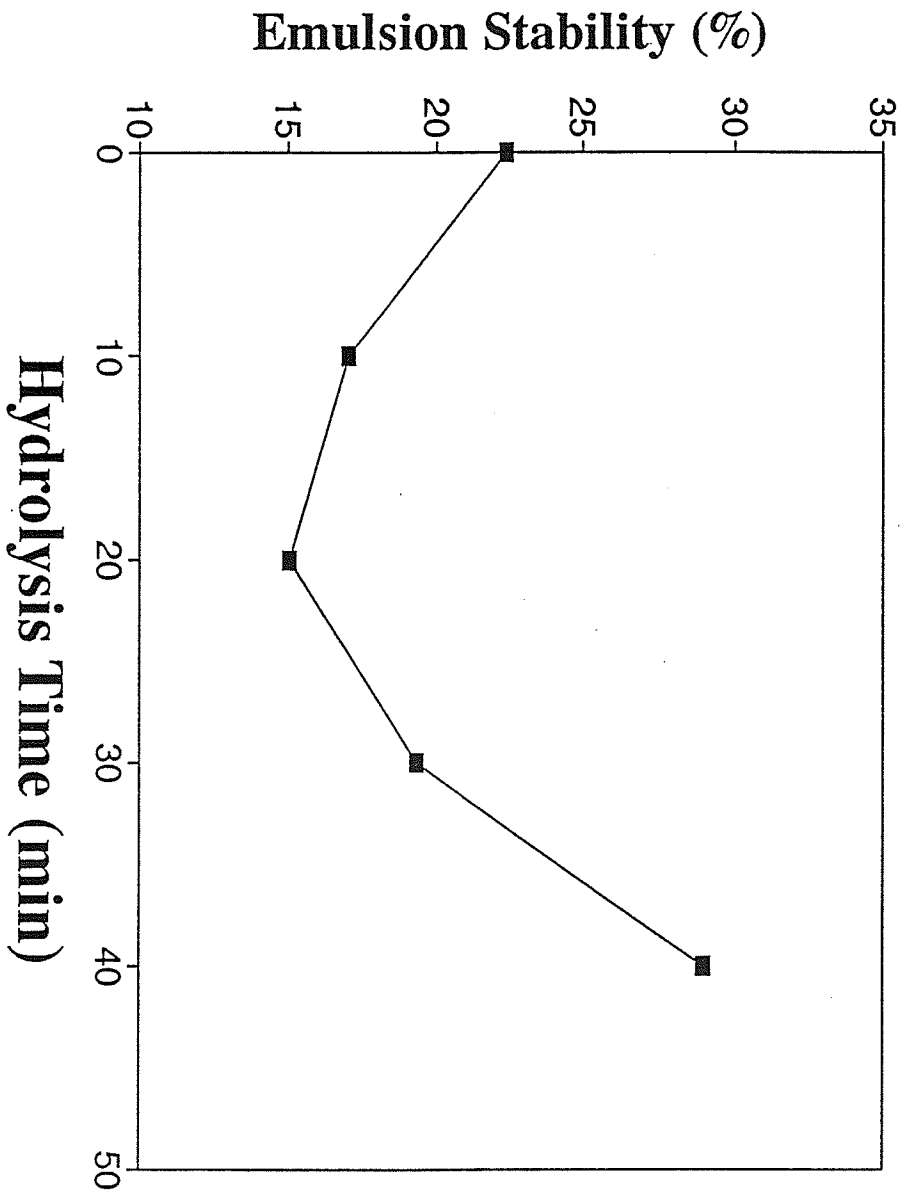
FIGURE 26. The emulsion stability (%) as a function of hydrolysis time (min) for alternate acid hydrolysates.
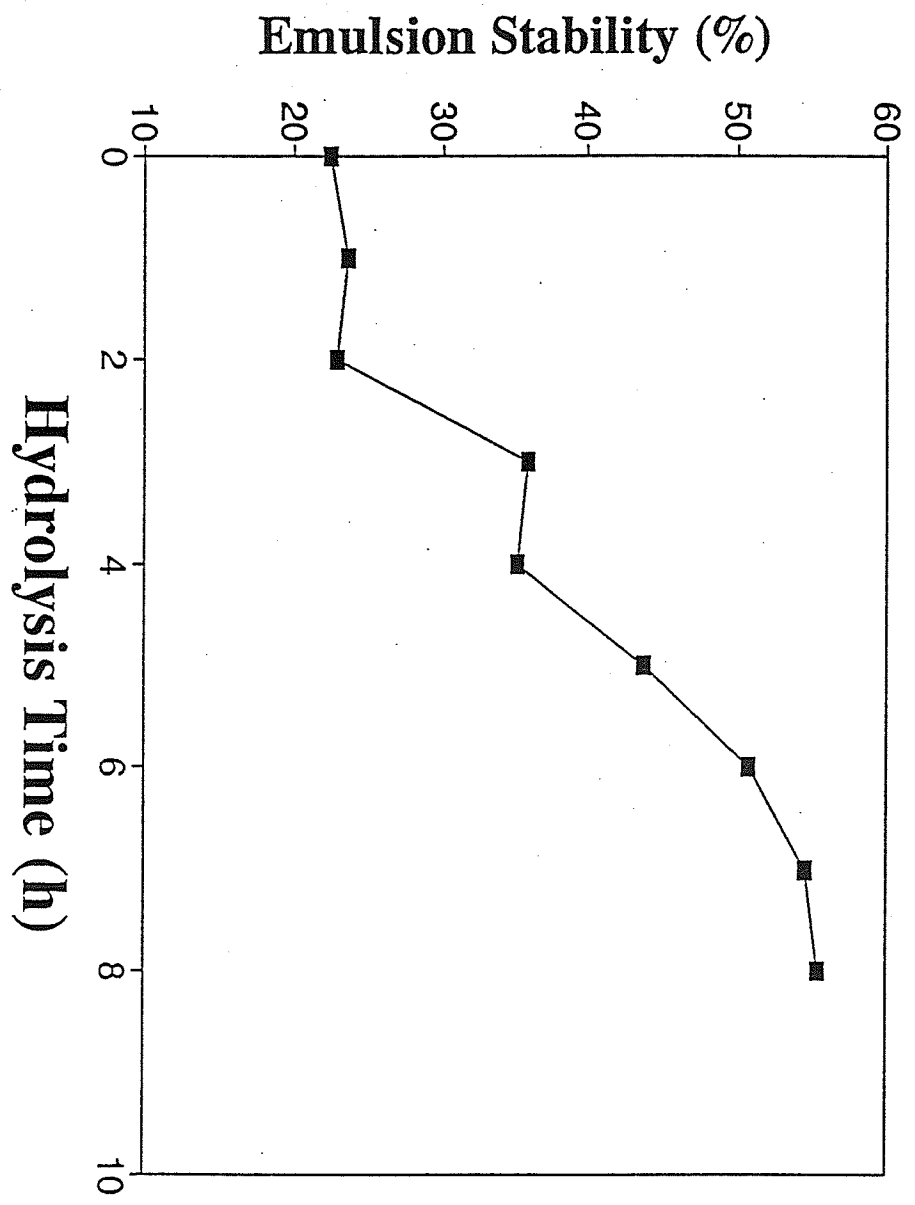
Figures 24, 25 and 26 show ES as a function of hydrolysis time. The ES values for PMM and all hydrolysates are tabulated in Appendix 18 and the analysis of variance data is given in Appendix 19.

PMM had an ES of 22.4%. From Fig. 24, all enzyme hydrolysates exhibited a decreased ES after the first 5 min of hydrolysis. The ES remained quite constant throughout the whole hydrolysis treatment for fungal and bacterial proteases. The analysis of variance data showed that no significant effect from hydrolysis time for both fungal and bacterial proteases treatments on the ES (F=0.81, p=0.5683 and F=7.88, p=0.0620, respectively).

Chymotrypsin treated samples showed the lowest ES (6.8%) at 5 min and then increased to 28.4% at 10 min (Fig. 24). Then the ES dropped significantly and remained constant. Hydrolysis time had a significant effect on the ES for chymotrypsin (F=179.53, p=0.0007) as well as for trypsin (F=10.7, p=0.0413). Overall, trypsin and chymotrypsin treatments gave a negative impact on ES values (with exception of the C10 sample) and a significant decrease in ES values was observed. Despite the large decrease in ES in trypsin hydrolysates during the initial 5 min of treatment, the ES remained quite constant. After 15 min, the ES increased significantly (Fig. 24).

With the mild acid treatment, the ES values decreased significantly during the initial 30 min and then improved significantly until the highest ES was observed with the A40 sample (29.0%) (Fig. 25). Significant effects of hydrolysis time on ES were noted (F=18.56, p=0.0193).

During the first 2 h of hydrolysis of the alternate acid treatment, the ES remained

constant and improved significantly when the hydrolysis time reached 3 h (Fig. 26). The ES remained constant until 4h of hydrolysis; however, the ES values increased significantly when the hydrolysis proceeded for more than 4 h. The effect of hydrolysis time was significant on ES for the alternate acid treatment ($F = 57.15$, $p = 0.001$).

A positive correlation between protein solubility and the ability of a protein to emulsify and stabilize an emulsion has been reported in many studies (Crenwelge *et al.*, 1974; Volkert and Klein, 1979; Yasumatsu *et al.*, 1972). Undissolved protein contributes very little to emulsification due to the fact that proteins must dissolve and migrate to the interface before their surface properties come into play. However, many authors point to evidence that emulsifying properties and solubility are not well correlated (Aoki *et al.*, 1980; McWatters and Holmes, 1979; Voutsinas *et al.*, 1983). In this study, ES values are found to have a significantly positive correlation with nitrogen solubility at pH 7.0 ($r = 0.8272$, $p = 0.0001$; Appendix 13).

All protein samples were solubilized in 0.1 M sodium phosphate buffer pH 7.0 in the ES testing procedure. The isoelectric point for PMM is pH 6.6. All enzyme hydrolysates had isoelectric points in the range of pH 6.3-6.7 whereas all acid hydrolysates possessed isoelectric points between the pH 4.4-5.7. From Appendix 11, it was noted that all enzyme hydrolysates exhibited poorer NS at pH 7.0 than most acid hydrolysates from both mild and alternate acid treatments. The higher ES values for acid hydrolysates are perhaps due to the higher soluble protein in the buffer which contributed to stabilizing of the surface charge of the oil droplets. As seen from Appendix 18, all acid hydrolysates exhibited higher ES values than all enzyme hydrolysates. This has

shown that initial solubility is important for the emulsion properties. Charge repulsion of emulsion droplets is minimal near the isoelectric point, resulting in decreased stability (Mangino, 1994) and this has clearly explained the poorer ES of the enzyme hydrolysates.

According to Cheftel *et al.* (1985), many factors influence the characteristics of emulsions and the results of emulsion tests. Some of the factors are : equipment type and geometry, rate of oil addition, oil phase volume, temperature, pH, ionic strength, kind of oil and concentration of soluble protein. Therefore, without standardization, emulsification results for a particular protein from different investigators cannot be compared.

The more hydrophobic the protein, the greater the concentration of protein at the interface, the lower the interfacial tension, and the more stable the emulsion (Cheftel *et al.*, 1985). As mentioned previously, surface hydrophobicity has been positively correlated with emulsifying activity (Nakai *et al.*, 1980). This close relationship can be explained by easier transfer of more hydrophobic protein molecules to the oil/water interface, thereby preventing the coalescence of oil droplets. However, Damodaran (1994) suggested this is not an absolute relationship. Shimizu *et al.* (1985) studied the emulsifying properties of β-lactoglobulin and they found that the emulsifying parameters are better above pH 7 than at pH 3, whereas the surface hydrophobicity is greater at pH 3 than at pH 7. Such discrepancies indicate that molecular factors other than surface hydrophobicity are also important in the expression of emulsifying properties. These include the disjoining forces arising from electrostatic, hydration repulsion, and steric

interaction between the loops of the adsorbed protein molecules at the interface (Damodaran, 1994).

Kato and Nakai (1980) used CPA as a measure of surface hydrophobicity; however, this is not necessarily directly correlated with the total content of hydrophobic amino acids in the protein molecule. Pearce and Kinsella (1978) measured emulsifying activity index (EAI, m^2/g) and emulsion stability index (ESI, min) for native and denatured samples of several seed proteins. They concluded that the emulsifying properties of proteins ultimately depend on the balance between the hydrophile and lipophile, and do not necessarily increase linearly as the proteins become more hydrophobic.

Matsudomi *et al.* (1985) concluded that the emulsifying properties of the mild acid treated soy protein were increased in proportion to the increase of aliphatic hydrophobicity. Vojdani and Whitaker (1994) studied the chemical and enzymatic modification of proteins and they concluded that emulsion activity is affected by surface hydrophobicity and emulsion stability is affected by electrostatic repulsive forces near the isoelectric point. In this study, ES values were not correlated with aromatic hydrophobicity but were correlated positively with aliphatic hydrophobicity for all six different hydrolysis treatments (r=0.4003, p=0.0314; Appendix 13).

