STUDIES ON THE ENZYMATIC HYDROLYSIS OF DOGFISH (Squalus acanthias) MUSCLE PROTEIN

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STUDIES ON THE ENZYMATIC HYDROLYSIS OF DOGFISH (*Squalus acanthias*) MUSCLE PROTEIN

by

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I hear and I forget,
I see and I remember,
I do and I understand.
Confucious

TO MY BELOVED PARENTS

ABSTRACT

Response surface methodology (RSM) was used to study the combined effects of pH, temperature and enzyme - substrate ratio (E/S) on the degree of hydrolysis (DH) of dogfish muscle protein. The effects of the factor variables which affect hydrolysis were described using a three-level Box-Behnken factorial design giving a mathematical model equation that showed the influence of each variable and their interactions. The optimization of the enzymatic process was performed with the RSM statistical technique having the recovery of soluble nitrogen (NR) from the original substrate as the process response. The optimum values for enzyme / substrate ratio, temperature and pH were found to be 3.7 % (w/w), 55.3 °C, and 8.3, respectively, resulting in a maximum NR of 77.68 %. The coefficient of determination (R^2_{adj}) was greater than 95 % at the 0.1 % probability level, and a lack-of-fit test revealed a non-significant value for the NR model equation, indicating that the regression equation was adequate for predicting the nitrogen recovery under any combination of values of the variables. The shark protein hydrolysate produced under these conditions contained a high crude protein concentration (> 85 %, dry weight basis), and its high nutritional value was indicated by the presence of all essential amino acids, and by high protein efficiency ratio (PER) values. The functional, physical and rheological properties of the optimized spray dried protein hydrolysate was compared to the protein hydrolysate obtained by autolytic degradation, and a control, freeze dried

muscle. The Alcalase®-assisted hydrolysis, in general, improved functional properties such as solubility and dispersibility, indicating that enzymatic hydrolysis under the experimental conditions described in this study can produce a new source protein ingredient with superior protein functionality. Physical and rheological product characteristics were also subjected to significant changes. The off-white protein hydrolysate had a low viscosity in aqueous dispersions even at high protein concentration.

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GLOSSARY

ABBREVIATIONS (SYMBOLS AND DEFINITION)

ANOVA	- Analysis of Variance
AOAC	- Association of Official Analytical Chemists
AOCS	- American Oil Chemists Society
α	- degree of dissociation
B	- base consumption (mL)
dF	- degree of freedom
DH	- degree of hydrolysis
DNMRT	- Duncan's New Multiple Range Test
Eqn	- equation
E/S	- enzyme - substrate ratio (%, w/w)
FAO/WHO	- Food and Agriculture Organization/World Health Organization
FPC	- Fish Protein Concentrate
FPH	- Fish Protein Hydrolysate
f_N	- Kjeldahl conversion factor
h	- number of peptide bonds cleaved
h_{tot}	- total number of peptide bonds in a protein ($meqv.g^{-1}N \times f_N$)
M	- molar
M_p	- mass (gram) of protein in the substrate

mEq	- miliequivalent
N	- Kjeldahl nitrogen content
N_b	- normality of NaOH
NR	- nitrogen recovery
P	- probability level
pK	- Average pK of α-NH_2 groups released during hydrolysis
R_{ad}^2	- adjusted coefficient of determination
rpm	- revolutions per minute
RSM	- Response Surface Methodology
RSREG	- Response Surface Regression
S	- substrate concentration (%)
SD	- standard deviation
SPH	- Shark Protein Hydrolysate
T	- hydrolysis temperature (°C)
T	- hydrolysis temperature (K)
t	- hydrolysis time (min)
TCA	- Trichloroacetic acid
X_1	- Hydrolysis pH
X_2	- Hydrolysis temperature
X_3	- Enzyme - substrate ratio in the hydrolysis
Y	- measured response variable

CHAPTER I

INTRODUCTION

Enzymatic hydrolysis of fish protein has been employed as an alternative approach for converting underutilized fish biomass, commonly used in making animal feed or fertilizer into edible protein products (Suzuki, 1981; Venugopal, 1994; Vieira *et al.*, 1995). Therefore, application of enzyme technology would make the low-cost but high-value protein of species such as dogfish (*Squalus acanthias*) available as a new and acceptable food source for human consumption.

In this process, proteolytic enzymes are used to solubilize the fish muscle protein, resulting in two distinguishable fractions, the soluble and the insoluble. The insoluble fraction may be used as animal feed (Mohr, 1978), and the soluble fraction, which contains the hydrolysed protein, may be converted into an ingredient for incorporation into food systems. The soluble hydrolysate is subjected to dehydration, resulting in a more stable, powdered form with a high protein concentration. Such a product is known as fish protein hydrolysate (FPH). Under controlled proteolysis, FPH possesses desirable functional properties and a high nutritional value (Mohr, 1978; Quaglia and Orban, 1987a; Rebeca *et al.*, 1991). The most important variables to be controlled in this complex enzymatic reaction have been reported to be concentration and specificity of the enzyme, temperature and pH of the reaction, the nature of the protein substrate, and the degree of hydrolysis attained (Adler-Nissen, 1986). The optimization of process

parameters for the enzymatic hydrolysis of fish protein is important in any attempt to develop an economical process (Martin and Porter, 1995).

Response surface methodology (RSM) is a useful technique for the investigation of complex processes. It has been successfully applied in optimization of food processing operations, including enzymatic processes (Thompson, 1982; Joglekar and May, 1987; Floros and Chinnan, 1988; Garrote *et al.*, 1993, 1994; Cui *et al.*, 1994; Oomah *et al.*, 1994; Govindasamy *et al.*, 1995). It consists of a group of mathematical and statistical procedures that can be used to study relationships between one or more responses (dependent variables) and a number of factors (independent variables). RSM defines the effect of the independent variables, alone and in combinations, and generates a mathematical model that accurately describes the overall process.

The effect of the process on the quality and nutritional value of the final product must also be considered in the development of new food products. In the case of fish protein hydrolysates, a high degree of hydrolysis (DH, %) can cause undesirable bitterness in the product (Quaglia and Orban, 1987b; Martin and Porter, 1995). Therefore, the degree of hydrolysis is not necessarily the only or best parameter to be considered when optimizing this process.

Not only the nutritional quality, but also the functional properties of a new source of protein are important in food formulation. These characteristics govern the ability to incorporate it as a food ingredient. Moreover, protein functionality is a primary source of information on how the material will behave in a food system

(Crenwelge *et al.*, 1974).

Solvent extraction methods, as well as alkaline and acidic hydrolyses, which produce protein concentrates lacking in functional properties (Sikorski and Naczk, 1982; Martin and Patel, 1991) and, hence, make a new source protein product unsuitable for food applications. Enzymatic hydrolysis of the original protein, however, leads to a protein hydrolysate with desirable functional properties. For this reason, the use of enzymatic methods in the production of fish protein hydrolysates has been presented by several authors (Venugopal, 1994; Martin and Porter, 1995; Shahidi *et al.*, 1995; Vieira *et al.*, 1995). Thus, it is fundamental to study what changes of the functional properties will be introduced from the enzymatic treatment once these changes reflect on which direction the new source protein ingredient will take to be feasible as a food component.

The main purpose of this study is therefore to investigate the digestion of dogfish (*Squalus acanthias*) muscle protein by means of enzymatic hydrolysis and examine the nutritional quality and functional properties of the recovered shark hydrolysate. Specific objectives of this project are as follows:

1. To apply the response surface methodology to study the combined effects of different hydrolysis parameters, namely pH, temperature and enzyme - substrate ratio, on degree of hydrolysis on the digestion of dogfish muscle protein using Alcalase®, a protease of microbial origin.
2. To optimize the processing conditions using response surface methodology which would result in maximum nitrogen recovery (NR%)

from the original substrate.

3. To estimate the nutritional quality and investigate the functional properties of the shark protein hydrolysate obtained from the conditions of maximum nitrogen recovery.

CHAPTER II

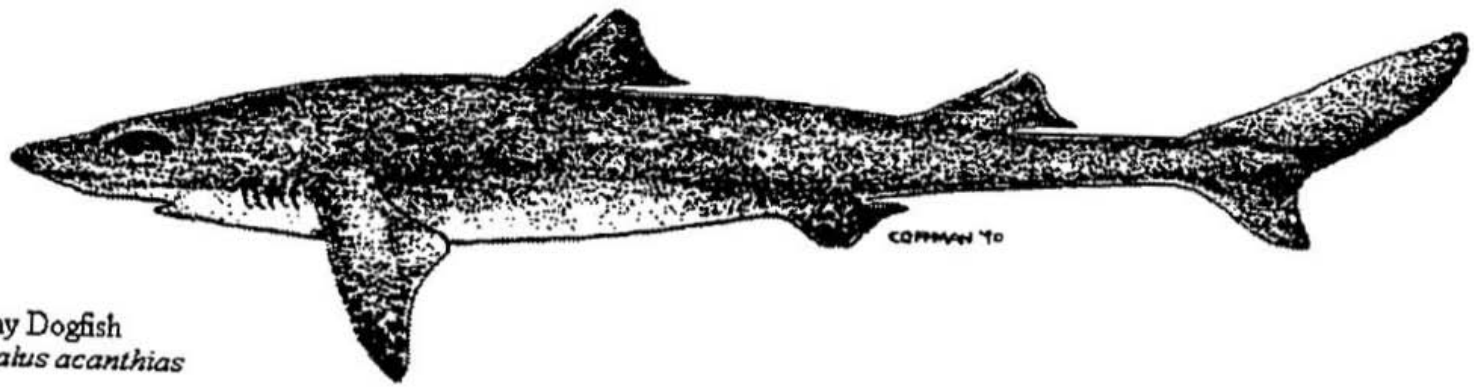
LITERATURE REVIEW

2.1 Dogfish: an underutilized species

The spiny dogfish (*Squalus acanthias*) belongs to the subclass Elasmobranchii of the class Chondrichthyes or cartilaginous fishes. Its scientific name comes from the Latin word *Squalus* or shark, and the Greek *akanthias* referring to the spines on the dorsal fins (Figure 2.1). Individuals reach a maximum length of about 1.2 m, and are found in the Northeast Pacific from Southern California to Northwestern Alaska, and in the western North Atlantic from about Georgia to Newfoundland (Nammack *et al.*, 1985; Salsbury, 1986).

The spiny dogfish is considered a nuisance by fishermen, damaging their fishing nets with its rough skin and sharp teeth and spines. Moreover, dogfish prey on more valuable fish and young shellfish (Boyd *et al.*, 1967; Anon., 1990; 1993). It is also not easily marketed for human consumption due to aesthetic reasons. This results in a low market price for its fillet, which varies from CDN$ 0.10 - 0.12/lb (Shiau and Chai, 1985; Salsbury, 1986; Hardy, 1993); even though it is a potential source of high-quality protein (Morris, 1975; Kreuzer, 1978; Ronsivalli, 1978). Therefore, efforts are being made to maximize its value and commercial viability by searching for more profitable uses (Shiau and Chai, 1985; Asgard and Austreng, 1985; Mowbray *et al.*, 1988; Heras *et al.*, 1994; Anon., 1995). Because of the low-cost but high value

Figure 2.1 The spiny dogfish (*Squalus acanthias*). Reprinted from Anonymous (1990).