Petersen (1991) stated that the emulsifying capacity of soy protein can be increased considerably by enzymic hydrolysis (Alcalase 0.6L); however, the control of the proteolytic process is needed so that the optimal functional properties can be obtained. Mietsch *et al.* (1989) concluded that the emulsifying activity of hydrolysates

with low DH values (below 10%) is greatly improved. It was also concluded that the ES values in this study were negatively correlated with the DH values ($r=-0.6737$, $p=0.0001$; Appendix 13). As observed from Table 9 and Appendix 18, samples with higher DH values had less ability to stabilize the emulsion compared to the samples with lower DH values. Puski (1975) also examined the emulsifying properties of soy protein treated with *A. oryzae* and he stated that emulsion capacity was increased whereas the emulsion stability was reduced. Puski (1975) stated that emulsion capacity increased with enzyme treatment whereas ES decreased. This is due to the fact that enzyme digestion of proteins will increase the number of peptide molecules available at the oil-water interface, and therefore a larger area may be "covered" resulting in the emulsification of more oil. However, since these peptides are smaller and less globular, they will form a "thinner" protein layer around the oil droplets resulting in an emulsion with less stability. In this study, all hydrolysates from all enzyme treatments (except the C10 sample) exhibited an decrease in ES values.

The ES values were found to be negatively correlated with the temperature of denaturation ($r=-0.7408$, $p=0.0001$; Appendix 13) and the enthalpy of denaturation ($r=-0.5196$, $p=0.0039$; Appendix 13). From Table 12, it was shown that all enzyme hydrolysates were denatured in the range of 79.4-83.0 °C whereas all acid hydrolysates could be denatured at much lower temperatures (62.2-68.6 °C). All acid hydrolysates exhibited higher ES values than all enzyme hydrolysates and therefore the suggestion can be made that less denatured proteins will exhibit poorer ES values.

5. Foaming Properties

Food foams are usually dispersions of gas bubbles in a continuous liquid or semisolid phase that contains a soluble surfactant (Cheftel *et al.*, 1985). According to Damodaran (1994), the basic requirements for a protein to be a good foaming agent are the ability to (a) rapidly adsorb at the air/water interface during whipping or bubbling, (b) undergo rapid conformation change and rearrangement at the interface, and (c) form a cohesive viscoelastic film via intermolecular interactions.

a. Foaming Capacity (FC). Figures 27, 28 and 29 show FC as a function of hydrolysis time. The FC values for PMM and all hydrolysates are tabulated in Appendix 20 and the analysis of variance is presented in Appendix 21.

Among all enzyme treatments, the effects of hydrolysis time on FC were significant for trypsin, chymotrypsin and bacterial protease treated samples ($F=69.66$, $p=0.0028$, $F=25$, $p=0.0127$ and $F=12$, $p=0.0354$, respectively) whereas fungal hydrolysates exhibited FC patterns which were not significantly affected by the hydrolysis time ($F=0.41$, $p=0.7599$ for fungal).

Bacterial hydrolysates showed a significant increase in FC during the initial 5 min and remained constant for another 10 min (Fig. 27). The FC then increased sharply when the hydrolysis time reached 40 min. Fungal hydrolysates also showed improved FC during the initial 5 min of hydrolysis (Fig. 27). The FC values then remained constant even with an increase in hydrolysis time. The highest FC among all enzyme hydrolysates was obtained in the 10 min trypsin hydrolysate (155%); however, the FC

FIGURE 27. The foaming capacity (%) as a function of hydrolysis time (min) for all enzyme hydrolysates.
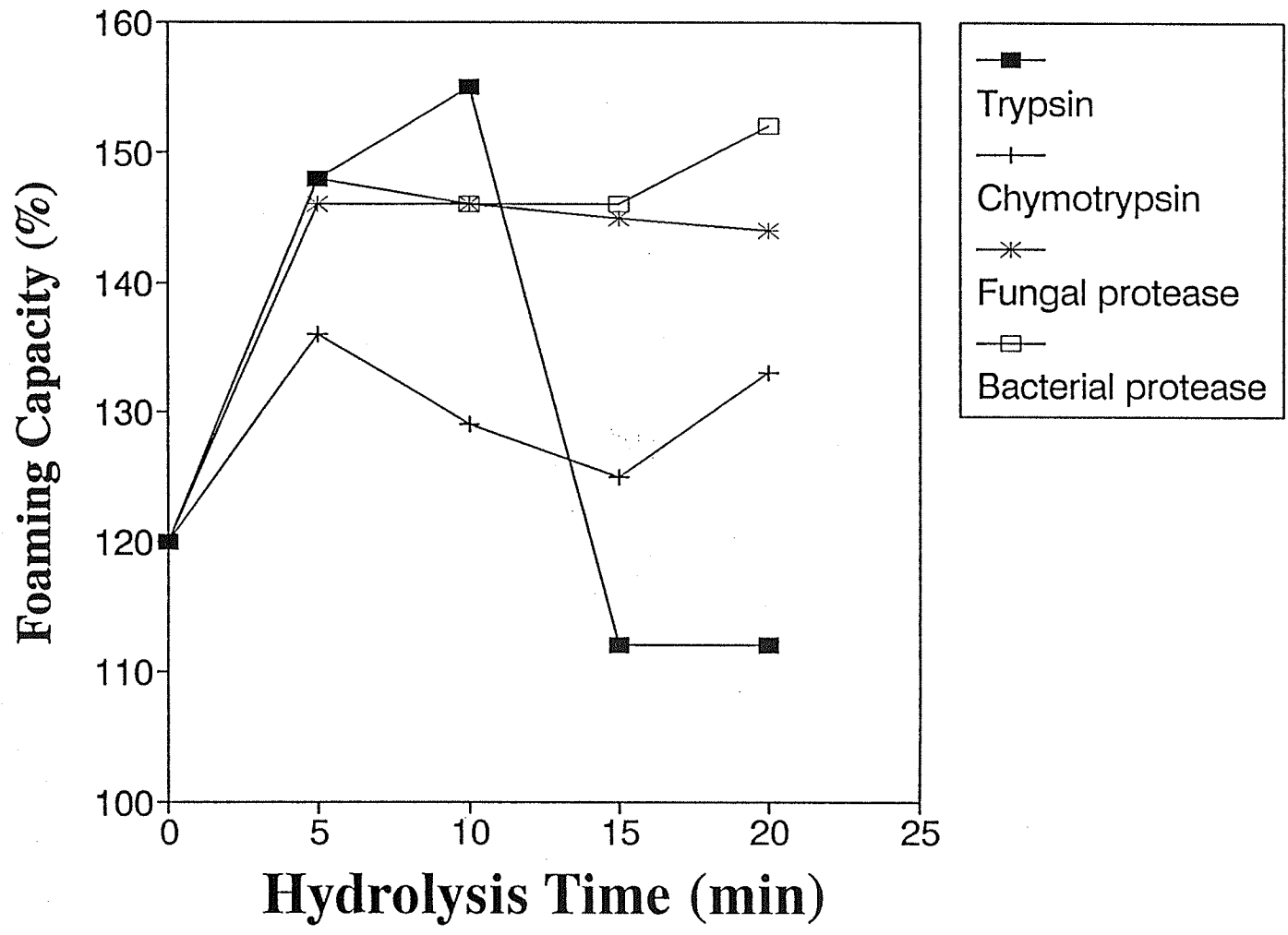
FIGURE 28. The foaming capacity (%) as a function of hydrolysis time (min) for mild acid hydrolysates.
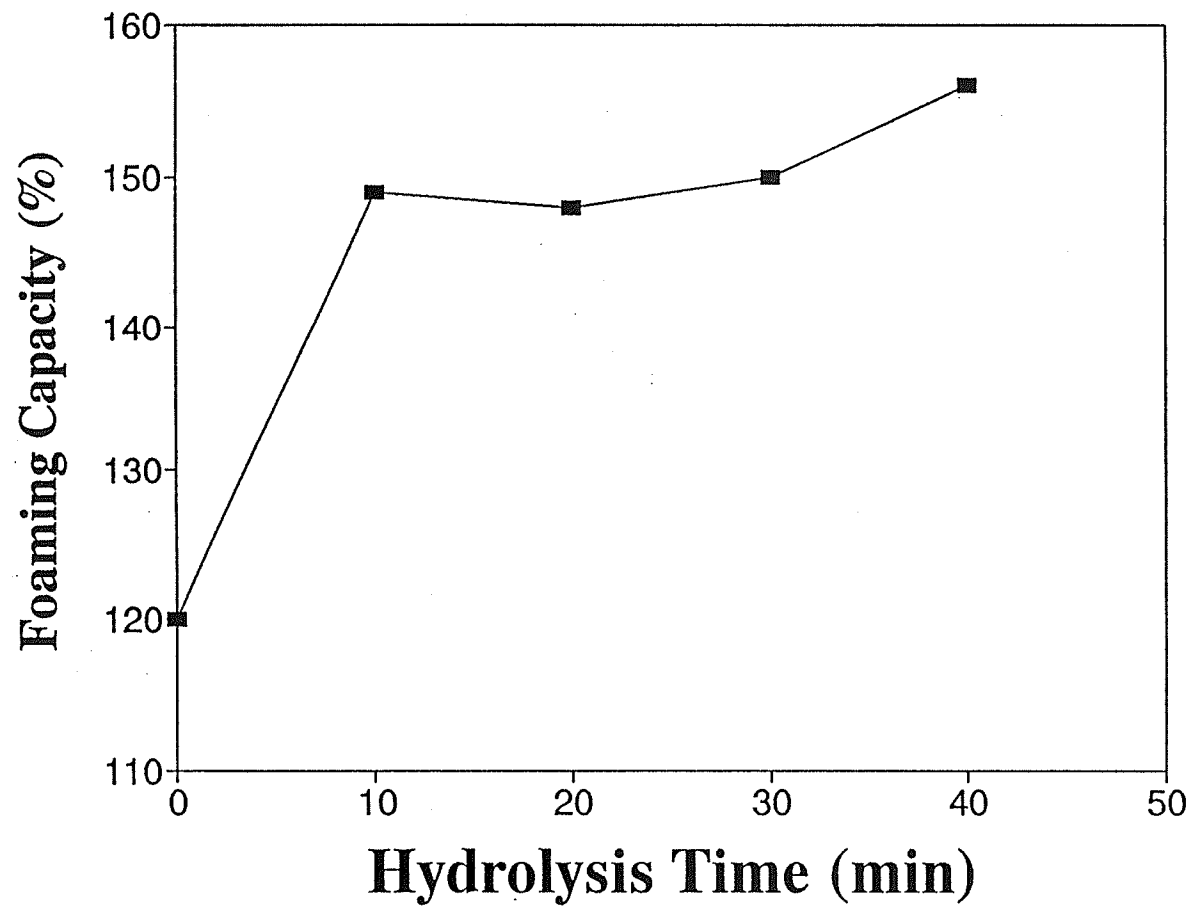
FIGURE 29. The foaming capacity (%) as a function of hydrolysis time (min) for alternate acid hydrolysates.
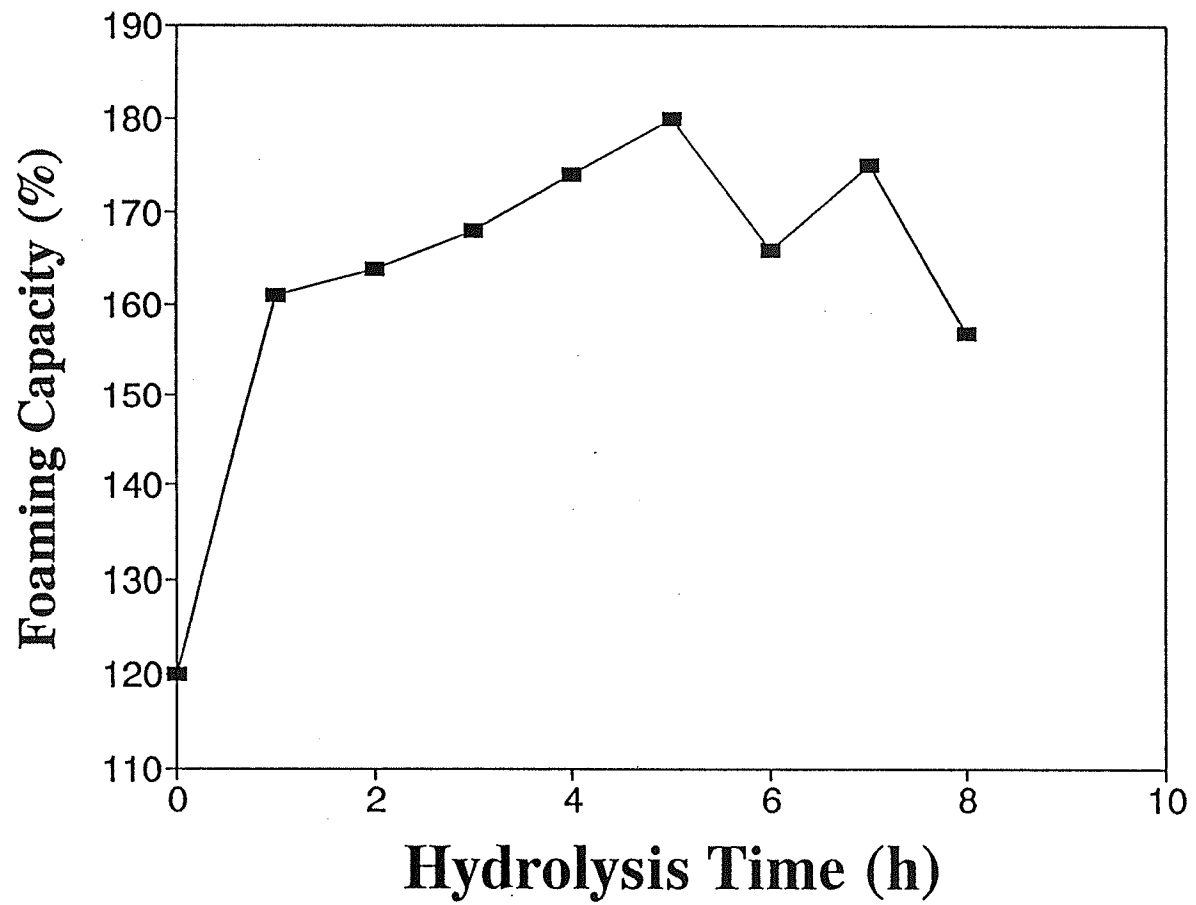
was reduced significantly when the hydrolysis was carried out for more than 10 min (Fig. 27). The FC was improved in chymotrypsin treatment during the initial 5 min, then the FC values reduced significantly. The FC value was improved when the hydrolysis reached 20 min.