Spiny Dogfish
Squalus acanthias

dogfish protein, alternative processing technologies, such as production of fish protein concentrates, could make this species more acceptable as a new food ingredient for human consumption.

2.2 Fish protein concentrate

Fish protein concentrates (FPC) use underutilized fish species, by-catches or even fish processing waste as raw material. Such a product can be defined as any stable wholesome fish product prepared by grinding fish or parts thereof, in which the protein and other nutrient materials are converted into a more highly concentrated form than in the "untreated" fish by removal of water and, in certain cases, of oil and bones (Windsor and Barlow, 1981; Finch, 1977; Bárzana and García-Garibay, 1994). Protein concentration may range from less than 75 % to as high as 95 % (Stillings, 1974). FPC can be manufactured by physical, chemical, and biological methods.

2.2.1 Physical method

Physical techniques are employed in this process. As an example, a periodical discharge of electrical current through a fish slurry followed by centrifugation could be applied to separate raw fish into its constituents. Major disadvantages include significant loss of protein in the extracted solubles, difficulty in maintenance of sanitary conditions during processing, and low quality of recovered oil (Dyke, 1986; Martin, 1986).

2.2.2 Chemical method

The chemical method mainly comprises the use of organic solvents to eliminate water and lipids from the raw materials. Minced fish is mixed with solvents, heated, and centrifuged. These steps are generally repeated several times to remove additional water and lipids. Heating is then applied to remove the solvent, and the residual material is dried, ground and packaged (Dyke, 1986). The most promising solvents used are isopropanol, methanol, ethanol and 1,2-dichloromethane (Bárzana and García-Garibay, 1994).

The major problem found in this procedure is the potential toxicity of residual solvent in the concentrate. Moreover, the end-product is generally lacking in important functional properties such as solubility, dispersability, and emulsifying capacity (Windsor and Barlow, 1981; Green and Mattick, 1979). In addition, solvent extraction involves treatments at extreme pHs and temperatures (Löffler, 1986) compromising the nutritive value of the FPC.

Another approach involves chemical hydrolysis of the fish protein by acid and alkalis. Many side reactions occur which influence the recovery of amino acids, such as the partial destruction of cysteine, tyrosine and tryptophan (Clegg and McMillan, 1974; Nair *et al.*, 1976; Ledward and Lawrie, 1984).

2.2.3 Biological method

Biological methods involve hydrolysis of the fish protein by enzymes either naturally present in the fish or added into it. Water-soluble peptides and amino

acids are produced. Two procedures are known: enzymatic and microbial. The first one is related to the direct addition of commercial enzymes to a minced fish substrate. The second procedure involves the addition of a crude enzyme preparation from a microbial source or living microorganisms into the substrate. The enzyme preparation or the living cells provide a mixture of biocatalysts (enzymes).

In comparison to chemical and physical methods, biological procedures offer the following advantages: (a) protein concentrates present more desirable functional properties, such as solubility; (b) fish hydrolysates are high in protein and minerals (ash), and low in fat; (c) enzymatic agents (including microorganisms) are relatively low in cost; (d) fish oil is easily recovered by centrifugation, and is thus of good quality because little or no heat is applied; (e) the species are adaptable to small scale production in remote areas (Spinelli *et al.*, 1972; Hale, 1974; Mackie, 1982; Adler-Nissen, 1986).

2.3 Enzymatic hydrolysis of fish proteins

The hydrolysis of fish protein entails the liquefaction of the fish tissue. Since hydrolysates are yielded from procedures which use acidic, alkaline or enzymatic hydrolysis, the fish protein concentrates produced are referred to as fish protein hydrolysates (FPH). However, this term is usually applied when

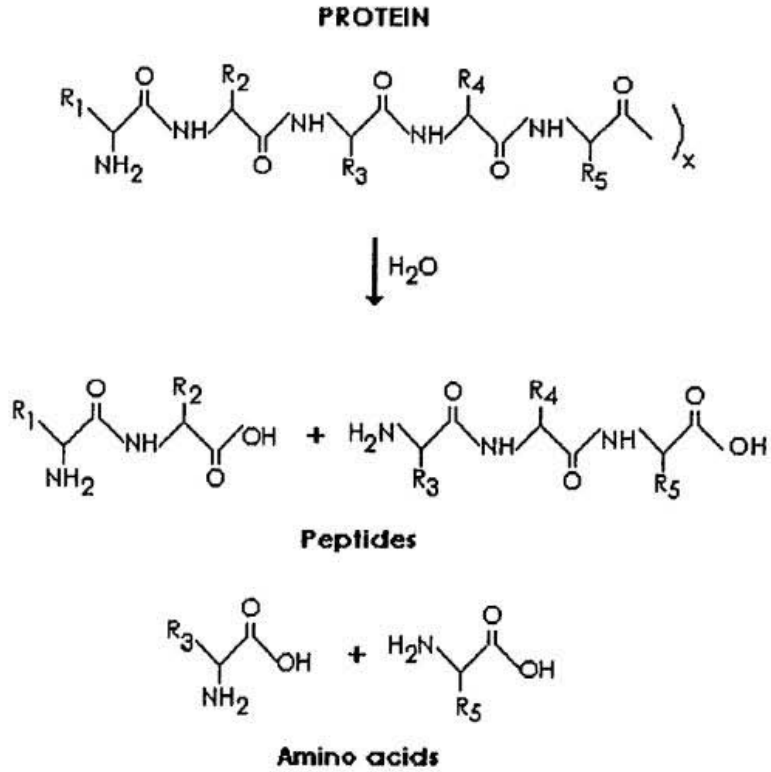
biological methods are employed to solubilize the fish tissue (Martin and Patel, 1991).

Enzymatic hydrolysis has several distinctive advantages over other processing methods including: (a) the unique specificity of action of the enzyme; (b) digestion under mild conditions, avoiding extreme pH and temperature; (c) the deactivation of the enzyme by subsequent heating, thus making the removal of enzymes unnecessary; (d) attractive functional characteristics such as solubility; and (e) no destruction of amino acids, so the protein tends to retain its nutritive value better than traditional acidic or alkaline hydrolysis (Clegg and McMillan, 1974; Petersen, 1981; Löffer, 1986; Lahl and Braun, 1994).

2.3.1 Peptide bond hydrolysis and the pH stat method

Amino acids form the basic unit of protein macromolecules and are bonded together by means of peptide linkages. This covalent carbon-nitrogen bond is the result of a condensation reaction between the amino group ($-NH_2$) of one of the twenty common amino acids constituents of proteins and the carboxyl group (–COOH) of another, with consequent elimination of water (Taylor, 1988). Therefore, the hydrolysis or cleavage of peptide bonds occurs through the addition of a water molecule (Scheme 1).

Scheme 1



It is relevant to mention that in aqueous media the excess of water favors this lytic process (Bárzana and García-Garibay, 1994). The catalytic agent responsible for the breakdown of the peptide bond is an enzyme which speeds up (catalysis) the reaction.

During the hydrolysis of proteins (Scheme 2) the NH_3^+ or COO^- polar groups increase in number, with a consequent decrease in molecular weight of the peptide chains. The molecular conformation also alters from that of the original protein molecule. These polar groups can become ionized under

certain pH conditions. The degree of dissociation of the α-amino and α-carboxyl groups are dependent on the pH relative to the pK_a values of the two groups.

Scheme 2

Opening of the peptide bond:

$$-CHR'-CO-NH-CHR''- + H_2O \xrightarrow{\text{enzyme}} -CHR'-COOH + -NH_2-CHR''$$

Proton exchange:

$$-CHR'-COOH + -NH_2-CHR'' \longrightarrow -CHR'-COO^- + -\overset{+}{N}H_3-CHR''$$

Titration of amino group:

$$-\overset{+}{N}H_3-CHR'' + OH^- \rightleftharpoons NH_2-CHR'' + H_2O$$

The α-amino groups in polypeptides have pK_a values estimated to be 7.5 - 7.8 at 25 °C. The pK_a values for α-carboxyl groups range from 3.1 to 3.6 (Rupley, 1967). For pH lower than 5.5 and higher than 6.5 the dissociation is significant and the pH-stat becomes a useful device to follow the hydrolytic reaction (Jacobsen *et al.*, 1957; Adler-Nissen, 1986). Therefore, the pH-stat technique is not useful between pH 5.5 and 6.5, because the dissociation constant does not sufficiently change. Moreover, the pH-stat cannot work at pH < 3 and pH >

11 due to the high buffering capacity of the protein. Consequently, where applicable, to maintain a constant pH it is necessary to add a considerable amount of acid or base to titrate the released α-amino and α-carboxyl groups. The principles of the pH-stat method are based on this relationship, and the technique has been used by many workers for kinetic studies and for monitoring the degree of hydrolysis attained by the enzymatic reaction on food proteins (Adler-Nissen, 1978, 1986; Rubio *et al.*, 1992, 1993; Moreno and Cuadrado, 1993; Gonzalez-Tello *et al.*, 1994; Mullally *et al.*, 1994; Shahidi *et al.*, 1995).

2.3.2 Enzymes for fish protein hydrolysis

General considerations

Proteolytic enzymes can be classified in various ways. The most simple is based on their primary source, e. g. proteolytic enzymes produced from animal glands are denominated animal proteases, those from plant tissue are plant proteases, and those produced by submerged fermentation using bacteria, yeast, or fungi are microbial proteases.

They may also be classified by their mode of action into endo- or exopeptidases. Endopeptidases cleave the peptide bonds randomly, which results in large peptide chains. Conversely, exopeptidases have as specific targets the terminal amino acids from protein or peptide chains,

and thus include carboxypeptidases and aminopeptidases, which respectively cleave either the carboxyl or amino group of the protein molecule (Figure 2.2).