For both acid treatments, the hydrolysis time had a significant effect on FC ($F=51.67$, $p=0.0044$ for mild acid; $F=13.14$, $p=0.0015$ for alternate acid treatment). The FC increased significantly during the initial 10 min of mild acid hydrolysis and then remained constant until the hydrolysis reached 40 min where a significant increase in FC was observed (Fig. 28). Overall, the alternate acid treatment yielded hydrolysates that had an excellent FC. The FC increased significantly during the first 1 h of hydrolysis and remained constant for another 1 h (Fig. 29). Then, a significant increase was observed in FC for the A3, A4 and A5 samples. However, a sharp decrease in FC was noted in the A6 sample and then the FC value was increased significantly in the A7 sample. Nevertheless, the FC decreased significantly when the hydrolysis reached 8 h.

b. Foaming Stability (FS). The FS values for PMM and all hydrolysates are tabulated in Appendix 22 and the relationships of FS as a function of hydrolysis time are presented in Fig. 30, 31 and 32. The analysis of variance data is shown in Appendix 23.

Before hydrolysis, PMM had an FS value of 11.7%. When PMM was subjected to different hydrolysis treatments, various FS patterns were observed. Both bacterial hydrolysates and fungal hydrolysates had the best FS values (Fig.30). The hydrolysis time had no significant effect on FS for fungal hydrolysates ($F=0.55$, $p=0.6804$) whereas a significant effect from hydrolysis time was observed in bacterial hydrolysates

FIGURE 30. The foaming stability (%) as a function of hydrolysis time (min) for all enzyme hydrolysates.
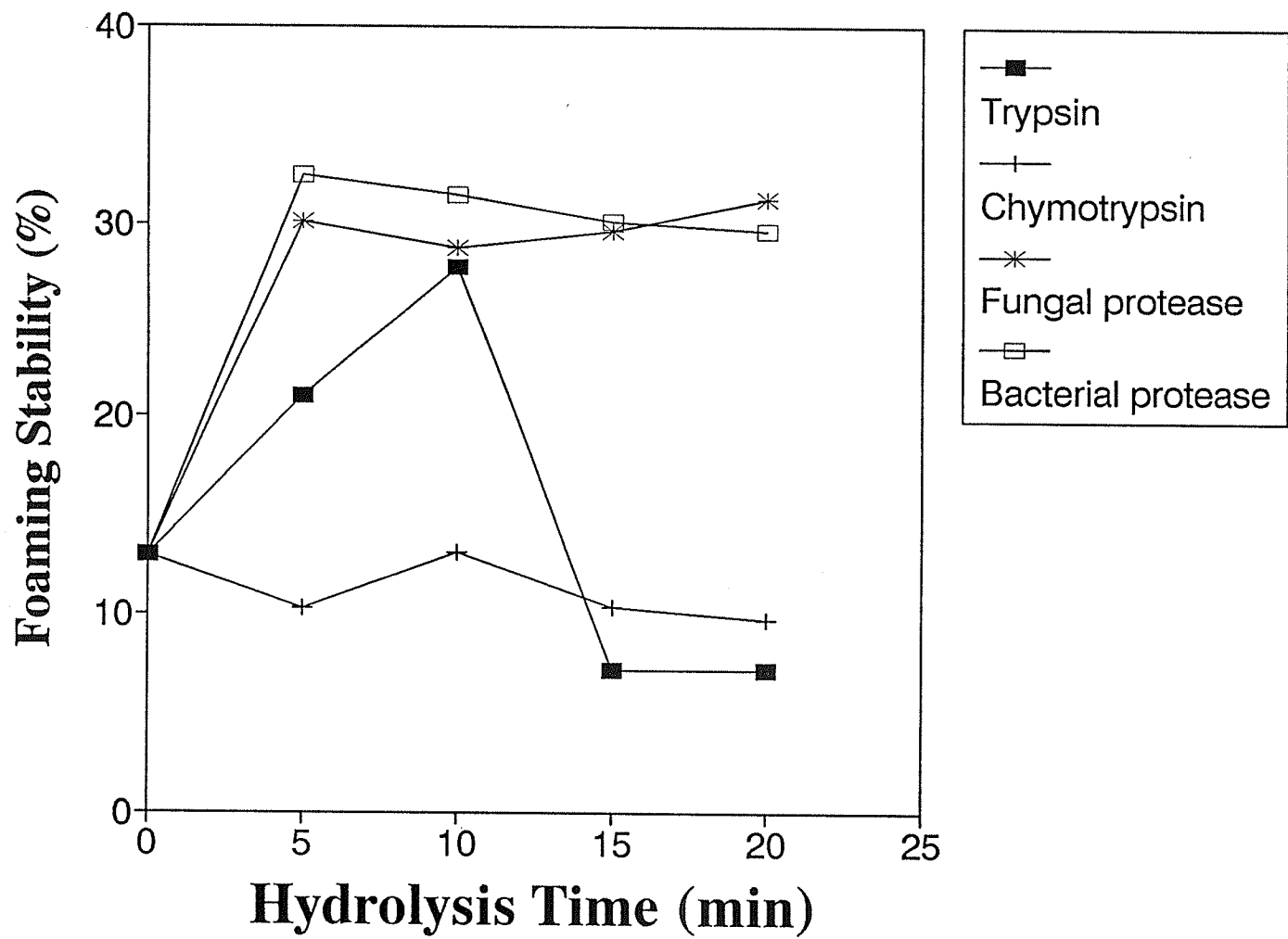
FIGURE 31. The foaming stability (%) as a function of hydrolysis time (min) for mild acid hydrolysates.
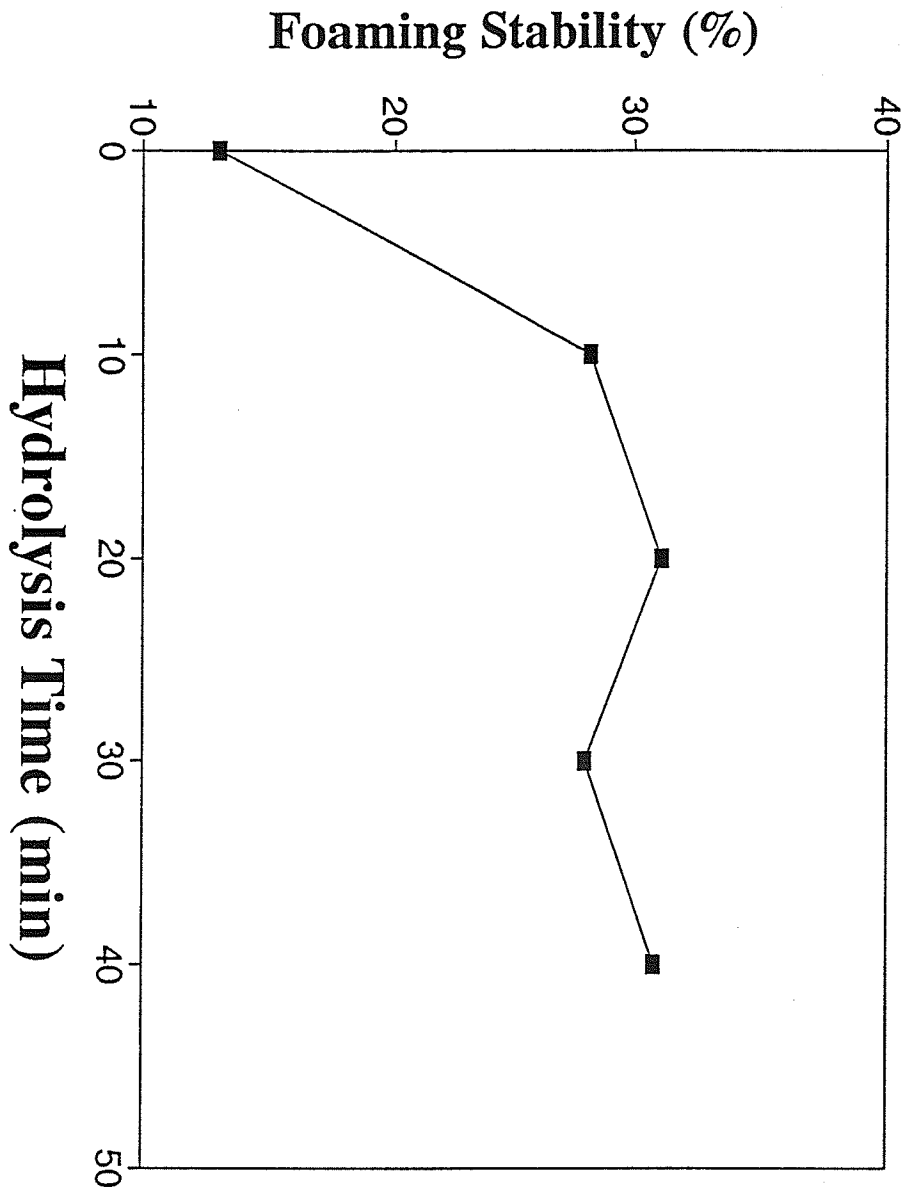
FIGURE 32. The foaming stability (%) as a function of hydrolysis time (min) for alternate acid hydrolysates.
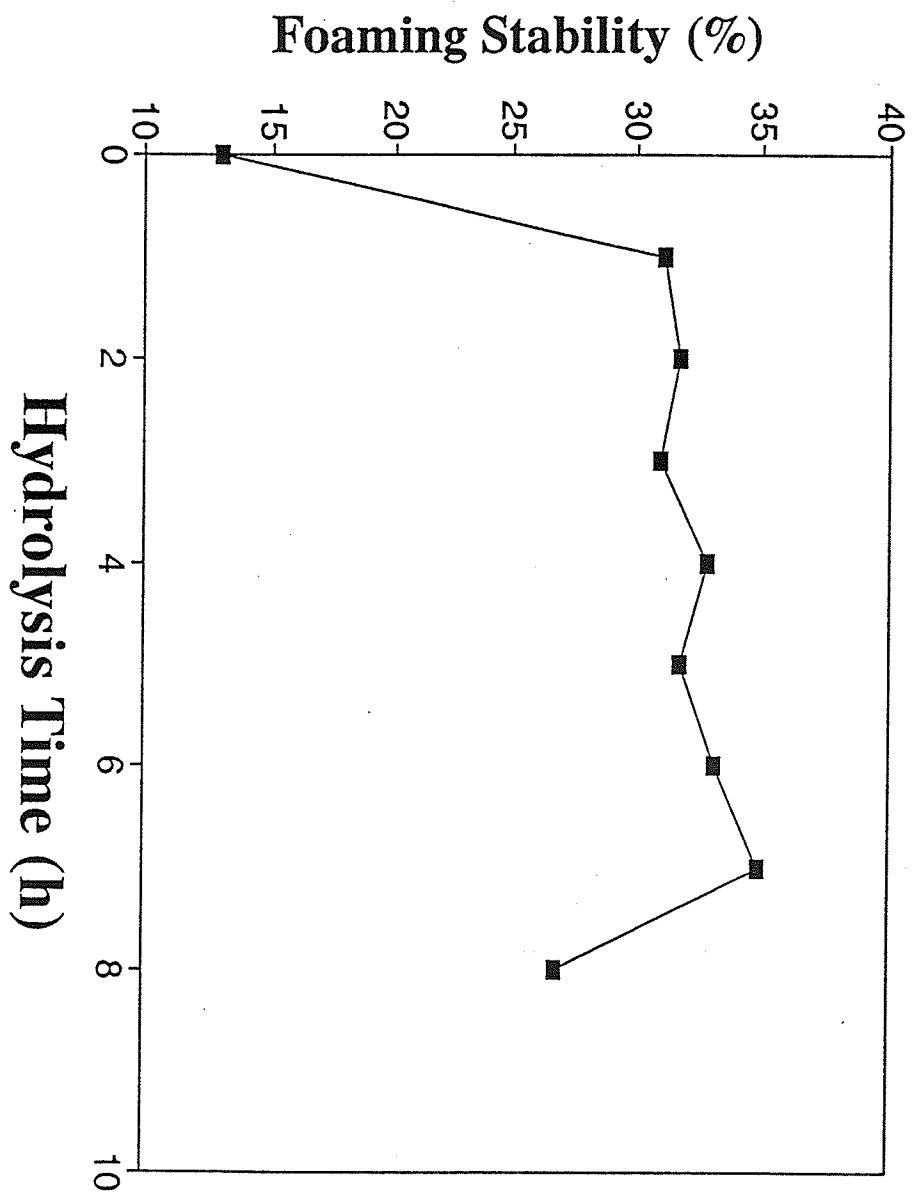
(F=15.39, p=0.0251).

Trypsin treated samples showed a significant increase in FS during the first 10 min of incubation; however, after 10 min of hydrolysis, the FS showed a significant decrease which remained constant after 15 min (Fig. 30). Chymotrypsin hydrolysates showed the lowest FS values. The effects of hydrolysis time on FS for trypsin and chymotrypsin hydrolysis were significant (F=6815.61, p=0.0001; F=31.44, p=0.0091, respectively).

Both acid treatments generally improved the FS values by a factor of approximately three for all acid hydrolysates (except A10, A30 and A8) (Figs. 31 and 32). Hydrolysis time exerted significant effects on the FS values obtained (F=272.92, p=0.0004 for mild acid; F=6.74, p=0.0111 for alternate acid).

According to Nakai and Li-Chan (1988), the foaming behaviour of proteins is affected by several factors including pH, temperature, the presence of salts, sugars, lipids, and the protein source. Cheftel *et al.* (1985) mentioned that many researchers emphasize the necessity of high protein solubility as a prerequisite to good foaming capacity and stability, it also seems that insoluble protein particles can play a beneficial role in stabilizing foams due to the increase in surface viscosity. Some proteins exhibited good FS (despite poor FC) at their isoelectric point due to the fact that at their isoelectric points, the electrostatic intermolecular attractions increase the thickness and rigidity of the protein films adsorbed at the air/water interface. However, an increase in foam stability of some proteins is also observed at extremes of pH due to the increase in viscosity (Cheftel *et al.*, 1985). In this study, it was found that there were correlations

between both foaming properties and nitrogen solubility at pH 7.0 (Appendix 13). However, FC values were more significantly correlated with nitrogen solubility at pH 7.0 ($r=0.7632$, $p=0.0001$) whereas FS values were less significantly correlated with nitrogen solubility at pH 7.0 ($r=0.4587$, $p=0.0123$).

Matsudomi *et al.* (1985) also concluded that the maximum FC and FS of the treated protein was reached by heat treatment for 30 min at 95°C. However, both foaming properties decreased when the heating time was prolonged further. In addition, it was also suggested that foaming properties are improved due to an increase in the surface hydrophobicity (Kato and Nakai, 1980). However, Townsend and Nakai (1983) found no significant relationship between aliphatic hydrophobicity of proteins and their FC; this also holds true in this study (Appendix 13). Furthermore, both FC and FS had no correlations with aromatic hydrophobicity. In addition, FS values were found to be correlated significantly with FAC values ($r=-0.7793$, $p=0.0001$) whereas FC values were less correlated with FAC values ($r=-0.4924$, $p=0.0067$). As seen from Appendices 16 and 22, samples exhibiting lower FAC values will have more tendency to stabilize the foam.

Bernadi Don *et al.* (1991) modified soy protein concentrates using fungal and bacterial proteases. Bacterial protease had more impact on FC whereas fungal protease had more effect on FS. The better FC of the bacterial protease treated sample could be related to the higher solubility and hydrophobicity.

Vojdani and Whitaker (1994) suggested that FC is affected by protein stability, while FS is influenced primarily by protein-protein interactions, which is modulated

negatively by charge repulsion. Puski (1975) treated soy protein with *A. oryzae* and he noted an increase in FC, but FS was zero in all instances that were tested. According to Turner (1969), in order to make a stable protein containing foam, partially hydrolysed protein is needed to increase foam expansion and some larger protein components are needed to stabilize the foam. In this study, it was found that there was correlation between FC and molecular weights of the hydrolysates. As seen from Table 10 and Appendix 20, the larger the protein fraction presented in the sample, the higher the FC values ($r=0.6393$, $p=0.0002$). However, in Puski's (1975) study, the heat treatment used may have denatured the larger protein components sufficiently so that they could not stabilize the foam. It was also found that the FC and FS values in this study were negatively correlated with Td ($r=-0.6166$, $p=0.0004$; $r=-3921$, $p=0.0354$). From Table 13 and Appendix 20, it was noted that samples which were more denatured exhibited a higher capacity to foam whereas FS values were not correlated with the thermal parameters. Both FC and FS values were found to be negatively correlated with DH value ($r=-0.6477$, $p=0.0001$ and $r=-0.5846$, $p=0.0009$, respectively). As observed from Table 9 and Appendices 20 and 22, hydrolysates with lower DH values exhibited higher ability to foam and to stabilize the foam. Therefore, hydrolysates that were cleaved to a lesser extent will exhibit better foaming capacity and stability.

V. GENERAL SUMMARY AND CONCLUSIONS

The objective of this research was to identify and characterize protein hydrolysates with optimum functional properties using the PMM procedure in combination with either chemical or enzymatic hydrolysis methods for different time intervals. In addition, the molecular bases for specific functional properties were investigated.

All enzyme hydrolysates had higher DH values than all acid hydrolysates as enzymes can cleave proteins to a greater extent at specific sites. Molecular weight distribution patterns of all hydrolysates were studied and it was found that P1 and P2 fractions (170,800 D and 7,800 D, respectively) from PMM were cleaved into much smaller units after all treatments. The major molecular weight components in trypsin and chymotrypsin hydrolysates were in the range of $3,000 \pm 200$ D whereas the major components in fungal and bacterial hydrolysates were in the range of $155,000 \pm 5,400$ D. Hydrolysates from both acid methods had major components in the range of $7,500 \pm 700$ D. After treatment with four different enzymes, the resultant hydrolysates exhibited isoelectric points that were shifted slightly from the isoelectric point of PMM (pH 6.6). The isoelectric range for all trypsin and chymotrypsin hydrolysates was from pH 6.2 to 6.6 whereas bacterial and fungal hydrolysates possessed isoelectric points in the pH range of 6.6-6.7. However, the isoelectric points of resultant hydrolysates shifted to the acidic side after mild and alternate acid treatments (pH 4.4-4.8 for mild acid hydrolysates and pH 4.4-5.7 for alternate acid hydrolysates). Fungal and bacterial

hydrolysates were less denatured than all other hydrolysates in terms of the enthalpy of denaturation and the thermal denaturation temperature. All acid hydrolysates had relatively low denaturation enthalphies as well as thermal denaturation temperatures. This showed that both acid hydrolysis methods were quite denaturing. In terms of surface hydrophobicity, the aliphatic hydrophobicity was increased for all hydrolysates, both enzymic and acidic. Aromatic surface hydrophobicity was only increased after trypsin and mild acid treatments.

On the whole, C20 exhibited the highest nitrogen solubility at pH 4.5 whereas A4 showed the highest nitrogen solubility at pH 7.0. The A7 hydrolysate had the best water holding capacity and C10 exhibited the highest fat absorption capacity. The protein sample A8 showed the best emulsion stability. The highest foaming capacity and foaming stability resulted from the A7 hydrolysate. In terms of identifying the optimum hydrolysis conditions, alternate acid hydrolysis for 7 h the showed three best functional properties (water holding capacity, foaming capacity and foaming stability) among the five tested.

The relationships between molecular characteristics and functional properties were also examined. Nitrogen solubility at pH 4.5 was found to have positive correlation with DH whereas nitrogen solubility at pH 7.0 was found to be negatively correlated to DH, ΔH, Td and aromatic surface hydrophobicity. As the protein was hydrolysed to a greater extent (higher DH values), the resultant hydrolysates showed better solubility at pH 4.5 and poorer solubility at pH 7.0. In terms of enthalpy of denaturation and the thermal denaturation temperature, proteins that were more denatured showed better nitrogen

solubility at pH 7.0. Proteins having higher DH values were expected to be denatured to a greater extent; however, in this study, it seemed that all hydrolysates with higher DH values had higher ΔH and Td values (ie. less denaturation). Therefore, it was possible that thermally stable protein fragments were produced from some hydrolysis methods. Water holding capacity was found to have a negative correlation with ΔH and Td plus a positive correlation with aliphatic surface hydrophobicity. Hydrolysates that were less denatured showed poorer water holding capacity and this functional property was improved with an increase in aliphatic surface hydrophobicity. Fat absorption capacity was found to be correlated positively only with DH; therefore, hydrolysates that had higher DH values showed higher fat absorption capacity values. Emulsification stability was correlated negatively with DH, ΔH and Td plus positively correlated with aliphatic surface hydrophobicity. Proteins that were hydrolysed to greater extent (higher DH values) and that were more denatured exhibited better emulsification stability. In addition, hydrolysates with higher aliphatic surface hydrophobicities showed better emulsification stability. Both foaming capacity and foaming stability were negatively correlated with DH, Td and aromatic surface hydrophobicity. Proteins with higher DH values exhibited poorer foaming capacity and foaming stability values. An increase in aromatic surface hydrophobicity also resulted in hydrolysates with poor foaming capacity and foaming stability.

In order to extend this study, future considerations could focus on producing hydrolysates with low levels of antinutritional factors. Substrate-enzyme ratios also could be examined into in order to produce hydrolysates with higher DH values (if desired).

Furthermore, functional properties at different pH values could be studied in order to have a broader idea for the possible use of canola protein hydrolysates in food systems.

REFERENCES

ADLER-NISSEN, J. 1976. Enzymatic hydrolysis of proteins for increased solubility. J. Agric. Food Chem. 24(6):1090-1093.

ADLER-NISSEN, J. 1986. Enzymic Hydrolysis of Food Proteins. Elsevier Applied Science Publishers. London and New York.

AOAC. 1975. Official Methods of Analysis. 12th ed., Association of Official Analytical Chemists, Washington, D.C.

APPELQVIST, L. and OHLSON, R. 1972. Rapeseed - Cultivation, Composition, Processing and Utililzation. Elsevier Publishing Co., Amsterdam.

ARNTFIELD, S.D. and MURRAY, E.D. 1981. The influence of processing parameters on food protein functionality. I. Differential scanning calorimetry as an indicator of protein denaturation. Can. Inst. Food Sci. Technol. J. 14(4):289-294.

ARNTFIELD, S.D., ISMOND, M.A.H. and MURRAY, E.D. 1985. The fate of antinutritional factors using the preparation of a fababean protein isolate using a micellization technique. Can. Inst. Food Sci. Tech. J. 18:137-143.

BELL, J.M. 1989. Nutritional characteristics and protein uses of oilseed meals. Pages 194-204. in : Oil Crops of the World : Their Breeding and Utilization. G. Röbbelem, R.K. Downey and A. Ashri eds. McGraw-Hill Publishing Co., New York.

BERNARDI DON, L.S., PILOSOF, A.M.R. and BARTHOLOMAI, G.B. 1991. Enzymatic modification of soy protein concentrates by fungal and bacterial proteases. J. Am. Oil Chem. 68(2):102-105.

BHATTY, R., MACKENZIE, S. and FINLAYSON, A. 1968. The protein of rapeseed (*Brassica napus* L.) soluble in salt solutions. Can. J. Biochem. 46:1191-1197.

BIGELOW, C.C. 1967. On the average hydrophobicity of proteins and the relation between it and protein structure. J. Theoret. Biol. 16:187-211.

BURGESS, D. 1991. Purification, characterization and the micelle response of the 12S canola globulin. M. Sc. Thesis, University of Manitoba, Winnipeg, MB.

CHEFTEL,J.C., COQ, J.L. and LORIENT, D. 1985. Amino acis, peptides, and

proteins. Pages 245-331 in: Food Chemistry. 2nd ed. O.R. Fennema ed. Marcel Dekker, Inc., New York.

CRENWELGE, D.D., DILL. C.W., TYBOR, P.T. and LANDMANN, W.A. 1974. A comparison of the emulsion capacities of some protein concentrates. J. Food Sci. 39(1):175-177.

DAMODARAN, S. 1994. Structure-function relationship of food proteins. Pages 1-38 in : Protein Functionality in Food Systems. N.S. Hettiarachchy and G.R. Ziegler eds. Marcel Dekker, Inc. New York.

ERICSON, M.L., RÖDIN, J., LENMAN, M., GLIMELIUS, K., JOSEFSSON, L. and RASK, L. 1986. Structure of the rapeseed 1.7S storage protein, napin, and its precursor. J. Biol. Chem. 261(31):14576-14581.

GEORGIOU, G. 1987. The role of noncovalent forces in the micellization phenomenon using legumin, as a study system. M. Sc. Thesis, University of Manitoba, Winnipeg, MB.

GODING, L., BHATTY, R. and FINLAYSON, A. 1970. The characterization of the 12S globulin from rapeseed and its glucoprotein component. Can. J. Biochem. 48:1096-1103.

HAMADA, J.S. 1992. Modification of food proteins by enzymatic methods. Pages 249-270 in: Biochemistry of Food Proteins. B.J.F. Hudson ed. Elsevier Applied Science.

HAYAKAWA, S. and NAKAI, S. 1985. Relationships of hydrophobicity and net charge to the solubility of milk and soy proteins. J. Food Sci. 50:486-491.

INGLIS, A.S. 1983. Cleavage of aspartic acid. Pages 324-334 in: Methods in Enzymology. Vol 91. C.H.W. Hirs and S.N. Timasheff eds. Academic Press, New York.

ISMOND, M.A.H. 1984. The role of noncovalent forces in food protein interactions - a study system using the legume storage protein vicilin. Ph. D. Thesis, University of Manitoba, Winnipeg, MB.

ISMOND, M.A.H., MURRAY, E.D. and ARNTFIELD, S.D. 1986a. The role of non-vocalent forces in micelle formation by vicilin from Vicia faba. The effects of pH variations on protein interactions. Food Chem. 20:305-318.

ISMOND, M.A.H., MURRAY, E.D. and ARNTFIELD, S.D. 1986b. The role of non-

vocalent forces in micelle formation by vicilin from Vicia faba. II. The effect of stabilizing and destabilizing anions on protein interactions. Food Chem. 21:27-46.

ISMOND, M.A.H. and WELSH, W.D. 1992. Application of new methodology to canola protein isolation. Food Chem. 45:125-127.

KATO, A. and NAKAI, S. 1980. Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. Biochim. Biophys. Acta. 624:13-20.

KARP, G. 1984. Cell Biology. MaGraw-Hill Book Co., New York.