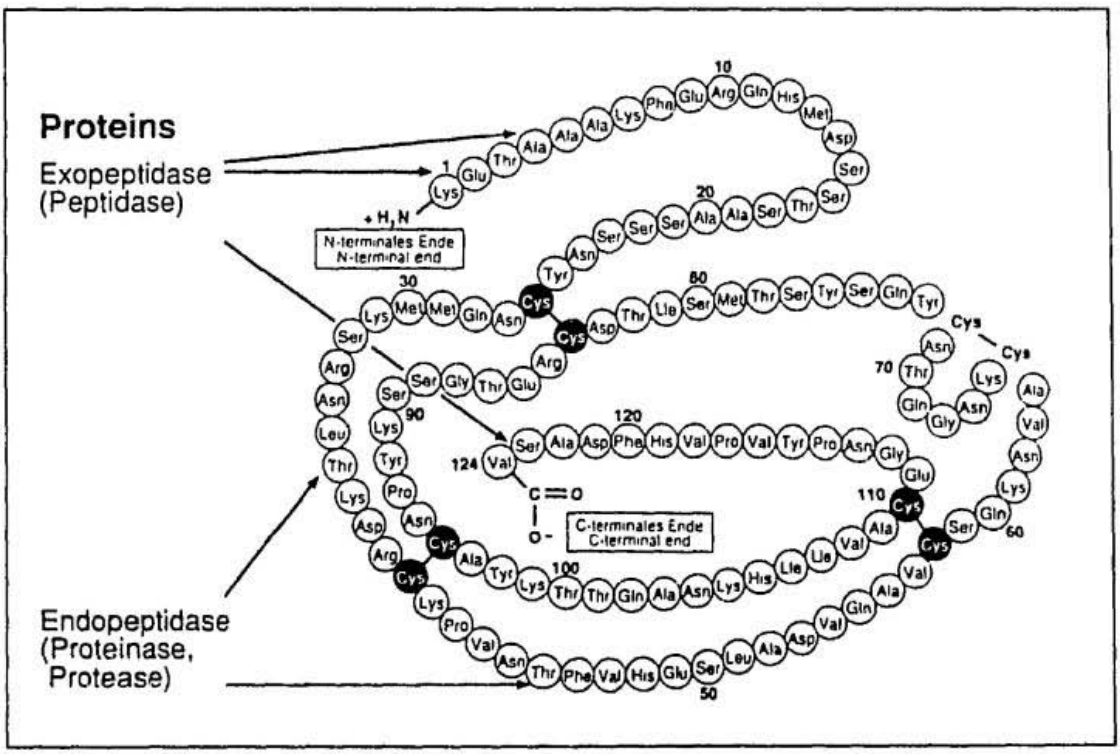
Proteases are also classified by the nature of their catalytic site. Alkaline or basic proteases are active in pH 7.0 - 11.0. Because the active site has an essential seryl group, these enzymes are appropriately known as serine proteases. Trypsin, chymotrypsin, elastase and subtilisins are examples from this group (Whitaker, 1974).

Sulfhydryl or cysteine proteases have a cysteinyl and hystidyl residues in their active site and exhibit maximum proteolytic activity at neutral pH. Papain, ficin, and bromelain have a sulfhydryl group at their catalytic site (Liener, 1974).

Metalloproteases or metal-containing proteases are those that require Zn^{+2} or Mn^{+2}, and optimum activity is detected at neutral pH. For instance, carboxypeptidase A and B, and carnosinase require Zn^{+2}, and prolidase and iminodipeptidase require Mn^{+2}. They are inhibited by metal-chelating agents (Riordan, 1974; Whitaker, 1994).

The acid proteases, as the name indicates, exhibit optimum activity at the acid pH range. They are more appropriately called aspartic proteases, because they always have an aspartic acid carboxyl group in the catalytic site. In this group, pepsin, renin, cathepsins D and E, are included (Adler-Nissen, 1986; Whitaker, 1994).

Figure 2.2 Mechanism of peptidase activity. Reprinted from Moll (1990).


Proteins
Exopeptidase
(Peptidase)
Endopeptidase
(Proteinase,
Protease)
N-terminales Ende
N-terminal end
C-terminales Ende
C-terminal end

Enzymes for fish hydrolysis

Most of the commercial enzymes used to solubilize fish protein are of plant, animal or microbial sources. Plant proteases used include mostly papain, bromelain, and ficin (Hevia *et al.*, 1976; Beddows and Ardeshir, 1979; Mackie, 1982; Quaglia and Orban, 1987b).

Sen *et al.* (1962) may have been the first to thoroughly examine the digestion of fish tissue with papain, a neutral plant protease. From their experiments it was observed that pH and temperature play an important role on the extent of hydrolytic degradation of the protein substrate. Low molecular weight peptide units were obtained at optimum pH, a reflection of the high proteolytic activity for papain. Mahesh *et al.* (1993) reported the preparation of a protein concentrate by enzymatic treatment using papain as the biocatalyst. It yielded an end-product with desirable functional properties, but with only a modest percentage of protein recovery. Levin *et al.* (1989) investigated the recovery of proteinaceous materials from cod frames using 0.05% papain at different reaction temperatures to produce peptones for microbial growth. The papain-assisted digestion recovered up to 50% of the total protein.

Yanez *et al.* (1976) produced a high quality protein hydrolysate from Chilean hake (*Merluccius igayi*) fillets using bromelain. The fish protein hydrolysate was successfully used as a supplement to cereal protein. Beddows *et al.* (1976) reported the use of bromelain in the hydrolysis of mackerel for the production of fermented fish flavour. Burica and Vitez (1981) investigated the hydrolysis of chicken meat proteins by bromelain. A soluble protein hydrolysate

possessing good taste and odor, as well a nitrogen concentration from 14 to 14.8 % was reported by the latter. Crude bromelain, obtained by comminuting pineapple pieces, has also been used to hydrolyse tuna canning wastes (Raghunath, 1993). The recovery of both solids and total nitrogen was considered good at optimum hydrolysis conditions, stated to be as follows: Enzyme concentration of 0.008 units enzyme/mg protein, pH 5.6 and reaction temperature 65 °C

Enzymes of animal origin such as the acidic protease, pepsin, have been studied in the hydrolysis of fish protein (McBride *et al.*, 1961; Tarky *et al.*, 1973). Pepsin was reported to be the most effective protease compared to bromelain and Rhozyme B-6, by McBride *et al.* (1961) for the proteolysis of herring (*Clupea harengus*). Tarky *et al.* (1973) reported a protein hydrolysate from English sole (*Parophyrus vetulus*) waste with comparable nutritional quality, evaluated by protein efficiency ratio studies, to casein. Pepsin is the most widely used among animal enzymes (Liu and Pigott, 1981), though chymotrypsin has also been studied (Montecalvo *et al.*, 1984).

Hale (1969) has thoroughly tested the relative proteolytic activities of commercially available enzymes with potential use on fish tissue.

Advances in fermentation technology and bioseparations during the last decade have resulted in a variety of commercial microbial proteases, mostly of bacterial origin, being available at low cost for use in the production of FPH (Mackie, 1982).

In comparison to animal- or plant-derived enzymes, microbial enzymes offer several advantages including the great variety of catalytic activities available, increased pH and temperature stabilities over corresponding plant- and animal-derived enzymes, and a more convenient production procedure than for plant and animal enzymes (Cheetham, 1985; Klacik, 1988).

Enzymes of microbial origin have been also applied to the hydrolysis of fish. Hale (1972) suggested that the use of a *Streptomyces* protease in the production of FPH has a potential application in formulating infant diets.

Several authors have observed that alkaline proteases (i. e. Alcalase®, from *Bacillus licheniformis*) exhibit higher proteolytic activity than neutral and acid enzymes such as papain and pepsin (Rebeca *et al.*, 1991; Hoyle and Merrit, 1994). Alcalase®, in particular, has an additional advantage of producing hydrolysates bland in taste, even at a relatively high degree of hydrolysis, where bitter peptides are produced causing bitterness. The hydrolysis of cod offal and herring (Lalasidis *et al.*, 1978; Lalasidis and Sjoberg, 1978; Hoyle and Merrit, 1994), menhaden (Hale and Bauersfield, 1978), tilapia (Yu and Tan, 1988), and sardine (Sugiyama *et al.*, 1991) with Alcalase® were, for example, reported to yield hydrolysates bland in taste or with no bitter taste.

Studies conducted by Hale (1974) indicated that fish hydrolysis at pH 8.5 using an alkaline protease from *Bacillus subtilis* resulted in a protein hydrolysate with high nutritive value and high product yield.

The ability of alkaline, neutral and acidic proteases to solubilize fish protein were compared by Sugiyama *et al.* (1991). The highest rate of protein

solubilization was reported for the alkaline proteases (Alcalase®, Actinase, and Proleather) compared to neutral (Neutrase®, papain) and acidic enzymes (Molsin, pepsin, and Newlase). In addition to the higher activity, the alkaline proteases had the higher tryptophan content.

Poulsen *et al.* (1990) carried out hydrolysis of chopped herring using Alcalase®, Neutrase®, pepsin, trypsin and papain. The results revealed higher values of protein yield and relatively higher values of peptide chain length for the Alcalase®-assisted hydrolysates.

Rebeca *et al.* (1991) examined the hydrolysis of eviscerated mullet (*Mugil cephalus*) by bacterial and fungal proteases. Since bacterial proteases (HT-200, Protease N, and Pescalase - 560) showed high activity on casein they were selected to study the hydrolysis of the fish protein. Pescalase - 560 presented the higher yield of protein recovery, 80 to 85 %, after a 2-hr hydrolysis.

From the technical and economical viewpoints, proteolytic microorganisms seem to be the most promising source of proteases. Methods used to produce microbial enzymes are cheap and easy to scale up. Environmental conditions can be monitored closely to have consistency in the enzyme purity, stability, and activity. In addition, microorganisms exhibit a wider variety of specific proteases than either plant or animals (Löffler, 1986). Protease-producing microorganisms are common among the genera *Bacillus, Clostridium, Pseudomonas, Proteus, Aspergillus, Streptomyces* and *Rhizopus* (Cheetham, 1985; Ward, 1985; Venugopal, 1994).

2.3.3 Factors affecting the hydrolysis and recovery of proteinaceuos substances from the fish

The hydrolysis of food proteins is a complex process that involves a great deal of variables. These factors markedly influence the amount of soluble nitrogen released during the hydrolysis of fish proteins. The recovery of proteinaceous substances and the rate of hydrolysis depend on the type of protein and the conditions of hydrolysis, such as pH, temperature, concentration and specificity of enzyme, and time of hydrolysis (Adler-Nissen, 1982; 1986). Ledward and Lawrie (1984) observed that not only yield is influenced by the hydrolysis parameters, but also composition and molecular weight distribution of the peptides in protein hydrolysates.

The pH of the hydrolysis mixture has an important role in the hydrolytic reaction. Enzyme and substrate molecules have their charge distribution and conformation changes with variation in pH, which will affect the individual rate constants to different extent, mainly because many titratable groups with different pK are involved. This suggests that pH would influence the properties of a hydrolysate at a fixed degree of hydrolysis (Adler-Nissen, 1982).

The concentration of the enzyme relative to the concentration of substrate (E/S) is usually more characteristic of the reaction velocity, and also influence the nitrogen recovery from the protein substrate. In fact, Rebeca *et al.* (1991) observed that increases in enzyme concentration improved the recovery of soluble nitrogen from the fish substrate.

Temperature markedly affects the conversion rate of the reaction. It should be varied within the optimal range for maximum protease activity and up to the point that the heat stability of the enzyme and the substrate is not compromised (Adler-Nissen, 1986).

The specificity of the enzyme and the nature and state of denaturation of the substrate will exert an influence on the molecular weight distribution of hydrolysates, ranging from still unconverted protein (unhydrolysed) to small peptides and free amino acids (Adler-Nissen, 1976).

Some workers have observed that an increase in hydrolysis time, or in the E/S ratio, will result in a decrease in the average chain length of the peptides in soluble fraction (hydrolysed protein) (Adler-Nissen, 1986; Rebeca *et al.*, 1991). Furthermore, prolonged proteolysis may result in the formation of highly soluble peptides, completely lacking the functional properties of the native proteins, and may promote the formation of undesirable bitter peptides (Quaglia and Orban, 1987a; Mohr, 1978).

In summary, the proper set of hydrolysis parameters leads to a final product with better functional properties, high nutritive value, and improved yield.

2.4 Functional properties of enzymatically hydroiyzed proteins

Functional properties of proteins can be defined as the physicochemical properties that affect the processing, storage stability, organoleptic quality, and biological efficacy of the formulated food (Kinsella, 1976; Vojdani and Whitaker,

1994). Therefore, functional properties characterize the overall performance of any protein in food systems, which consequently govern the acceptability of a new protein food.

As pointed out by Damodaran (1994), these physicochemical and structural properties are size, shape, amino acid composition and sequence, net charge, charge distribution, hydrophobicity/hydrophilicity ratio, secondary, tertiary, and quaternary structural arrangements, number of microdomain structures, and adaptability of domain structures or the structure of the whole molecule to changes in environmental conditions.

Solubility, dispersibility, emulsifying and foaming capacities, binding of water or fat, and the rheological characteristics of protein dispersions are some functional properties of relevant interest in food formulation (Table 2.1) (Hidalgo, 1978; Adler-Nissen, 1986; Giese, 1994).

A lack of standard procedures for measuring any specific functionalproperty has been noted and this has generated a great deal of debate and efforts to bring conciseness to the assessment methodology (Adler-Nissen, 1986).

Fish protein hydrolysates have been repeatedly reported to possess desirable functional properties (Adler-Nissen, 1986; Quaglia and Orban, 1987b; Rebeca *et al.*, 1991; Shahidi *et al.*, 1995). For example, fish hydrolysates often present a remarkable dispersibility in water (Sikorski and Naczk, 1981) and high solubility over a wide pH range (Adler-Nissen, 1976; Quaglia and Orban, 1987a), which is a consequence of the enzymatic degradation of the original

protein to smaller peptide units. Surface properties, such as emulsification and foaming capacities are in general more than acceptable (Sikorski and Naczk, 1981), this being related to the degree of hydrolysis that the original protein has undergone during the reaction (Quaglia and Orban, 1990). Overall, the enzymatic process enhances the functionality of the native protein.

Table 2.1 Desired functional properties of proteins in food systems

Function	Mode of action	Food system example	Protein source (most common)
Emulsification	Adsorption at interfaces, film formation	Sausages, bologna, soup, cakes, dressing	Muscle proteins, egg proteins, milk protein
Fat absorption and binding	Hydrophobic bonding, entrapment	Simulated meats, bakery, doughnuts	Milk protein, egg proteins
Foaming	Interfacial adsorption, film formation to entrap gas	Whipped toppings, ice cream, angel cakes, desserts	Egg proteins, milk protein
Solubility	Hydrophilicity, protein solvation	Beverages	Whey proteins
Viscosity	Water binding, hydrodynamic size, shape	Soups, gravies, salad dressings	
Water absorption and binding	H-bonding, hydration	Meat sausages, cakes, breads	Muscle proteins, egg proteins

Source: Kinsella, 1982 and Kinsella *et al.*, 1985.

2.5 Nutritional quality of fish protein hydrolysates

It has been reported that enzymatic hydrolysis of fish tissue maintains the nutritional quality of the original substrate (Mackie, 1982). As previously pointed out by Bárzana and García-Garibay (1994), FPH, generally, have an essential amino acid content similar or even superior to the reference protein pattern indicated by FAO/WHO (1973) (Figure 2.3).

Lalasidis *et al.* (1978) compared the protein efficiency ratio of FPH obtained from cod offal to the control casein and observed that the protein hydrolysate was as nutritive as the milk protein. Moreover, protein efficiency ratio can surpass the control casein. It has also been reported that fish hydrolysate can be more nutritious than casein, with respect to the PER. For instance, Yañez *et al.* (1976) found that the protein hydrolysate obtained from hake had 15 % higher PER value than the control. Rebeca *et al.* (1991) tested the nutritional quality of mullet protein hydrolysate with the same feeding trials with rats (PER) and noticed that the product had a nutritive value similar to casein.

2.6 Applications of fish protein hydrolysates

FPH could find use in human consumption. Also, fish hydrolysates have proven to be effective as a milk replacer for calves and piglets (Hale and Bauersfield, 1978; Goldhor *et al.*, 1990; Mackie, 1994) and as a protein supplement in fish feed (Poulsen *et al.*, 1990; Uchida *et al.*, 1990), poultry feed

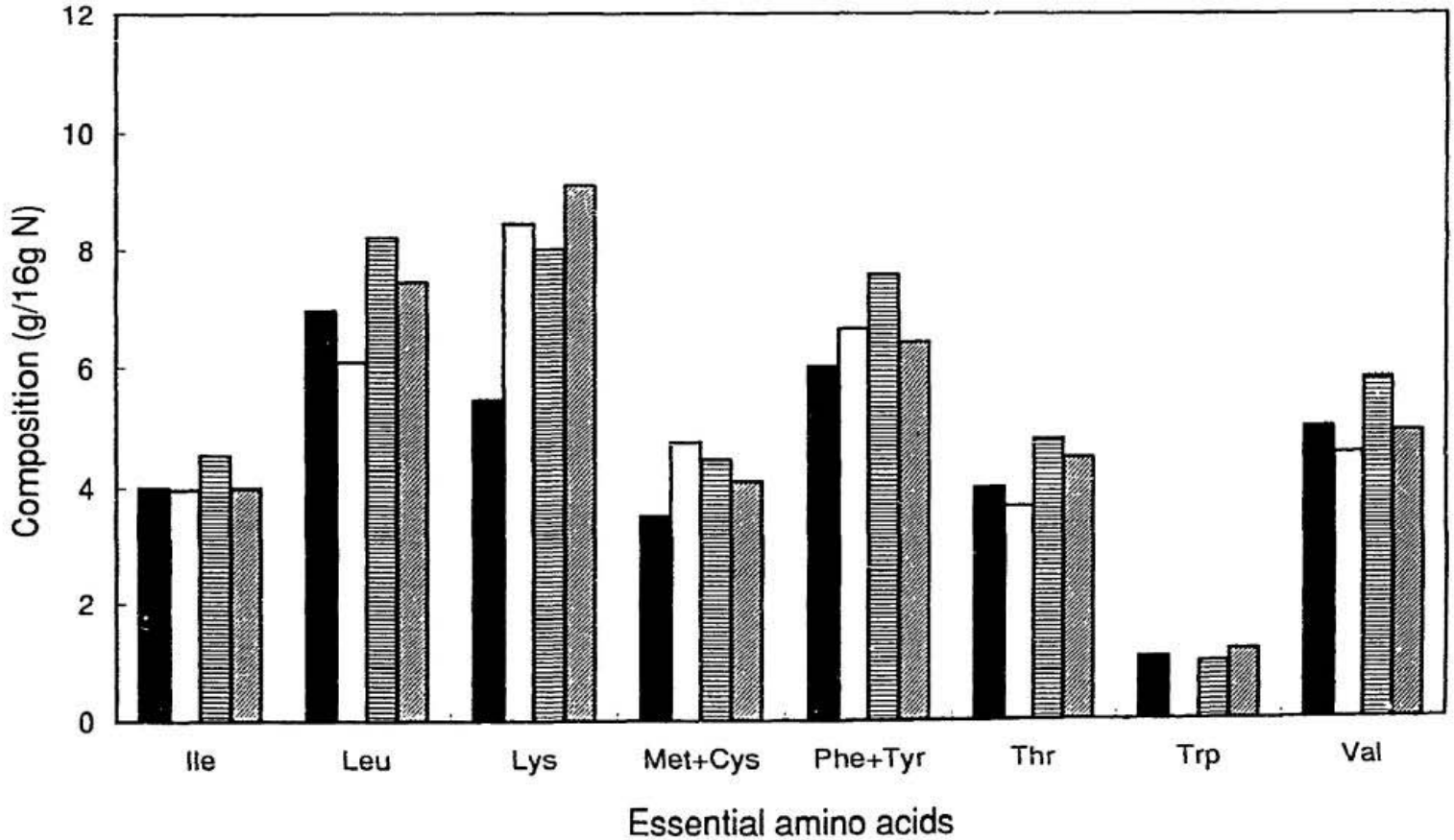
Figure 2.3 Essential amino acid content of the reference protein and of fish protein hydrolysates from different species.

■ : Reference protein. FAO/WHO, 1973.

□ : FPH from mullet. Rebeca *et al.*, 1991.

▤ : FPH from cod offal. Lalasidis *et al.*, 1978.

▨ : FPH from herring. Lalasidis, 1978.


12
10
8
6
4
2
0
Composition (g/16g N)
Ile
Leu
Lys
Met+Cys
Phe+Tyr
Thr
Trp
Val
Essential amino acids

(Kilara, 1985), and pet food (Willard, 1990).

Non-food applications of FPH have been reported by many researchers, especially as a nitrogen source for microbial growth (Beuchat, 1974; Gildberg *et al.*, 1989; Levin *et al.*, 1989; Vecht-Lifshitz *et al.*, 1990).

As products for human consumption FPH might find similar outlets as fish protein concentrates. Kvitka and Chen (1982) reported a feasible application of fish protein concentrate from hake and albacore as a protein supplement in home baked products, such as whole wheat bread and muffins, peanut butter cookies, and date nut bars. It was concluded that FPC can replace up to 5 % of flour maintaining standard quality and consumer acceptability. Shehata *et al.* (1989) also indicated that FPC can favorably replace up to 6 % of wheat flour in the production of pastries. A hamburger type product made from minced beef and FPC was reported by Vareltzis *et al.* (1990). The manufactured food product obtained acceptable sensory quality ratings, even though a fishy-flavor was consistently detected in the samples.

FPC has also been successfully examined as a protein supplement for pasta products, such as macaroni (Crisan, 1970) and noodles (Woo and Erdman, 1971).

The evaluation of FPH as food ingredients has been carried out as well. Bertullo and Pereira (1970) may have been the first investigators to approach the use of FPH for human consumption, using a yeast to degrade the protein substrate. Later, Yañez *et al.* (1976) produced a high quality protein

hydrolysate successfully used as a supplement to cereal protein. Protein hydrolysates produced from tilapia (*Oreochromis mossambicus*) were incorporated into fish crackers (keropok) with promising results. The final product had higher nitrogen content than the control sample and the overall acceptability was not significantly different from the control (Yu and Tan, 1990). Shoji (1990) introduced a fish protein hydrolysate, in the form of a paste, obtained either from pink salmon, pollock, or snow crab that can be incorporated into foods such as surimi, tofu, seafood soup, and snacks. Spinelli *et al.* (1975) suggested the use of a succinylated fish hydrolysate for gelatin dessert and toppings, due to its good whipping properties. The enzymatic hydrolysis of seafood is also used for the production of fish flavour (In, 1990). The use of protein hydrolysates as a protein supplement in protein-enriched drinks has been suggested (Frøkjaer, 1994), mainly due to the high dispersibility and solubility of the hydrolysate. Owens and Mendoza (1985) have thoroughly reviewed the use of enzymatic hydrolysis in the development of fishery products.