KIM, S.Y., PARK, P.S.W. AND RHEE, K.C. 1990. Functional properties of proteolytic enzyme modified soy protein isolate. J. Agric. Food Chem. 38:651-656.

KIMBALL, M.E., HSIEH, D.S.T. and RHA, C.K. 1981. Chymotrypsin hydrolysis of soybean protein. J. Agric. Food Chem. 29(4):872-874.

LEE, C.H., KIM, H.R., YANG, H.C., LEE, M.Y. and BAE, C.C. 1982. Effect of external conditions on the emulsifying properties of proteins. Korean J. Food Sci. Technol. 14:49-53.

LI-CHAN, E., NAKAI, S. and WOOD, D.F. 1985. Relationship between functional (fat binding, emulsifying) and physicochemical properties of muscle proteins. Effectsf of heating, freezing, pH and species. J. Food Sci. 50:1034.

LIN, M.J.Y. and HUMBERT, E.S. 1974. Certain functional properties of sunflower meal products. J. Food Sci. 39:368-370.

LOWRY, O.H., ROSEBROUGH, N.H., FARR, A.L. and RANDALL, R.R. 1951. Protein measurements with the folin phenol reagent. J. Biol. Chem. 193:265-275.

MACKENZIE, S.L. and BLAKELY, J.A. 1972. Purification and characterization of seed globulins from Brassica juncea, B. nigra and B. hirta. Can. J. Bot. 50:1825-1834.

MACKENZIE, S.L. 1975. Subunit structure of the 12S protein from seeds of Brassica juncea. Can. J. Bot. 53:2901-2907.

MATSUDOMI, N., SASAKI, T., KATO, A. and KOBAYASHI, K. 1985.

Conformational changes and functional properties of acid-modified soy protein. Agric. Biol. Chem. 49(5):1251-1256.

MIETSCH, F., FEHER, J. and HALÁSZ, A. 1989. Investigation of functional properties of partially hydrolyzed proteins. Die Nahrung 33(1):9-15.

MIETH, G., SCHWENKE, K.D., RAAB, B. and BRÜCKNER, J. 1983. Rapeseed : Constituents and protein products. Part I. Composition and properties of proteins and glucosinolates. Die Nahrung. 27(7):675-697.

MURRAY, E.D., MYERS, C.D., BARKER, L.D. and MAURICE, T.J. 1981. Functional attributes of proteins. A non-covalent approach to processing and utilizing proteins. Pages 158-176 in: Utilization of Protein Resources. D.W. Stanley, E.D. Murray and D.H. Lees eds. Food and Nutritional Press, Inc., Westport, Ct.

NAKAI, S., HO, L., HELBIG, N., KATO, A. and TUNG, M.A. 1980. Relationship between hydrophobicity and emulsifying properties of some plant proteins. Can. Inst. Food Sci. Technol. J. 13:23-27.

NAKAI, S. and LI-CHAN, E. 1988. Hydrophobic Interactions in Food Systems. CRC Press Inc., Florida.

NG, P.K.W., SCANLON, M.G. and BUSHUK, W. 1988. A catalog of biochemical fingerprints of registered Canadian wheat cultivars by electrophoresis and High-Performance Liquid Chromatography. Food Science Department, University of Manitoba, Winnipeg, MB.

NORTON, G. 1989. Nature and Biosynthesis of storage proteins. Pages 165-191 in: Oil Crops of the World: Their Breeding and Utilization. G. Röbbelen, R.K. Downey and A. Ashri eds. McGraw-Hill Publishing Co., New York.

OLSMAN, H. 1979. Hydrolyzed and autolysed vegetable proteins as functional food ingredients. J. Am. Oil Chem. Soc. 3(56):375-376.

PEARCE, K.N. and KINSELLA, J.E. 1978. Emulsifying properties of proteins : evaluation of a turbidimetric technique. J. Agric. Food Chem. 26(3):716-723.

PETERSEN, B.R. 1991. The impact of the enzymic hydrolysis process on recovery and use of proteins. Pages 149-175 in: Enzymes and Food Processing. G.G. Birch, N. Blakebrough and K.J. Parker eds. Applied Science Publishers.

PRAKASH, V. and RAO, M.S.N. 1986. Physicochemical properties of oilseed

proteins. CRC Crit. Rev. Biochem. 20:265-363.

PUSKI, G. 1975. Modification of functional properties of soy proteins by proteolytic enzyme treatment. Am. Assoc. Cereal Chem. 52:655-664.

REICHELT, R., SCHWENKE, K.D. and KÖNIG, T. 1980. Electron microscopic studies of the quaternary structure of 11S/12S globulin from plant seeds. Studia Biophysica. 79:177-178.

REYNOLDS, J.A. 1979. The role of micelles in protein detergent interactions. Pages 58-62 in: Methods in Enzymology. Vol 61. C.H.W. Hirs and S.N. Timasheff eds. Academic Press, New York.

SCHWENKE, K., RAAB, B., LINOW, K., PAHTZ, W. and ULHING, J. 1981. Isolation of the 12S globulin from rapeseed (Brassica napus L.) and characterization as "neutral" protein. On seed proteins. Part 13. Die Nahrung. 25:271-280.

SCHWENKE, K., RAAB, B., PLIETZ, P. and DAMASCHUN, G. 1983. The structure of the 12S globulin from rapeseed (Brassica napus L.). Die Nahrung. 27:165-175.

SHIH, F.F. 1992. Modification of food proteins by non-enzymatic methods. Pages 235-248 in: Biochemistry of Food Proteins. B.J.F. Hudson ed. Elsevier Applied Science.

SHIMIZU, M., SAITO, M. and YAMAUCHI, K. 1985. Hydrophobicity and emulsifying activity of milk proteins. Agric. Biol. Chem. 50(3):791-792.

SKLAR, L.A., HUDSON, B.S. and SIMONI, R.D. 1977. Conjugated polyene fatty acids as fluorescent probes : binding to bovine serum albumin. Biochem. 16:5100-5108.

TANFORD, C. 1973. The hydrophobic effect: Formation of micelles and biological membranes. J. Wiley and sons, New York.

THOMPSON, L.U., LIU, R.F.K. and JONES, J.D. 1982. Functional properties and food applications of rapeseed protein concentrate. J. Food Sci. 47:1175-1180.

TOWNSEND, A.A. and NAKAI, S. 1983. Relationships between hydrophobicity and foaming characteristics of food proteins. J. Food Sci. 48:588-594.

TURNER, J.R. 1969. Modified soy protein and the preparation thereof. U.S. Patent

No. 2,489,2

VOJDANI, F. and WHITAKER, J.R. 1994. Chemical and enzymatic modification of proteins for improved functionality. Pages 261-309. in : Protein Functionality in Food Systems. N.S. Hettiarachchy and G.R. Ziegler eds. Marcel Dekker, Inc. New York.

VOUTSINAS, L.P. and NAKAI, S. 1983. A simple turbidimetric method for determining the fat binding capacity of proteins. J. Agric. Food Chem. 31(1):5

WELSH, W.D. 1988. Application of new methodology to canola protein isolation. M. Sc. Thesis, University of Manitoba, Winnipeg, MB.

WINTER, A. and ANDERSON, V. 1977. Analytical electrofocusing in thin layers of polyacrylamide gels. LKB application Note No. 250.

APPENDIX 1.   Calibration curve for molecular weight determination of PMM and all hydrolysates by gel filtration.

$$y = 10.43 - 1.357x$$

$$r2 = 0.9908$$
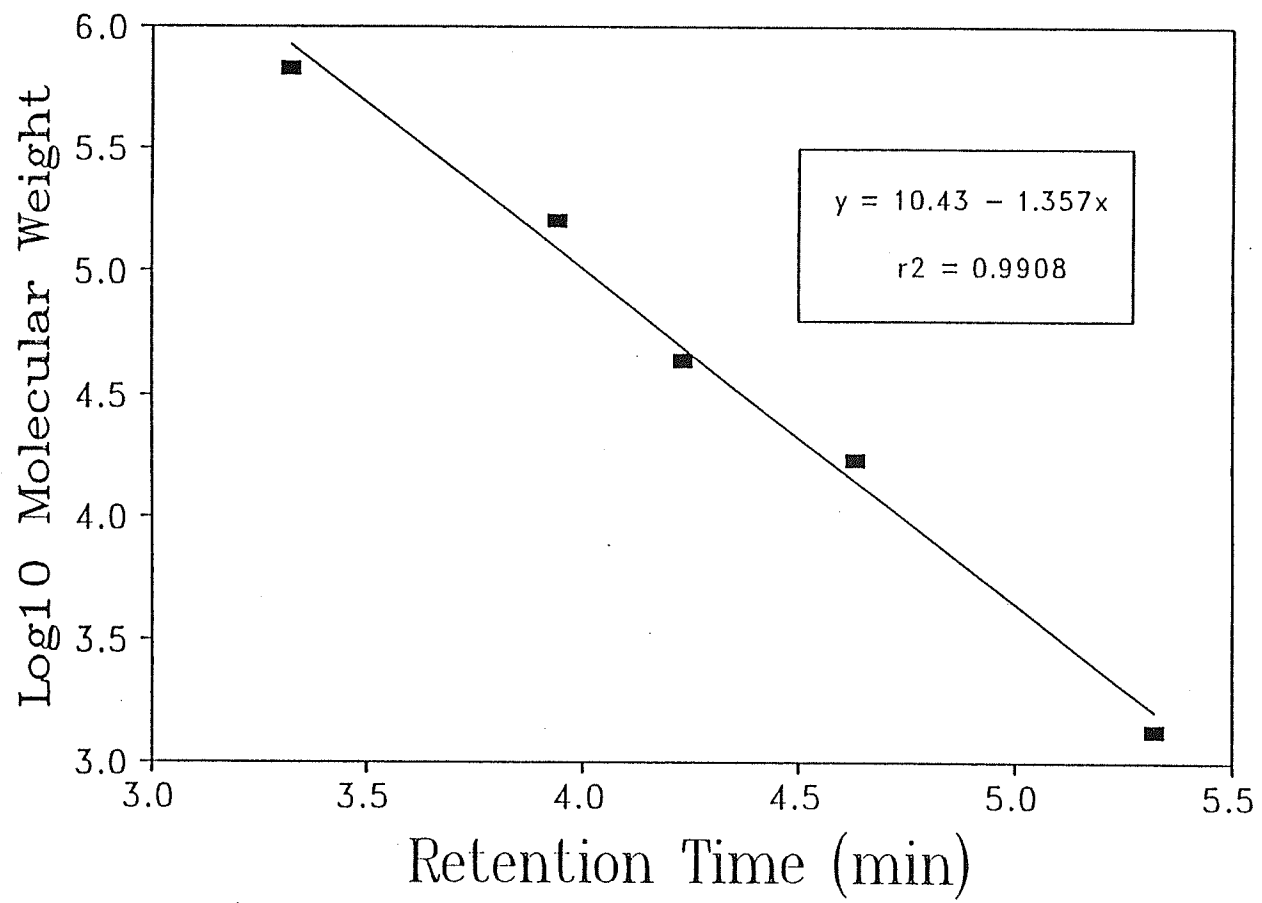
APPENDIX 2.   Calibration curve for subunit molecular weight determination of PMM and all hydrolysates by SDS-PAGE.

$y = 5.44 - 0.115x$

$r2 = 0.9854$
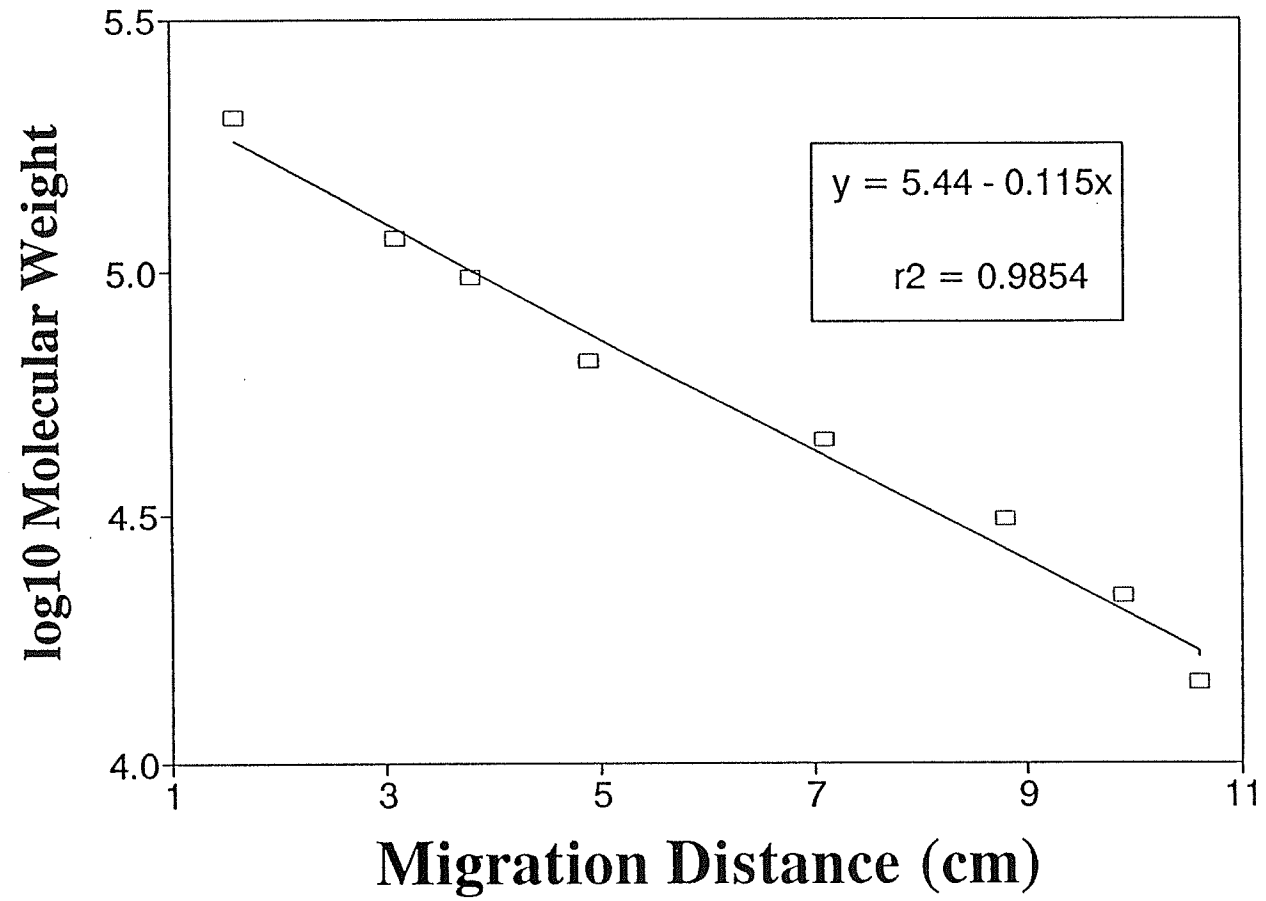
APPENDIX 3.   Calibration curve for isoelectric point determination of PMM and all hydrolysates by isoelectric focusing.

$$y = 9.53 - 0.630x$$

$$r2 = 0.9963$$
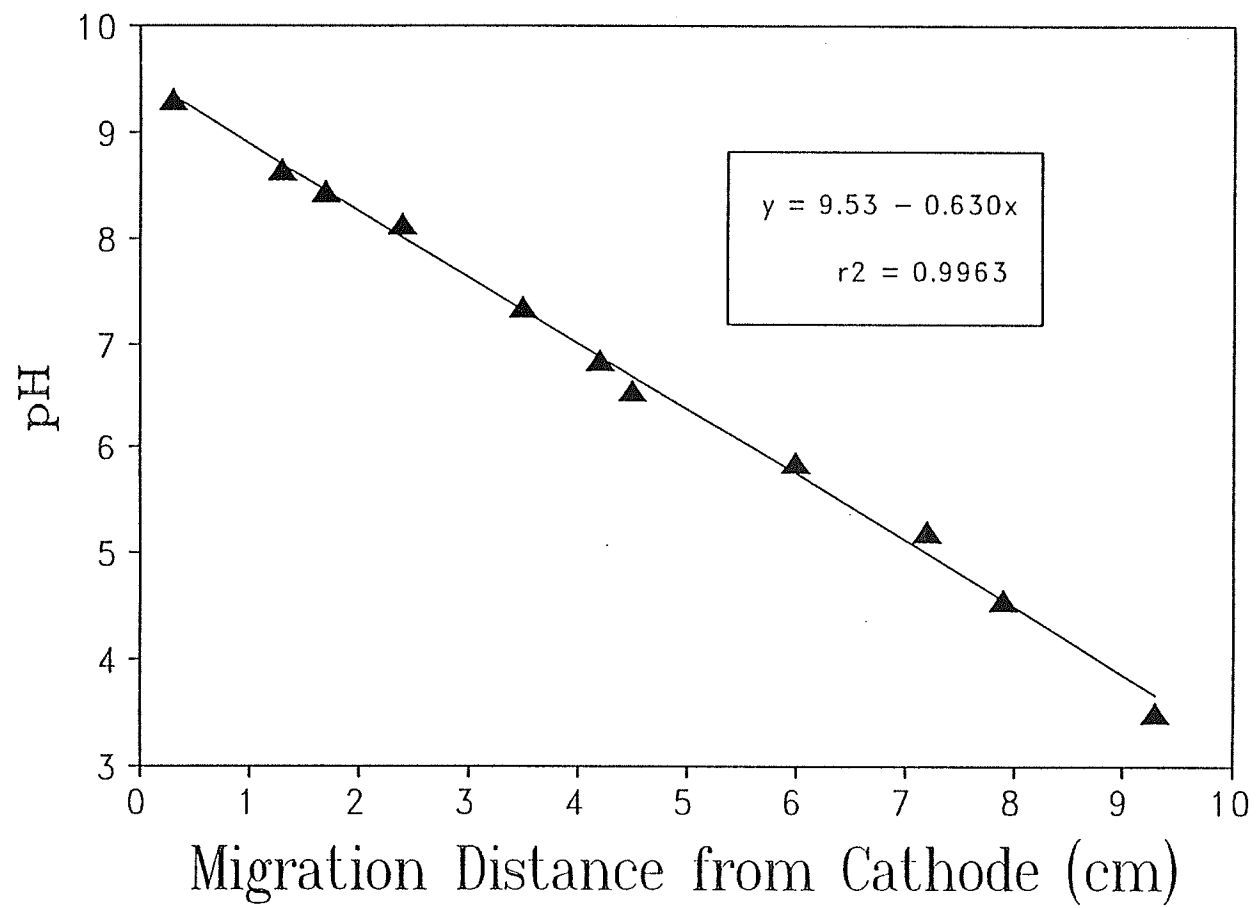
APPENDIX 4.   Analysis of variance of the degree of hydrolysis.

ANALYSIS OF VARIANCE OF DEGREE OF HYDROLYSIS

Source	df	SS	MS	F	Pr > F
TRYPSIN					
Model	4	0.1470	0.0490	2.52**	0.1968
Error	3	0.0778	0.0194		
Corrected Total	7	0.2248			
Time	3	0.1470	0.0490	2.52**	0.1554
Rep	1	0.66	0.66	21.16*	0.0193
CHYMOTRYPSIN					
Model	4	14.84	3.71	5.00**	0.1122
Error	3	0.44	2.15		
Corrected Total	7	15.28			
Time	3	13.99	4.66	1.44**	0.1191
Rep	1	0.84	0.84	5.7*	0.0970
FUNGAL PROTEASE					
Model	4	8.38	2.10	0.55**	0.7142
Error	3	11.35	3.78		
Corrected Total	7	19.74			
Time	3	6.28	2.09	0.55**	0.6804
Rep	1	2.10	2.10	0.56**	0.5103
BACTERIAL PROTEASE					
Model	4	0.29	0.07	2.42**	0.2470
Error	3	0.09	0.03		
Corrected Total	7	0.38			
Time	3	0.27	0.09	2.98**	0.1970
Rep	1	0.02	0.02	0.74**	0.4528
MILD ACID					
Model	4	0.54	0.42	1.11**	0.2530
Error	3	0.34	0.38		
Corrected Total	7	0.88			
Time	3	0.52	0.44	1.92**	0.2830
Rep	1	0.27	0.40	1.00**	0.3910
ALTERNATE ACID					
Model	8	76.00	9.50	22.92*	0.0151
Error	7	11.27	1.61		
Corrected Total	15	87.27			
Time	7	75.96	10.85	22.92*	0.0111
Rep	1	0.04	0.04	0.02**	0.8792

*　　significant at 0.05 level.
**　　not significant at 0.05 level.

APPENDIX 5.   Analysis of variance of the enthalpy of denaturation (ΔH).

ANALYSIS OF VARIANCE OF $_\Delta$II

Source	df	SS	MS	F	Pr > F
TRYPSIN					
Model	5	1.10	0.22	0.86**	0.5550
Error	6	1.53	0.26		
Corrected Total	11	2.63			
Time	3	0.95	0.32	1.24**	0.3734
Rep	2	0.15	0.07	0.29**	0.7591
CHYMOTRYPSIN					
Model	5	0.90	0.18	1.02**	0.4809
Error	6	1.06	0.18		
Corrected Total	11	1.96			
Time	3	0.81	0.27	1.54**	0.2993
Rep	2	0.09	0.04	0.25**	0.7895
FUNGAL PROTEASE					
Model	5	2.02	0.40	1.15**	0.4278
Error	6	2.11	0.35		
Corrected Total	11	4.13			
Time	3	1.33	0.44	1.26**	0.3692
Rep	2	0.69	0.35	0.98**	0.4269
BACTERIAL PROTEASE					
Model	5	2.11	0.42	1.63**	0.2826
Error	6	1.55	0.26		
Corrected Total	11	3.67			
Time	3	0.72	0.24	0.93**	0.4815
Rep	2	1.39	0.69	2.69**	0.1469
MILD ACID					
Model	5	0.49	0.10	19.80*	0.0011
Error	6	0.03	0.01		
Corrected Total	11	0.52			
Time	3	0.49	0.16	32.88*	0.0004
Rep	2	0.01	0.01	0.19**	0.8315
ALTERNATE ACID					
Model	10	3.66	0.37	5.43*	0.0015
Error	16	1.08	0.07		
Corrected Total	26	4.74			
Time	8	3.41	0.43	6.32*	0.0009
Rep	2	0.25	0.25	1.87**	0.1863

*   significant at 0.05 level.
**   not significant at 0.05 level.

APPENDIX 6.   Analysis of variance of the denaturation temperature (Td).

ANALYSIS OF VARIANCE OF Td

Source	df	SS	MS	F	Pr > F
TRYPSIN					
Model	5	5.91	10.18	8.86*	0.0097
Error	6	0.80	0.13		
Corrected Total	11	6.71			
Time	3	5.54	1.85	13.84*	0.0042
Rep	2	0.37	0.18	1.38**	0.3203
CHYMOTRYPSIN					
Model	5	3.93	0.78	52.02*	0.0001
Error	6	0.09	0.02		
Corrected Total	11	4.02			
Time	3	3.84	1.28	84.79*	0.0001
Rep	2	0.09	0.04	2.85**	0.1346
FUNGAL PROTEASE					
Model	5	6.54	1.31	0.70**	0.6437
Error	6	11.21	1.87		
Corrected Total	11	17.75			
Time	3	3.29	1.10	0.59**	0.6457
Rep	2	3.25	1.63	0.87**	0.4658
BACTERIAL PROTEASE					
Model	5	1.29	0.26	1.97**	0.2169
Error	6	0.79	0.13		
Corrected Total	11	2.08			
Time	3	1.14	0.38	2.91**	0.1233
Rep	2	0.15	0.07	0.56**	0.5977
MILD ACID					
Model	5	6.09	1.22	0.13**	0.9810
Error	6	58.04	9.67		
Corrected Total	11	64.14			
Time	3	1.55	0.52	0.05**	0.9821
Rep	2	4.54	2.27	0.23**	0.7978
ALTERNATE ACID					
Model	10	131.66	13.17	4.62*	0.0034
Error	16	45.56	2.85		
Corrected Total	26	177.22			
Time	8	128.32	16.04	5.63*	0.0017
Rep	2	3.34	1.67	0.59**	0.5682

*   significant at 0.05 level.
**   not significant at 0.05 level.

APPENDIX 7.   Analysis of variance of the aromatic surface
hydrophobicity.

ANALYSIS OF VARIANCE OF AROMATIC HYDROPHOBICITY

Source	df	SS	MS	F	Pr > F
TRYPSIN					
Model	4	542.13	131.03	24.54*	0.0125
Error	3	16.02	5.34		
Corrected Total	7	540.15			
Time	3	512.96	170.99	32.02*	0.0089
Rep	1	11.16	11.16	2.09**	0.2440
CHYMOTRYPSIN					
Model	4	74.38	18.60	2.13**	0.2800
Error	3	26.16	8.72		
Corrected Total	7	100.54			
Time	3	71.82	23.94	2.75**	0.2144
Rep	1	2.57	2.57	0.29*	0.6252
FUNGAL PROTEASE					
Model	4	24.84	6.21	17.32*	0.0206
Error	3	1.08	0.36		
Corrected Total	7	25.92			
Time	3	24.03	8.01	22.34*	0.0149
Rep	1	0.81	0.81	2.27**	0.2292
BACTERIAL PROTEASE					
Model	4	3.91	0.98	0.25**	0.8937
Error	3	11.79	3.93		
Corrected Total	7	15.70			
Time	3	0.9247	0.31	0.08**	0.9674
Rep	1	2.99	2.99	0.76**	0.4473
MILD ACID					
Model	4	597.01	149.25	463.98*	0.0002
Error	3	0.96	0.32		
Corrected Total	7	597.97			
Time	3	596.22	198.74	617.82*	0.0001
Rep	1	0.79	0.79	2.45**	0.2156
ALTERNATE ACID					
Model	9	154.19	17.13	1.96**	0.18
Error	8	70.07	8.76		
Corrected Total	17	224.25			
Time	8	154.03	19.25	2.20**	0.1430
Rep	1	0.15	0.15	0.02**	0.8987

*   significant at 0.05 level.
**   not significant at 0.05 level.

APPENDIX 8.   Analysis of variance of the aliphatic surface
hydrophobicity.