2.7 Response surface methodology

Response surface methodology (RSM) is a useful statistical technique for investigation of complex processes and has been successfully applied to optimize food processing operations (Thompson, 1982; Joglekar and May, 1987; Floros and Chinnan, 1988; Smith *et al.*, 1988; Garrote *et al.*, 1993, 1994; Cui *et*

al., 1994; Haltrich *et al.*, 1994; Oomah *et al.*, 1994; Govindasamy *et al.*, 1995). It consists of a group of mathematical and statistical procedures that can be used to study relationships between one or more responses (dependent variables) and a number of factors or parameters (independent variables). RSM defines the effect of the independent variables, alone and in combinations (interactions), on the process. In addition to analysing the effects of the factor variables, this experimental methodology determines the settings of variables that should be changed in order to improve the process performance. Moreover, RSM generates a mathematical model equation that accurately describes the overall process and determines an optimal process level, indicating what should be done to achieve this optimum performance level (Haaland, 1989).

In the field of enzymatic modification of food proteins, RSM has proved to be a powerful methodology characterized by efficiency and realism. Surówka and Fik (1992) applied RSM to optimize the recovery of proteinaceous substances by a Neutrase®-assisted hydrolysis of chicken heads. Because the statistical approach was successful they maintained the same experimental methodology to investigate, two years later, the hydrolysis of the same protein substrate, but this time using an animal-origin enzyme, pepsin (Surówka and Fik; 1994). RSM has also been used to describe the hydrolysis of seal (Shahidi *et al.*, 1994), capelin (Shahidi *et al.*, 1995), oyster (Cha *et al.*, 1995), and canola meal (Ma and Ooraikul, 1986).

The use of RSM to investigate the extraction of protein from brewer's spent

grain (Diptee *et al.*, 1989), and hydrolytic depolymerization of amaranth starch (Guzmán-Maldonado *et al.*, 1993) has also been carried out with success.

All these studies confirm the application of RSM as a reliable statistical problem-solving technique for complex processes which might be subjected to the effects of various experimental variables.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Raw material

Spiny dogfish (*Squalus acanthias*) was supplied by Fishery Products International Ltd., St. John's, Newfoundland. The fish was caught along the Southwest coast of Newfoundland, Canada, in August 1995, and immediately eviscerated on board. The dogfish was transported to the laboratory under chilled conditions and then stored at -25 °C until use. Dogfish fillets were prepared by removing the fins, head, belly flap and, finally, the skin. Small pieces of the fillets were washed and soaked in cold water to remove blood and other soluble substances, particularly urea. Excess water was removed from the flesh by squeezing pieces in the hand. Randomly selected pieces were then run twice through an electrical meat grinder (OMAS Model TS8, Mississauga, ON) using a cutter plate with 5 mm diameter holes. The final ground muscle material was mixed thoroughly and used in the hydrolysis experiments.

3.1.2 Enzyme and Chemicals

Alcalase®, a serine bacterial endopeptidase (generic name, Subtilisin Carlsberg) prepared from a strain of *Bacillus licheniformis*, was provided by Novo

Industri A/S, Bagsvaerd, Denmark. The food-grade enzyme, having a specific activity of 2.4 Anson units · g^{-1} (Novo Industri, 1988), was stored at 5 °C until it was used for the hydrolysis experiments. Its optimum enzymatic activity occurs at temperatures between 50 and 70 °C, and in pH-range between 6 and 10. Its density is given as 1.18 g · mL^{-1}, and its deactivation temperature is 85 °C for 10 min (Novo Industri, 1988, 1991).

All reagents were of analytical grade.

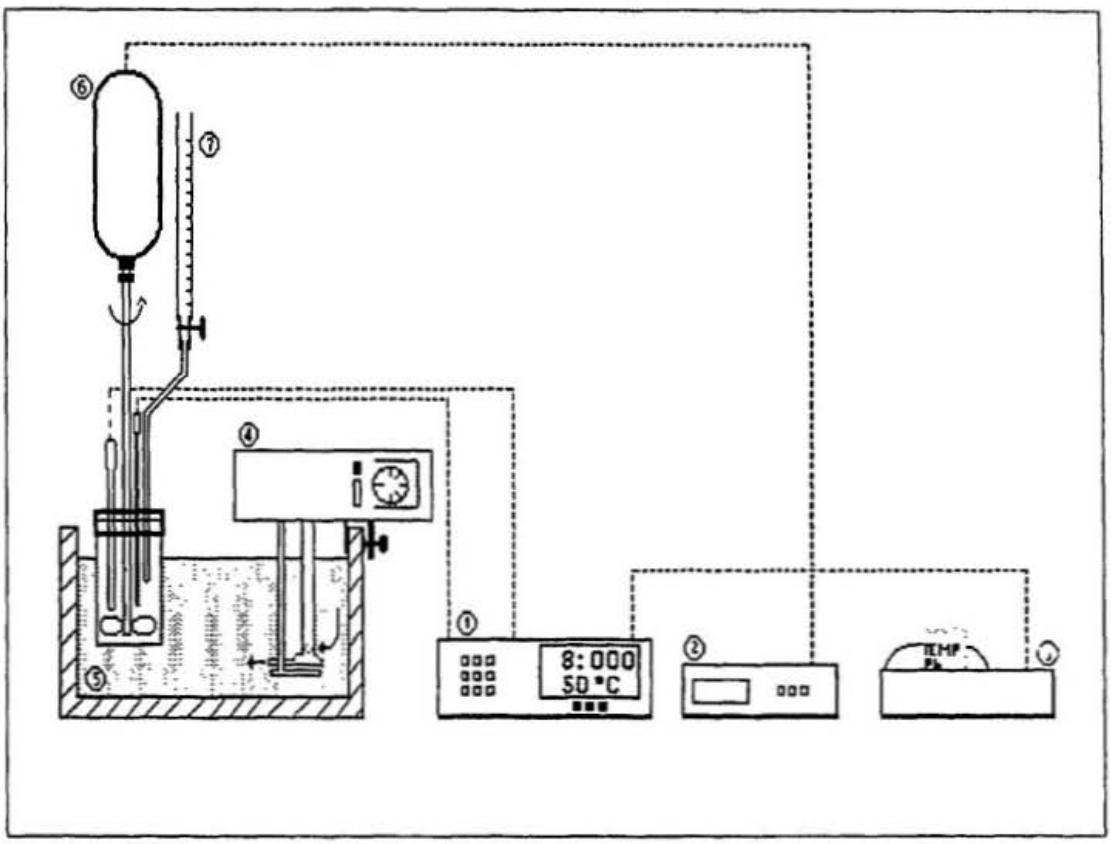
3.2 Experimental procedures

3.2.1 Enzymatic hydrolysis of dogfish muscle: pH-stat method

All hydrolysis experiments were accomplished using an apparatus specially designed and properly assembled to resemble the pH-stat device described by Jacobsen *et al.* (1957) and Boyce (1986). The pH-stat device can be broken down into four basic units, consisting of: 1) a reaction vessel with stopper and entries on it to a pH electrode, temperature probe, hose for addition of NaOH and a mixer shaft; 2) a printer to record the temperature, pH and time of hydrolysis; 3) pH meter; and 4) mixer controller for monitoring the shaft rotation.

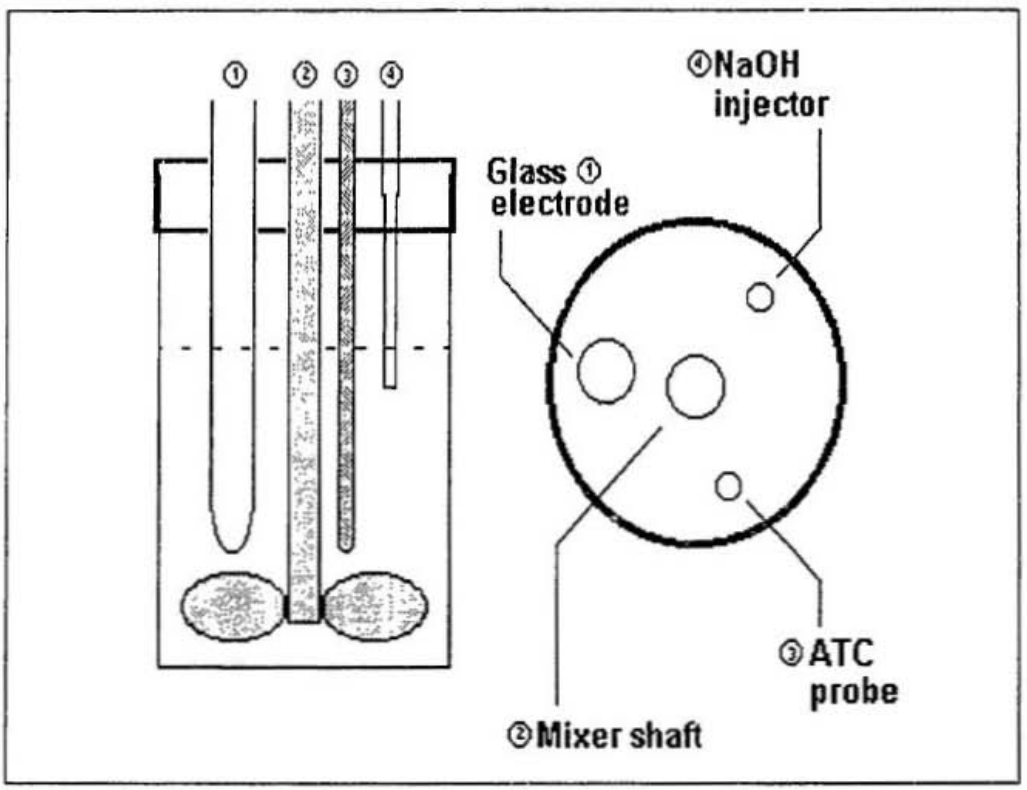
Hydrolytic reactions to investigate the effect of hydrolysis variables were performed in a 250-mL polyethylene vessel immersed in a constant temperature water bath (Figure 3.1). The reaction vessel was wrapped with a cover that had holes cut in it for an automatic temperature compensator (ATC

Figure 3.1 Experimental setup for the enzymatic hydrolysis of the dogfish muscle protein. (1) pH meter; (2) Mixer controller; (3) Recorder; (4) Thermostat - reflux controller; (5) Water bath; (6) Mixer head; and (7) Burette (50 mL).


⑥
⑦
④
⑤
①
8:000
50 °C
②
TEMP
③

probe), a pH glass electrode, a shaft mixer, and the addition of alkali (Figure 3.2). During reactions pH was maintained at a desired value by addition of 0.2 N NaOH. The reaction flask, with ground dogfish muscle, deionized water and phosphate buffer, making up an initial substrate concentration (S %) of 8 % of protein (N x f_N), was placed into a previously heated water bath. A 5-min homogenization was allowed for adjustment of pH, through the addition of NaOH, and of temperature to the desired values described for each particular set of experiments. Then, enzyme was added and reaction was allowed to proceed under constant agitation, at 200 rpm. The volume of base needed to keep pH constant during the hydrolytic reaction was recorded to allow calculation of degree of hydrolysis. Control experiments were performed without enzyme addition, but under the same experimental conditions. Reactions were terminated by heating the solution to 90 °C for 15 min, which assured the inactivation of the enzyme (Novo Industri, 1988) and the separation of the oil from substrate. The resultant slurry was centrifuged at 2800 x *g* for 20 min and the lipid layer was skimmed off. The volume of supernatant was recorded and analyzed for nitrogen content by the Kjeldahl method.