ANALYSIS OF VARIANCE OF ALIPHATIC HYDROPHOBICITY

Source	df	SS	MS	F	Pr > F
TRYPSIN					
Model	4	316.80	79.20	5.74**	0.0913
Error	3	41.37	13.79		
Corrected Total	7	358.17			
Time	3	313.44	104.48	7.58**	0.0652
Rep	1	3.35	3.35	0.24**	0.6557
CHYMOTRYPSIN					
Model	4	1075.74	268.93	58.19*	0.0036
Error	3	13.86	4.62		
Corrected Total	7	1089.60			
Time	3	1051.02	350.34	75.80*	0.0025
Rep	1	24.71	24.71	5.35**	0.1038
FUNGAL PROTEASE					
Model	4	23.36	5.84	0.84**	0.5790
Error	3	20.75	6.92		
Corrected Total	7	44.11			
Time	3	23.03	7.68	1.11**	0.4669
Rep	1	0.32	0.32	0.05**	0.8425
BACTERIAL PROTEASE					
Model	4	89.39	22.35	3.07**	0.1920
Error	3	21.85	7.28		
Corrected Total	7	111.24			
Time	3	89.21	29.74	4.08**	0.1391
Rep	1	0.17	0.17	0.02**	0.8879
MILD ACID					
Model	4	646.81	161.70	21.80*	0.0149
Error	3	22.26	7.42		
Corrected Total	7	669.07			
Time	3	646.23	215.41	29.03*	0.0102
Rep	1	0.58	0.58	0.08**	0.7983
ALTERNATE ACID					
Model	9	696.16	77.35	16.71*	0.0003
Error	8	37.03	4.63		
Corrected Total	17	733.19			
Time	8	691.56	86.44	18.67*	0.0002
Rep	1	4.60	4.60	0.99**	0.3480

*   significant at 0.05 level.
**   not significant at 0.05 level.

APPENDIX 9. Nitrogen solubility (%) at pH 4.5 for PMM and all hydrolysates.

SAMPLE	NITROGEN SOLUBILITY AT pH 4.5 (%)[1]
PMM	100%[2]
T5	116.3 ± 0.9[a]
T10	113.9 ± 0.9[b]
T15	116.0 ± 0.9[a]
T20	110.8 ± 0.40[c]
C5	201.3 ± 3.0[a]
C10	191.5 ± 1.0[b]
C15	195.9 ± 1.0[ab]
C20	210.4 ± 2.1[c]
F5	120.9 ± 4.2[a]
F10	136.2 ± 3.0[bc]
F15	136.7 ± 0.7[b]
F20	129.2 ± 1.6[c]
B5	154.7 ± 0.3[a]
B10	156.8 ± 0.2[b]
B15	162.7 ± 0.5[c]
B20	164.1 ± 0.4[c]
A10	68.9 ± 0.8[a]
A20	65.8 ± 0.5[b]
A30	69.6 ± 0.3[a]
A40	75.5 ± 0.0[c]
A1	93.0 ± 1.2[a]
A2	110.5 ± 0.7[b]
A3	127.1 ± 1.7[c]
A4	146.5 ± 2.1[d]
A5	151.5 ± 0.8[d]
A6	166.9 ± 1.3[e]
A7	184.5 ± 2.4[f]
A8	165.0 ± 5.6[e]

[1] Each value represents a mean of two determinations.
[2] Nitrogen solubility of PMM at pH 4.5 is 0.95 mg/ml and it is assumed as 100% solubility.
[3] Values followed by the same letter are not significantly different (p ≤ 0.05) within each hydrolysis group.

APPENDIX 10.   Analysis of variance of nitrogen solubility at pH 4.5.

ANALYSIS OF VARIANCE OF NITROGEN SOLUBILITY AT pH 4.5

Source	df	SS	MS	F	Pr > F
TRYPSIN					
Model	4	42.82	10.71	90.80*	0.0018
Error	3	0.35	0.12		
Corrected Total	7	43.18			
Time	3	38.17	12.72	107.91*	0.0015
Rep	1	4.65	4.65	39.45*	0.0082
CHYMOTRYPSIN					
Model	4	410.47	102.62	17.83*	0.0198
Error	3	17.26	5.75		
Corrected Total	7	427.74			
Time	3	397.72	132.57	23.04*	0.0142
Rep	1	12.75	12.75	2.22**	0.2333
FUNGAL PROTEASE					
Model	4	370.80	92.70	19.02*	0.0181
Error	3	14.62	4.87		
Corrected Total	7	385.43			
Time	3	327.09	109.03	22.37*	0.0148
Rep	1	43.71	43.71	8.97*	0.0579
BACTERIAL PROTEASE					
Model	4	122.59	30.65	127.04*	0.0011
Error	3	0.72	0.24		
Corrected Total	7	123.32			
Time	3	122.44	40.81	169.18*	0.0008
Rep	1	0.15	0.15	0.63**	0.4863
MILD ACID					
Model	4	99.95	24.99	57.44*	0.0036
Error	3	1.30	0.43		
Corrected Total	7	101.25			
Time	3	99.54	33.18	76.28*	0.0025
Rep	1	0.40	0.40	0.9300**	0.4058
ALTERNATE ACID					
Model	8	13393.39	1674.17	330.36*	0.0001
Error	7	35.47	5.07		
Corrected Total	15	13428.87			
Time	7	13332.94	1904.71	375.85*	0.0001
Rep	1	60.45	60.45	11.93*	0.0106

*   significant at 0.05 level.
**   not significant at 0.05 level.

APPENDIX 11.   Nitrogen solubility (%) at pH 7.0 for PMM and all hydrolysates.

SAMPLE	NITROGEN SOLUBILITY AT pH 7.0 (%)[1]
PMM	100%
T5	84.4 ± 0.2[a]
T10	89.3 ± 0.2[b]
T15	76.7 ± 0.9[c]
T20	66.3 ± 0.7[d]
C5	97.5 ± 1.4[a]
C10	88.8 ± 1.0[b]
C15	89.0 ± 1.2[b]
C20	101.7 ± 1.2[c]
F5	87.3 ± 0.9[a]
F10	82.9 ± 0.9[bc]
F15	85.3 ± 0.4[ab]
F20	80.5 ± 0.1[c]
B5	92.7 ± 0.3[a]
B10	93.8 ± 0.8[ab]
B15	92.7 ± 0.6[a]
B20	95.8 ± 0.0[b]
A10	96.4 ± 0.8[a]
A20	100.2 ± 1.1[b]
A30	71.7 ± 1.0[c]
A40	193.7 ± 0.6[d]
A1	387.3 ± 2.6[ab]
A2	351.1 ± 2.6[c]
A3	417.7 ± 2.6[d]
A4	427.8 ± 6.2[e]
A5	393.4 ± 3.6[a]
A6	367.4 ± 1.5[f]
A7	378.9 ± 5.8[b]
A8	350.7 ± 2.2[c]

[1] Each value represents a mean of two determinations.
[2] Nitrogen solubility of PMM at pH 7.0 is 2.05 mg/ml and it is assumed as 100% solubility.
[3] Values followed by the same letter are not significantly different (p ≤ 0.05) within each hydrolysis group.

APPENDIX 12.   Analysis of variance of nitrogen solubility at pH 7.0.

ANALYSIS OF VARIANCE OF NITROGEN SOLUBILITY AT pH 7.0

Source	df	SS	MS	F	Pr > F
TRYPSIN					
Model	4	602.01	150.50	186.19*	0.0006
Error	3	2.42	0.81		
Corrected Total	7	604.44			
Time	3	601.89	200.63	248.20*	0.0004
Rep	1	0.12	0.12	0.15**	0.7204
CHYMOTRYPSIN					
Model	4	258.11	64.53	1548.66*	0.0001
Error	3	0.12	0.04		
Corrected Total	7	258.23			
Time	3	247.06	82.35	1976.52*	0.0001
Rep	1	11.04	11.04	265.08*	0.0005
FUNGAL PROTEASE					
Model	4	52.06	13.02	11.80*	0.0352
Error	3	3.31	1.10		
Corrected Total	7	55.37			
Time	3	52.04	17.35	15.72*	0.0244
Rep	1	0.02	0.02	0.02**	0.9014
BACTERIAL PROTEASE					
Model	4	13.03	3.26	4.74**	0.1160
Error	3	2.06	0.69		
Corrected Total	7	15.10			
Time	3	13.03	4.34	6.32**	0.0822
Rep	1	0.0012	0.0012	0.00**	0.9687
MILD ACID					
Model	4	17255.47	4313.87	36584.05*	0.0001
Error	3	0.35	0.12		
Corrected Total	7	17255.83			
Time	3	17249.52	5749.84	48761.90*	0.0001
Rep	1	5.95	5.95	50.47*	0.0057
ALTERNATE ACID					
Model	8	11381.74	1422.72	79.54*	0.0001
Error	7	125.21	17.89		
Corrected Total	15	11506.96			
Time	7	11285.21	1612.17	90.13*	0.0001
Rep	1	96.53	96.53	5.40**	0.0532

* significant at 0.05 level.
** not significant at 0.05 level.

APPENDIX 13. The correlation matrix for molecular characteristics and functional properties.

	HEAT	Td	ARO	ALI	NS45	NS70	WHC	FAC	ES	FC	FS	MW
DH	0.2785	*0.7299	*0.5507	-0.1617	*0.3926	*-0.7001	-0.1628	*0.4767	*-0.6737	*-0.6477	*-0.5846	*-0.5852
	0.1434	0.0001	0.0020	0.4021	0.0351	0.0001	0.3987	0.0089	0.0001	0.0001	0.0009	0.0009
HEAT		*0.7288	-0.1973	*-0.4248	0.1815	*-0.1854	*-0.8797	-0.3365	*-0.5196	-0.2666	0.1018	*-0.4061
		0.0001	0.3049	0.0216	0.3462	0.0076	0.0001	0.0742	0.0039	0.1621	0.5994	0.0288
Td			0.2917	-0.3274	0.2911	*-0.7614	*-0.6203	0.1693	*-0.7408	*-0.6166	* *-0.3921	*-0.6627
			0.1247	0.0829	0.1713	0.0001	0.0003	0.3799	0.0001	0.0004	0.0354	0.0001
ARO				0.2345	-0.3225	*-0.4270	0.1472	0.1239	-0.3455	-0.3442	-0.3462	*-0.4015
				0.2208	0.0880	0.0209	0.4462	0.5220	0.0664	0.0675	0.0658	0.0309
ALI					-0.0330	0.2767	*0.3711	-0.0273	*0.4003	0.1695	-0.0408	0.3378
					0.8652	0.1462	0.0475	0.8883	0.0314	0.3795	0.8336	0.0731
NS45						0.0799	0.1061	*0.4979	0.1005	-0.0359	-0.2463	0.2049
						0.6804	0.5840	0.0060	0.6040	0.8533	0.1978	0.2863
NS70							*0.5764	-0.2052	*0.8272	*0.7632	*0.4587	*0.9149
							0.0011	0.2857	0.0001	0.0001	0.0123	0.0001
WHC								0.3572	*0.5830	0.3328	-0.0827	*0.5559
								0.0571	0.0009	0.0778	0.6699	0.0017
FAC									-0.1067	*-0.4924	*-0.7793	-0.1808
									0.5819	0.0067	0.0001	0.3480
ES										0.5993	0.3269	*0.8416
										0.0006	0.0835	0.0001
FC											*0.8459	*0.6393
											0.0001	0.0002
FS												0.3551
												0.0587

first column r = Pearson correlation coefficients
second column p = probability, N = 28

APPENDIX 14.   Water holding capacity (%) for PMM and all hydrolysates.

SAMPLE	WATER HOLDING CAPACITY (%)[1]
PMM	111.5 ± 5.8
T5	188.3 ± 2.2^a
T10	233.3 ± 2.0^b
T15	193.4 ± 0.7^c
T20	205.9 ± 5.3^d
C5	194.1 ± 1.1^a
C10	200.2 ± 0.7^b
C15	205.8 ± 0.0^c
C20	191.5 ± 0.8^d
F5	130.9 ± 0.6^a
F10	133.1 ± 0.2^b
F15	137.0 ± 1.0^c
F20	116.9 ± 0.1^d
B5	120.4 ± 0.1^a
B10	113.5 ± 0.2^b
B15	108.9 ± 1.7^b
B20	138.6 ± 1.6^c
A10	187.7 ± 0.9^a
A20	176.4 ± 0.9^b
A30	181.9 ± 1.1^c
A40	171.4 ± 0.3^d
A1	211.0 ± 2.7^{ab}
A2	214.1 ± 4.3^{ab}
A3	202.4 ± 0.1^b
A4	212.5 ± 4.6^{ab}
A5	212.8 ± 2.3^{ab}
A6	231.4 ± 5.0^a
A7	260.8 ± 12.5^c
A8	208.3 ± 8.3^b

[1]   Each value represents a mean of two determinations.

[2]   Values followed by the same letter are not significantly different ($p \leq 0.05$) within each hydrolysis group.

APPENDIX 15.   Analysis of variance of water holding capacity.

ANALYSIS OF VARIANCE OF WATER HOLDING CAPACITY

Source	df	SS	MS	F	Pr > F
TRYPSIN					
Model	4	2486.58	621.65	251.46*	0.0004
Error	3	7.42	2.47		
Corrected Total	7	2493.99			
Time	3	2435.09	828.43	335.11*	0.0003
Rep	1	1.28	1.28	0.52**	0.5238
CHYMOTRYPSIN					
Model	4	249.20	62.30	147.39*	0.0009
Error	3	1.27	0.42		
Corrected Total	7	250.47			
Time	3	245.68	81.89	193.75*	0.0006
Rep	1	3.51	3.51	8.31**	0.0634
FUNGAL PROTEASE					
Model	4	459.62	114.90	252.87*	0.0004
Error	3	1.36	0.45		
Corrected Total	7	460.98			
Time	3	458.07	152.69	336.03*	0.0003
Rep	1	1.55	1.55	3.41**	0.1620
BACTERIAL PROTEASE					
Model	4	1021.70	255.42	102.93*	0.0015
Error	3	7.44	2.48		
Corrected Total	7	1029.14			
Time	3	1021.64	340.55	137.23*	0.0010
Rep	1	0.06	0.06	0.02**	0.8900
MILD ACID					
Model	4	296.53	74.13	36.25*	0.0071
Error	3	6.13	2.04		
Corrected Total	7	302.66			
Time	3	296.41	98.80	48.32*	0.0049
Rep	1	0.12	0.12	0.06**	0.8242
ALTERNATE ACID					
Model	8	4952.64	619.08	7.91*	0.0066
Error	7	548.10	78.30		
Corrected Total	15	5500.74			
Time	7	4902.83	700.40	8.95*	0.0049
Rep	1	49.81	49.81	0.64**	0.4513

*   significant at 0.05 level.
**   not significant at 0.05 level.