Figure 3.2 Experimental setup. Layout of the reaction vessel.


①
②
③
④
④NaOH
injector
Glass ①
electrode
③ATC
probe
②Mixer shaft

3.2.2 Determination of degree of hydrolysis

Degree of hydrolysis (DH) is generally used as a proteolysis monitoring parameter when the pH-stat method is employed, since it is a more practical measurement under industrial applications (Petersen, 1981; Turgeon *et al.*, 1991). The pH-stat reaction allows the estimation of DH based on the consumption of alkali to maintain a constant pH at the desired value. As defined by Adler-Nissen (1976, 1977, 1986), degree of hydrolysis is the percent ratio between the number of peptide bonds cleaved (h) and the total number of peptide bonds in the substrate studied (h_{tot}), which was estimated by adding up the mmols of each individual amino acid per gram of protein (N × 6.25), this being found by determining the amino acid composition of the dogfish muscle, as described below. The equation for determination of DH is as follows:

$$DH\ (\%) = \frac{h}{h_{tot}} \times 100 \qquad \text{Eqn [1]}$$

The parameter h is determined based on the principle that hydrolysis reactions conducted at pH values higher than 6.5 have a significant dissociation of α-NH_2 groups, and the number of peptide bonds cleaved is directly proportional to the alkali consumption during hydrolysis (Adler-Nissen, 1977; Petersen, 1981). Therefore, the reaction can be monitored by the consumption of alkali added to neutralize the hydrogen ions released during digestion and keep the pH of the

solution constant. This relationship can be expressed as:

$$h = B \cdot N_b \cdot \frac{1}{\alpha} \cdot \frac{1}{M_p} \qquad \text{Eqn [2]}$$

where B is the amount of alkali consumed to maintain the pH constant during the reaction, N_b is the normality of the alkali, M_p is the mass of substrate (protein, determined as N $\times$ 6.25) in the reaction, and α is the average degree of dissociation of α-NH_2 groups released during hydrolysis. The degree of dissociation is estimated from:

$$\alpha = \frac{10^{(pH-pK)}}{1+10^{(pH-pK)}} \qquad \text{Eqn [3]}$$

where the pK value varies with the temperature of the hydrolysis reaction. According to Steinhard and Beychok (1964), pK value can be estimated for the temperature range used in the hydrolysis experiments by:

$$pK = 7.8 + \frac{(298 - T)}{298 \cdot T} \times 2400 \qquad \text{Eqn [4]}$$

where T is the hydrolysis temperature in Kelvin. These values are depicted in Table 3.1.

Table 3.1 The degree of dissociation as a function of the pH and pK values, and the hydrolysis temperature (°C), calculated according equations [3] and [4].

T (°C)		40	45	50	55	60	65
	pK	7.41	7.29	7.17	7.06	6.95	6.84
pH							
6.5		0.11	0.14	0.18	0.22	0.26	0.31
7.0		0.28	0.34	0.40	0.47	0.53	0.59
8.0		0.80	0.84	0.87	0.90	0.92	0.93
9.0		0.97	0.98	0.99	0.99	0.99	0.99
9.5		0.99	0.99	1.00	1.00	1.00	1.00

3.2.3 Determination of nitrogen recovery

Nitrogen recovery, the percentage ratio of nitrogen content in the hydrolysate to that in the original substrate, was used as an index of nitrogen solubilization to describe the hydrolysis yield. For each hydrolysis reaction, the nitrogen in the centrifuged soluble fraction was determined by the Kjeldahl method (A.O.A.C., 1990, Method 955.04). The volume of the soluble fraction was recorded after it was filtered through a Whatman #1 filter paper, and 10 mL-aliquots were analysed for nitrogen. Total soluble nitrogen in the supernatant was obtained by the following calculation:

$$\frac{\text{mg N}}{\text{10 mL aliquot}} = \frac{x}{\text{supernatant volume}} \qquad \text{Eqn [5]}$$

where x is the total mg N per volume of extract. Nitrogen recovery was expressed as a percentage of nitrogen content in the soluble fraction and in the initial substrate as shown by the following equation:

$$\text{NR (\%)} = \frac{\text{Total nitrogen in the supernatant (mg)}}{\text{Total nitrogen in the substrate (mg)}} \times 100 \qquad \text{Eqn [6]}$$

3.2.4 Influence of reaction variables in the hydrolysis of dogfish muscle: Preliminary experiments

Hydrolytic reactions involve mainly four independent factors (variables): temperature, pH, enzyme - substrate ratio and time, besides the selected enzyme and protein substrate (Adler-Nissen, 1986). It is important to know which of these are critical in the enzymatic process, since optimization of the process would lead to a large number of experiments, and keeping a large number of variables under control in commercial operations is costly (Adler-Nissen, 1984). Therefore, preliminary experiments were carried out to examine the prevailing effects of hydrolysis variables on the enzymatic reaction of dogfish muscle using Alcalase®. Furthermore, these experiments provided a more specific range, within each reaction condition, to efficiently apply a factorial design that described the overall enzymatic process with more realism.

The procedure to examine the influence of each hydrolysis variable was, at first, varying the temperature of hydrolysis (40 to 65 °C). The temperature that resulted in the highest % of nitrogen recovery was tested at various pH values (6.5 to 9.5). The best result was used for testing the effect of E/S ratio (1 to 4.5 %) on NR and DH, and subsequently for the effect of time (up to 150 min). These ranges were selected based on the suggestion of the manufacturer, and data from previous studies. Experiments were performed according to Table 3.2. The ranges for each parameter which produced the highest nitrogen recoveries and % hydrolyses, were then used in the hydrolysis experiments planned on the response surface methodology.

Table 3.2 Regimes used in the preliminary experiments [a].

Temperature (°C)	pH	E/S (%, w/w)	Time (min)
40 - 65	8.0	2.0	120
55	6.5 - 9.5	2.0	120
55	8.0	1 - 4.5	120
55	8.0	4	0 - 150

[a] Experiments were conducted in triplicate.

3.2.5 Design of the experiments using Response Surface Methodology

Response surface methodology (RSM) was used to investigate the combined effects of the hydrolysis variables pH, temperature and enzyme - substrate ratio (%, w/w) on the digestion of dogfish muscle using the microbial protease Alcalase®, and to optimize these variables regarding the maximum nitrogen recovery from the original substrate.

This methodology allows the modelling of a second-order equation that describes the process *per se*. It was assumed that two mathematical functions, f_k (k = 1,2), exist for each response variable Y_k (degree of hydrolysis and nitrogen recovery) in terms of three independent process variables.

$$Y_k = f_k\ (X_1, X_2, X_3) \qquad \text{Eqn [7]}$$

where X_1, X_2 and X_3 represent, respectively, pH, temperature and the ratio between enzyme and substrate on weight basis (E/S).

The true function f_k can be approximated by a known and simple second-order polynomial (Eqn [8]) using multiple regression through the least squares method for estimation of the coefficients β.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_{ii} + \sum_{i=1}^{2} \sum_{j=2}^{3} \beta_{ij} X_i X_j \qquad \text{Eqn [8]}$$

where Y is the measured response variable; β_0, β_1, β_2 and β_3 are constant, linear,

quadratic and crossproduct regression coefficients of the model (mathematical function), respectively; and where X_i and X_j represent the independent variables (hydrolysis parameters) in coded values.

A Box-Behnken factorial design with three factors and three levels, including three replicates at the center point (Box and Behnken, 1960), was used for fitting a second order response surface in both cases (response variables, Y_k). Table 3.3 gives the factors (reaction variables) and their coded and uncoded values. Table 3.4 depicts the experimental design which shows a total of 15 combinations of settings (runs) for the process variables.

Actual values of independent variables were coded according to the following formula:

$$X = \frac{A_C - \Delta_M}{\Delta_S} \qquad \text{Eqn [9]}$$

where X is the coded value, A_c is the actual value, Δ_M represents the average between the highest and lowest values for the variable in the design, and Δ_s is the increment of the actual value corresponding to one unit of X.

Table 3.3 Hydrolysis variables and experimental design levels for response surfaces

Independent variables	Symbols		Levels		
	Coded	Uncoded	-1	0	1
Hydrolysis pH	X_1	pH	7.0	8.0	9.0
Hydrolysis temperature (°C)	X_2	T	40	50	60
Enzyme/substrate ratio (%, w/w)	X_3	E/S	2.0	3.0	4.0

Table 3.4 Box-Behnken design matrix for the Alcalase®-assisted hydrolysis of dogfish muscle

Design point[a]	Independent variables		
	pH	Temperature (°C)	E/S/ ratio (%)
	X_1	X_2	X_3
1	1	1	0
2	1	-1	0
3	-1	1	0
4	-1	-1	0
5	0	0	0
6	1	0	1
7	1	0	-1
8	-1	0	1
9	-1	0	-1
10	0	0	0
11	0	1	1
12	0	1	-1
13	0	-1	1
14	0	-1	-1
15	0	0	0

[a] Experiments were conducted in a random order.

3.2.6 Spray drying

Dehydration is a method of improving the stability of a product in storage, and spray drying is widely used to preserve liquefied products as powders (Matzinos and Hall, 1993). Therefore, the liquefied shark protein hydrolysate was spray dried in a Büchi 190 Mini Spray Drier (Büchi Laboratoriums - Tecknik AG, Flawil / Schweiz) with inlet and outlet temperatures of 120 and 90 °C, respectively. The feed rate was 0.4 L/hr. The spray-dried powder was stored at 5 °C.

3.3 General analytical methodology

The grounded dogfish muscle and the protein hydrolysate were examined for chemical composition.

3.3.1 Moisture

Moisture content was determined according to A.O.A.C. (1990), Method 950.01, by drying a known amount of material (2 - 5 g), in a pre-weighed aluminum pan placed in a forced air oven (Blue M Electric Co., Blue Island, IL) at 103 - 105 °C until it reached a constant weight. Samples were then transferred to a desiccator, cooled and weighed immediately. The moisture content was calculated as a percentage of weight loss of the sample due to drying.

3.3.2 Ash

Samples, weighing between 3 - 5 g, were transferred into clean porcelain crucibles, charred using a flame and then placed in a temperature-controlled

muffle furnace (Blue M Electric Co., Blue Island, IL) which was preheated to 550 °C. Crucibles with lids were held at this temperature until grey ash remained, and then were transferred to a desiccator, cooled and weighed immediately. Ash was calculated as percentage weight of the remaining matter (A.O.A.C., 1990, Method 938.08).