APPENDIX 16.   Fat absorption capacity (%) for PMM and all hydrolysates.

SAMPLE	FAT ABSORPTION CAPACITY (%)[1]
PMM	104.9 ± 1.8
T5	111.5 ± 5.2[a]
T10	106.5 ± 6.5[a]
T15	136.6 ± 0.2[b]
T20	109.5 ± 0.2[a]
C5	162.0 ± 1.3[a]
C10	182.4 ± 0.3[b]
C15	175.4 ± 1.2[c]
C20	167.9 ± 0.4[d]
F5	85.7 ± 1.8[a]
F10	91.9 ± 0.1[b]
F15	73.6 ± 0.2[c]
F20	80.3 ± 4.6[d]
B5	86.0 ± 0.7[a]
B10	76.2 ± 2.6[b]
B15	94.1 ± 0.1[c]
B20	92.4 ± 1.5[c]
A10	104.8 ± 0.2[a]
A20	87.9 ± 1.0[b]
A30	89.6 ± 0.9[b]
A40	109.1 ± 1.1[a]
A1	100.2 ± 3.8[a]
A2	94.6 ± 1.8[a]
A3	94.4 ± 5.6[a]
A4	94.2 ± 1.9[a]
A5	95.0 ± 8.8[a]
A6	96.4 ± 3.6[a]
A7	104.0 ± 4.0[a]
A8	98.1 ± 5.8[a]

[1] Each value represents a mean of two determinations.
[2] Values followed by the same letter are not significantly different (p ≤ 0.05) within each hydrolysis group.

APPENDIX 17.   Analysis of variance of fat absorption capacity.

ANALYSIS OF VARIANCE OF FAT ABSORPTION CAPACITY

Source	df	SS	MS	F	Pr > F
TRYPSIN					
Model	4	1155.34	288.83	6.33**	0.0807
Error	3	136.81	45.60		
Corrected Total	7	1292.15			
Time	3	1154.55	384.85	8.44**	0.0566
Rep	1	0.79	0.79	0.02**	0.9038
CHYMOTRYPSIN					
Model	4	475.95	118.99	90.42*	0.0019
Error	3	3.95	1.32		
Corrected Total	7	479.90			
Time	3	473.18	157.73	119.86*	0.0013
Rep	1	2.77	2.77	2.11**	0.2425
FUNGAL PROTEASE					
Model	4	365.49	91.37	43.7*	0.0054
Error	3	6.27	2.09		
Corrected Total	7	371.77			
Time	3	364.48	121.49	58.11*	0.0037
Rep	1	1.02	1.02	0.49**	0.5360
BACTERIAL PROTEASE					
Model	4	396.22	99.06	15.54*	0.0240
Error	3	19.12	6.38		
Corrected Total	7	415.34			
Time	3	396.06	132.02	20.71*	0.0166
Rep	1	0.16	0.16	0.03**	0.8843
MILD ACID					
Model	4	684.87	171.22	90.10*	0.0019
Error	3	5.70	1.90		
Corrected Total	7	690.57			
Time	3	684.33	228.11	120.03*	0.0013
Rep	1	0.54	0.54	0.28**	0.6307
ALTERNATE ACID					
Model	8	229.97	28.75	0.62**	0.7389
Error	7	322.43	46.06		
Corrected Total	15	552.40			
Time	7	168.62	4.09	0.52**	0.7941
Rep	1	61.35	61.35	1.33**	0.2863

*   significant at 0.05 level.
**   not significant at 0.05 level.

APPENDIX 18.   Emulsion stability (%) for PMM and all hydrolysates.

SAMPLE	EMULSION STABILITY $(\%)^1$
PMM	22.4 ± 2.0
T5	7.6 ± 0.4^a
T10	7.5 ± 0.3^a
T15	6.8 ± 1.0^a
T20	15.1 ± 2.4^b
C5	6.8 ± 0.2^a
C10	28.4 ± 1.3^b
C15	6.9 ± 0.1^a
C20	8.6 ± 0.5^a
F5	7.5 ± 0.5^a
F10	7.1 ± 0.3^a
F15	7.5 ± 1.0^a
F20	8.8 ± 0.9^a
B5	8.1 ± 0.2^{ab}
B10	7.4 ± 0.5^b
B15	7.4 ± 0.8^b
B20	9.6 ± 0.1^a
A10	17.0 ± 2.1^a
A20	15.0 ± 1.9^a
A30	19.3 ± 1.6^a
A40	29.0 ± 1.0^b
A1	23.6 ± 2.2^a
A2	22.9 ± 2.2^a
A3	35.9 ± 2.6^b
A4	35.2 ± 0.5^b
A5	43.8 ± 2.1^c
A6	50.7 ± 0.2^d
A7	54.6 ± 0.9^d
A8	55.4 ± 0.1^d

[1] Each value represents a mean of two determinations.
[2] Values followed by the same letter are not significantly different ($p \leq 0.05$) within each hydrolysis group.

APPENDIX 19.   Analysis of variance of emulsion stability.

ANALYSIS OF VARIANCE OF EMULSION STABILITY

Source	df	SS	MS	F	Pr > F
TRYPSIN					
Model	4	97.10	24.28	8.50**	0.0549
Error	3	8.57	2.86		
Corrected Total	7	105.67			
Time	3	91.69	30.56	10.70*	0.0413
Rep	1	5.41	5.41	1.89**	0.2624
CHYMOTRYPSIN					
Model	4	662.08	165.52	134.69*	0.0010
Error	3	3.69	1.23		
Corrected Total	7	665.77			
Time	3	661.86	220.62	179.53*	0.0007
Rep	1	0.21	0.21	0.17**	0.7042
FUNGAL PROTEASE					
Model	4	3.42	0.86	0.61**	0.6867
Error	3	4.23	1.41		
Corrected Total	7	7.65			
Time	3	3.41	1.14	0.81**	0.57
Rep	1	0.01	0.01	0.01**	0.9301
BACTERIAL PROTEASE					
Model	4	7.43	1.86	6.77**	0.0741
Error	3	0.82	0.27		
Corrected Total	7	8.25			
Time	3	6.49	2.16	7.88*	0.0062
Rep	1	0.95	0.95	3.44**	0.1605
MILD ACID					
Model	4	239.68	59.92	14.57*	0.0263
Error	3	12.34	4.11		
Corrected Total	7	252.02			
Time	3	228.96	76.32	18.56*	0.0193
Rep	1	10.72	10.72	2.61**	0.2048
ALTERNATE ACID					
Model	8	2368.80	296.10	50.05*	0.0001
Error	7	41.41	5.92		
Corrected Total	15	2410.21			
Time	7	2366.61	388.09	57.15*	0.0001
Rep	1	2.19	2.19	0.37**	0.5621

*   significant at 0.05 level.
**   not significant at 0.05 level.

APPENDIX 20.   Foaming capacity (%) for PMM and all hydrolysates.

SAMPLE	FOAMING CAPACITY (%)[1]
PMM	120 ± 0
T5	148 ± 2^a
T10	155 ± 1^a
T15	112 ± 4^b
T20	112 ± 2^b
C5	136 ± 0^a
C10	129 ± 1^{bc}
C15	125 ± 5^c
C20	133 ± 7^{ab}
F5	146 ± 0^a
F10	146 ± 2^a
F15	145 ± 1^a
F20	144 ± 2^a
B5	148 ± 2^a
B10	146 ± 0^a
B15	146 ± 0^a
B20	152 ± 2^b
A10	149 ± 1^a
A20	148 ± 0^a
A30	150 ± 0^a
A40	156 ± 0^b
A1	161 ± 1^{ab}
A2	164 ± 0^{ab}
A3	168 ± 0^{bc}
A4	174 ± 2^{cd}
A5	180 ± 4^d
A6	166 ± 0^b
A7	175 ± 1^{cd}
A8	157 ± 5^a

[1]   Each value represents a mean of two determinations.

[2]   Values followed by the same letter are not significantly different ($p \leq 0.05$) within each hydrolysis group.

APPENDIX 21.   Analysis of variance of foaming capacity.

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ANALYSIS OF VARIANCE OF FOAMING CAPACITY

Source	df	SS	MS	F	Pr > F
TRYPSIN					
Model	4	3174.00	793.50	52.32*	0.0042
Error	3	45.50	15.17		
Corrected Total	7	3219.50			
Time	3	3169.50	1056.50	69.66*	0.0028
Rep	1	4.50	4.50	0.30**	0.6238
CHYMOTRYPSIN					
Model	4	138.00	34.50	18.82*	0.0183
Error	3	5.50	1.83		
Corrected Total	7	143.50			
Time	3	137.50	45.83	25.00*	0.0127
Rep	1	0.50	0.50	0.27**	0.6376
FUNGAL PROTEASE					
Model	4	10.00	2.50	0.56**	0.7133
Error	3	13.50	4.50		
Corrected Total	7	23.50			
Time	3	5.50	1.83	0.41**	0.7599
Rep	1	4.50	4.50	1.00*	0.3910
BACTERIAL PROTEASE					
Model	4	48.00	12.00	9.00**	0.0509
Error	3	4.00	1.33		
Corrected Total	7	52.00			
Time	3	48.00	16.00	12.00*	0.0354
Rep	1	0	0	0	1
MILD ACID					
Model	4	78.00	19.50	39.00*	0.0064
Error	3	1.50	0.50		
Corrected Total	7	79.50			
Time	3	77.50	25.83	51.67*	0.0044
Rep	1	0.50	0.50	1.00*	0.3910
ALTERNATE ACID					
Model	8	868.00	108.50	11.91*	0.0019
Error	7	63.75	9.11		
Corrected Total	15	931.75			
Time	7	837.75	119.68	13.14*	0.0015
Rep	1	30.25	30.25	3.32**	0.1112

*   significant at 0.05 level.
**   not significant at 0.05 level.

APPENDIX 22.   Foaming stability (%) for PMM and all hydrolysates.

SAMPLE	FOAMING STABILITY (%)
PMM	11.7 ± 0.0
T5	21.0 ± 3.7[a]
T10	27.8 ± 0.4[b]
T15	7.2 ± 0.3[c]
T20	7.2 ± 0.1[c]
C5	10.3 ± 0.0[a]
C10	13.2 ± 0.7[b]
C15	10.4 ± 0.4[a]
C20	9.8 ± 0.3[a]
F5	30.2 ± 1.3[a]
F10	28.8 ± 0.4[a]
F15	29.7 ± 1.8[a]
F20	31.3 ± 1.1[a]
B5	32.5 ± 0.4[a]
B10	31.5 ± 0.0[ab]
B15	30.1 ± 0.0[bc]
B20	30.2 ± 1.3[c]
A10	28.2 ± 0.2[a]
A20	31.1 ± 0.0[b]
A30	28.0 ± 0.0[a]
A40	30.8 ± 0.0[b]
A1	31.1 ± 0.2[a]
A2	31.7 ± 1.2[ab]
A3	31.0 ± 1.2[a]
A4	32.8 ± 0.2[ab]
A5	31.7 ± 1.3[ab]
A6	33.1 ± 0.6[ab]
A7	34.9 ± 0.4[b]
A8	26.8 ± 0.8[c]

[1] Each value represents a mean of two determinations.
[2] Values followed by the same letter are not significantly different (p ≤ 0.05) within each hydrolysis group.

APPENDIX 23.   Analysis of variance of foaming stability.

ANALYSIS OF VARIANCE OF FOAMING STABILITY

Source	df	SS	MS	F	Pr > F
TRYPSIN					
Model	4	639.63	159.91	5117*	0.0001
Error	3	0.09	0.03		
Corrected Total	7	639.72			
Time	3	638.96	212.99	6815.61*	0.0001
Rep	1	0.66	0.66	21.16*	0.0193
CHYMOTRYPSIN					
Model	4	14.84	3.71	25.00*	0.0122
Error	3	0.44	0.15		
Corrected Total	7	15.28			
Time	3	13.99	4.66	31.44*	0.0091
Rep	1	0.84	0.84	5.7*	0.0970
FUNGAL PROTEASE					
Model	4	8.38	2.10	0.55**	0.7142
Error	3	11.35	3.78		
Corrected Total	7	19.74			
Time	3	6.28	2.09	0.55**	0.6804
Rep	1	2.10	2.10	0.56**	0.5103
BACTERIAL PROTEASE					
Model	4	9.93	2.48	11.55*	0.0363
Error	3	0.64	0.21		
Corrected Total	7	10.57			
Time	3	9.92	3.31	15.39*	0.0251
Rep	1	0.01	0.01	0.02**	0.8885
MILD ACID					
Model	4	16.40	4.10	204.94*	0.0005
Error	3	0.06	0.02		
Corrected Total	7	16.46			
Time	3	16.38	5.46	272.92*	0.0004
Rep	1	0.02	0.02	1.00**	0.3910
ALTERNATE ACID					
Model	8	76.00	9.50	5.90*	0.0151
Error	7	11.27	1.61		
Corrected Total	15	87.27			
Time	7	75.96	10.85	6.74*	0.0111
Rep	1	0.04	0.04	0.02**	0.8792

*   significant at 0.05 level.
**   not significant at 0.05 level.