3.3.3 Total lipids

Total lipids content was determined by extraction with chloroform:methanol:water mixture using the method of Bligh and Dyer (1959) described by Ke *et al.* (1990). A 10 g, for FPH, or 50 g for the fish muscle, was homogenized in chloroform:methanol:water (1:1:1, v/v/v) and held overnight for separation. Aliquots (10 mL) from the chloroform layer, which contained the extracted lipids, were transferred to preweighed aluminum pans. Excess solvent was removed by evaporation, first at room temperature for two hours, and then by heating at 103 - 105 °C for 1 h.

3.3.4 Nitrogen determination

Total nitrogen content

Nitrogen determinations were based on the Kjeldahl method described by A.O.A.C. (1990), Method 955.04. Samples weighing approximately 250 mg, on nitrogen-free papers, were placed in the digestion tubes of a Büchi 426 digestor (Büchi Laboratoriums - Technik AG, Flawil/Schweiz). The samples were

digested with two Kjeltabs catalyst pellets (Profamo, Quebec) and 20 mL of concentrated H_2SO_4 in the Kjeldahl digestor until a clear solution was obtained. After 30 min of cooling time, the digested samples were diluted with distilled water (60 mL), 150 mL of 25% NaOH were added and the released ammonia was steam distilled (Distillation unit Büchi 315) into a 4% H_3BO_3 (50 mL) containing 12 drops of an end point indicator (N-point indicator, EM Science, Evanston, NJ) until 150 mL distillate were collected. The content of nitrogen in the distillate was determined by titrating it against 0.10 N H_2SO_4. The content of crude protein in samples was calculated by multiplying the percentage of nitrogen by a factor (f_N) of 6.25.

Non-protein nitrogen

The non-protein nitrogen (NPN) content of samples was measured as the percent fraction of the total Kjeldahl nitrogen that was not precipitated in a 10 % trichloroacetic acid (TCA) solution, according to the method of Ke *et al.* (1990). Ground shark muscle (50 g) was blended with 100 mL of 10% TCA solution, at 20 °C for 1 h using a commercial Waring blender. The insoluble residue was removed by centrifugation at 2000 x *g* for 15 min at 5 °C. The supernatant was collected and an aliquot of 10 mL was taken for determination of soluble nitrogen using the Kjeldahl method (A.O.A.C., 1990, Method 955.04). NPN of the protein hydrolysate sample (5 g) was determined by blending with 25 mL of TCA (10%) following the same procedure.

3.3.5 Amino acid analysis

The amino acid analysis was performed, using a 121 MB Amino Acid Analyzer (Beckman Instruments Inc., Palo Alto, CA), on the lyophilised dogfish muscle and spray dried, enzymatically-hydrolysed shark muscle. The samples (2 - 5 mg) were digested in 6N HCl under vacuum at 110 °C (Blackburn, 1978). The analyzer was interfaced with a model HP 3395 integrator (Hewlett-Packard, Boise, Idaho) enabling accurate peak area analysis in the nanomole range.

The sulphur-containing amino acids, methionine and cysteine, were measured separately as methionine sulphone and cysteic acid, after performic acid oxidation and 6N HCl hydrolysis as described by Blackburn (1978).

Tryptophan was determined by UV absorption after hydrolysis with 3N mercaptoethanesulfonic acid at 110 °C according to the method of Penke *et al.* (1974).

3.4 Estimation of nutritional quality of the shark protein hydrolysate

3.4.1 Amino acid score

As suggested by Morr (1981), an indication of the nutritional value of the protein could be provided by the amino acid score. The scoring procedure takes into consideration the essential amino acids of the hydrolysate relative to the amino acids in the FAO/WHO (1973) recommended protein standard. Amino acid score (AS) was calculated using the following equation:

$$AS\ (\%) = \frac{\text{Tested essential amino acid concentration}}{\text{Reference essential amino acid concentration}} \times 100 \qquad \text{Eqn[10]}$$

3.4.2 Protein Efficiency Ratio

Protein efficiency ratio (PER) or the weight (grams) gained by rats per gram of protein consumed (A.O.A.C., 1990) is usually applied to evaluate the nutritional quality of new protein sources. Mathematical equations developed by Alsmeyer *et al.* (1974) and Lee *et al.* (1978) can be used to predict PER values within a standard error of 0.2 of the PER values obtained by the original bioassay method (A.O.A.C., 1990), indicating that they are reliable estimators. These equations have also been applied to predict the nutritional value of beefstocks, prepared from meat and poultry products (Zarkadas *et al.*, 1995), fish muscle and the corresponding enzymatic fish hydrolysate (Shahidi *et al.*, 1991, 1995). Therefore, protein quality was estimated through PER by applying the prediction equations in (Table 3.5).

Table 3.5 Prediction equations of protein efficiency ratio (PER)

Regression equation	Reference
$Y = -0.684 + 0.456[Leu] - 0.047[Pro]$	Alsmeyer *et al.*, 1974
$Y = -0.468 + 0.454[Leu] - 0.105[Tyr]$	Alsmeyer *et al.*, 1974
$Y = -1.816 + 0.435[Met] + 0.780[Leu] + 0.211[His] - 0.944[Tyr]$	Alsmeyer *et al.*, 1974
$Y = 0.08084\ X_7 - 0.1094$	Lee *et al.*, 1978
$Y = 0.06320\ X_{10} - 0.1539$	Lee *et al.*, 1978

Y : PER

X_7 : Thr + Val + Met + Ile + Leu + Phe + Lys

X_{10} : X_7 + His + Arg + Trp

3.5 Characterization of the protein functionality of shark protein hydrolysate (SPH)

Functional properties of a new source of protein serve as a primary basis for information on how the food ingredient will behave in a food system (Crenwelge *et al.*, 1974). Therefore, the hydration and surface properties of SPH were examined in order to evaluate its incorporation as an ingredient in food formulations. The physical and rheological characteristics of the protein hydrolysate were investigated as well.

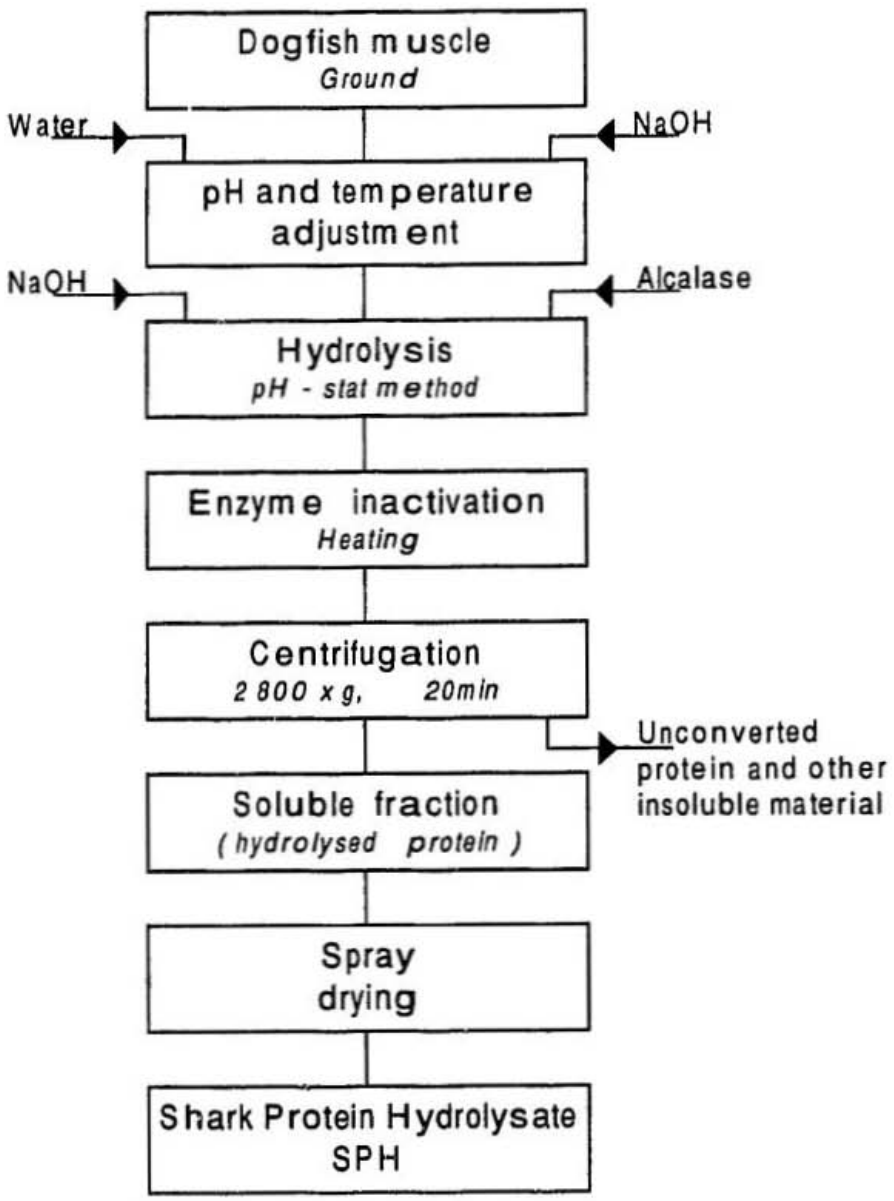
3.5.1 Preparation of protein hydrolysates

The shark protein hydrolysate was prepared according to the same experimental set-up previously described, using the optimized conditions for maximum nitrogen recovery. Autolytic degradation by the endogenous enzymes, was accomplished using the optimized experimental conditions. Each of the soluble hydrolysates was spray dried in a Büchi 190 Mini Spray Drier (Büchi Laboratoriums - Tecknik AG, Flawil/Schweiz, Switzerland) at inlet and outlet temperatures of 120 and 90 °C, respectively. The SPH and the hydrolysate obtained from a two-hour autolytic reaction which was conducted under the same process conditions for the production of the SPH except by the addition of Alcalase®, as well as a powdered freeze dried muscle (control, non-hydrolysed sample) that contained concentrated native dogfish protein, were stored at 5 °C until used in the functional property tests. Samples were coded as described in Table 3.6. Figure 3.3 outlines the overall process employed in the production of the shark hydrolysate.

Table 3.6 Characteristics used for assigning codes to the samples employed in functional properties evaluations.

Sample code	Sample characteristics
Control	The dogfish muscle was freeze dried and powdered. This represented the non-hydrolysed sample that contained the native dogfish protein .
Autolysis	Sample was obtained from a two-hour autolytic reaction which was conducted under the same process conditions for the production of the SPH except the addition of Alcalase®.
SPH	The dogfish muscle was hydrolysed under conditions of maximum nitrogen recovery.

Figure 3.3 Schematic diagram of the process used in the production of protein hydrolysate from spiny dogfish (*Squalus acanthias*).


Dogfish muscle
Ground
Water
NaOH
pH and temperature adjustment
NaOH
Alcalase
Hydrolysis
pH - stat method
Enzyme inactivation
Heating
Centrifugation
2 800 x g, 20min
Unconverted protein and other insoluble material
Soluble fraction
(hydrolysed protein)
Spray drying
Shark Protein Hydrolysate SPH

3.5.2 Functional property tests

Nitrogen solubility index

The solubility of samples was measured by the nitrogen solubility index (NSI) using the method of the American Oil Chemists Society (1989). Samples were dispersed in distilled water (10 g / L) and the pH of the solution was adjusted to the desired values (3.0, 5.0, 7.0 and 9.0) with either 0.5 N HCl or 0.5 N NaOH, while stirring for 45 min continuously. At the end of this period a 25 mL aliquot was centrifuged at 3750 × *g* for 30 min. A 15 mL aliquot of the supernatant was analyzed for nitrogen content by the Kjeldahl method and the NSI was calculated according to the following formula:

$$\text{NSI (\%)} = \frac{\text{Supernatant nitrogen concentration}}{\text{Sample nitrogen concentration}} \times 100 \qquad \text{Eqn [11]}$$

Dispersibility

Dispersibility was determined according to the procedure described by Rakesh and Metz (1973) with minor changes. A protein sample was dispersed in distilled water (10 g / L), the pH was adjusted to 7.0 by addition of 0.5 N HCl or 0.5 N NaOH, and it was then mixed for 60 min using a magnetic stirrer. Then, the solution was allowed to stand for 120 min. An aliquot of 5 mL of supernatant was dried at 103 °C to constant weight. Dispersibility was expressed as the percent

ratio of the suspended solids to the initial weight of the sample.

Water holding capacity

Water holding capacity (WHC) was determined according to the centrifugation method described by Cobb and Hyder (1972). Duplicate samples (0.5 g) were rehydrated with 20 mL of water in centrifuge tubes and dispersed with a vortex mixer for 30 sec. The mixture was allowed to stand at room temperature for 6 h, and it was then centrifuged at 2000 × *g* for 30 min. The supernatant was filtered through Whatman #1 filter paper and the volume recovered was accurately measured. The difference between the initial volume of distilled water added to the protein sample and the volume of the supernatant was determined, and the results were reported as mL of water absorbed per gram of the protein sample.

Oil holding capacity

Oil holding capacity (OHC) was measured as the volume of edible oil held by 50 mg of material, as described by Haque and Mozaffar (1992). Each powdered sample was added to 1 mL of commercial canola oil in a 15 mL glass centrifuge tube, and mixed for 30 sec in a vortex mixer. The oil dispersion was centrifuged at 2000 × *g* for 30 min at room temperature. The volume of oil separated from the hydrolysate was measured and OHC was calculated as the amount of oil (mL) absorbed by 50 mg of sample.

Bulk density

Bulk density was estimated following the procedure of Wang and Kinsella (1976), in which approximately 3 g of each of the control and spray dried hydrolysates were packed into 25 mL graduated cylinders by gently tapping them on the lab bench 10 times. The volume was recorded and bulk density was reported as g/mL of the sample.

Emulsification capacity

Emulsification capacity was measured using the procedure described by Rasekh and Metz (1973). A 0.5 g quantity of sample and 30 mL of cotton-seed oil (Sigma Chemical Co., St. Louis, MO) were added to 60 mL of NaCl solution (30 g / L) and mixed using an Ultra-turrax T25 homogenizer (Janke & Kunkel, Ika - Labortechnik, Berlin, Germany) at 9500 rpm for 30 min. Then, another 30 mL of oil were added over 1.5 min and mixed further for 30 sec. The mixture was transferred to centrifuge tubes, held in a water bath at 85 °C for 15 min, and then centrifuged at 3000 × *g* for 30 min. Emulsification capacity was calculated according the following equation:

$$\text{EC (oil emulsified)} = \frac{V_A - V_R}{W_S} \qquad \text{Eqn [12]}$$

where V_A is the volume of cotton-seed oil added to form an emulsion, V_R is the volume of oil released after centrifugation, and W_S is the weight of the sample.

Foaming properties

Foaming capacity was evaluated by the method of Bernardi Don *et al.* (1991), with minor modifications. A 30 mL quantity of 30 g / L aqueous dispersion was mixed thoroughly using an Ultra-turrax T25 homogenizer at 9500 rpm for 3 min in a 250 mL graduated cylinder. Foaming capacity was calculated as the percent increase in volume of the protein dispersion upon mixing. Foam stability (FS) was estimated as the percent of foam remaining after 60 min.

Rheological characteristics

The viscosity of control and protein hydrolysate sample dispersions at concentrations varying from 20 to 150 g / L water, at 20 °C, was measured with a cone and plate viscometer (Wells-Brookfield model RVTDV - II cp - 200) interfaced with data acquisition software (Brookfield DV gather software). A CP-40 spindle was used to monitor viscosity at shear rates of 150, 750 and 1500 s^{-1}. Viscosity results were reported in Pa·s as mean values from three readings for each determination.

Hunter colour measurements

Sample colours were evaluated according to the Hunter colour parameters, in which *L* is a measure of lightness (white = 100, black = 0), *a* represents the chromatic scale from green (- *a*) to red (+ *a*), and *b* represents the chromatic scale from blue (- *b*) to yellow (+ *b*), using a XL-20 Colorimeter (Gardner Laboratory, Inc., Bethesda, MD). The instrument was standardized to measure the

colour difference with Plate No. XL-20-167C with an *L* value of 92.0, an *a* value of -1.1, and a *b* value of 0.7. Total colour difference (ΔE) was determined by the following equation (Hunter, 1975):

$$\Delta E = \sqrt{(L_R - L_S)^2 + (a_R - a_S)^2 + (b_R - b_S)^2} \qquad \text{Eqn [13]}$$

where subscripts *R* and *S* represent the Hunter colour values for the reference and sample, respectively.

3.6 Statistical analysis

All analytical determinations were run in triplicates with at least three determinations for each experiment.

Data from the Box-Behnken factorial design for the degree of hydrolysis and nitrogen recovery was subjected to a second-order multiple regression analysis using the least squares regression methodology to obtain the parameter estimators of the mathematical model. Canonical analysis, which is used to predict the shape of the curve generated by the model (Watts, 1995), was done as well. These analyses were performed by the response surface regression (RSREG) procedure using the Statistical Analysis System (SAS/STAT, 1990). Graphical representations, *3D*-response surfaces and *2D*-contour plots were drawn using Microsoft Excel 5.0. These experiments were conducted in duplicate.

Experiments on functional prcperties were repeated three times, with duplicate determinations. The data obtained were subjected to one-way analysis of variance (ANOVA) using Minitab Statistical Software, release 6.1. Duncan's New Multiple Range Test (DNMRT) was performed to determine significant differences between samples at the 5 % probability level (Steel and Torrie, 1960).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Proximate composition of dogfish muscle

The composition of the spiny dogfish (*Squalus acanthias*) muscle is presented in Table 4.1. The ash content of the shark muscle was slightly higher than 1 %. This agrees with the findings of Jhaveri and Constantinides (1981), and Shiau and Chai (1985), who reported 0.99 - 1.02 % and 0.7 - 1.0 % of ash, respectively, for the spiny dogfish.

The lipid content of dogfish flesh normally shows considerable variation. It has been reported that although *S. acanthias* usually has a high lipid content which varies greatly according to season, maturity and fishing area, among other factors; therefore, certain variability can be expected (Morris, 1975). The author thoroughly reviewed the data on the composition of spiny dogfish, and reported variations in lipid content from 5.4 to 14 g per 100 grams edible portion (wet weight basis). In this work the lipid content accounted for 5.7 g per 100 grams of muscle.

Moisture content (74.6 %) was found between the expected range of 67.8 to 76.1 % suggested by Jhaveri and Constantinides (1981) for this species. Such variation is attributed to seasonality.

Total nitrogen accounted for 2.98 g per 100 grams of muscle, corresponding to 18.63 % of crude protein. The relatively low amount of NPN (0.45 g/100g, which represents 15 % of the total Kjeldahl nitrogen, in this work) in

Table 4.1 Proximate composition of dogfish (*Squalus acanthias*) muscle [a].

Components	Composition (g/100g)
Ash	1.07 ± 0.03 (4.21)[b]
Lipids	5.69 ± 0.1 ; (22.39)
Moisture	74.59 ± 0.22
Total nitrogen	2.98 ± 0.09 (11.74)
Non-protein nitrogen	0.45 ± 0.03 (1.76)

[a] Mean values of three determinations of three replicate samples ± standard deviations.

[b] Values in parenthesis represent proximate composition on dry weight basis.

the dogfish flesh is not unexpected, since washing and soaking the small pieces of fillet is known to decrease not only the urea content, which accounts for a large part of the NPN in shark, but also other NPN constituents (Morris, 1975; Chari and Sreenivasan, 1980). It is pertinent to mention that in elasmobranchs about 75 % of the water-soluble nitrogen is nonprotein nitrogen (Simidu, 1961).

Table 4.2 reports the amino acid composition of the dogfish (*Squalus acanthias*) muscle protein. The shark muscle has high levels of glutamic and aspartic acid, lysine and leucine. Sulphur-containing amino acids and tryptophan, on the other hand, were present at lower values. Essential amino acids were present at 40.89 % of total amino acids. Hydrophobic amino acids (valine, phenylalanine, tryptophan, leucine and isoleucine) accounted for 23.63 % of the total amino acid content.

The total number of peptide bonds (h_{tot}) determined in this work from the amino acid composition of the shark muscle, 7.2 mEq (g N × 6.25)$^{-1}$, is close to that given by the enzyme manufacturer (Novo Industri, 1978) for fish muscle: 7.3 mEq (g N × 6.25)$^{-1}$. Therefore, 7.2 mEq (g N × 6.25)$^{-1}$ was used in the calculation of degree of hydrolysis throughout this study.

Table 4.2 Total amino acid composition of dogfish (*Squalus acanthias*) muscle protein[a]

Amino acid	Composition (g/100 g Protein)
Alanine	5.33 ± 0.05
Arginine	6.14 ± 0.04
Aspartic acid + Asparagine	9.89 ± 0.17
Cysteine	0.90 ± 0.06
Glutamic acid + Glutamine	14.19 ± 0.01
Glycine	5.34 ± 0.06
Histidine	2.09 ± 0.02
Isoleucine *	4.75 ± 0.08
Leucine *	8.10 ± 0.08
Lysine *	8.94 ± 0.18
Methionine *	2.65 ± 0.16
Phenylalanine *	3.96 ± 0.11
Proline	4.28 ± 0.04
Serine	3.98 ± 0.03
Threonine *	4.59 ± 0.12
Tryptophan *	0.84 ± 0.03
Tyrosine	3.26 ± 0.16
Valine *	4.50 ± 0.10
EAA / TAA [b]	40.89 %

* Essential amino acids

[a] Values represent means of two determinations ± standard deviations.

[b] $\frac{\text{Essential amino acid}}{\text{Total amino acid}} \times 100$

4.2 Influence of reaction conditions on the hydrolysis of dogfish muscle: Single effects

4.2.1 Effect of temperature, pH, E/S and time of hydrolysis

The effect of temperature on the hydrolysis of shark muscle at pH 8.0 is shown in Figure 4.1. It can be seen that with increasing temperature the DH increased to a distinct maximum (55 °C), and then decreased. Nitrogen recovery is also maximized at same temperature value, for the two-hour hydrolytic reaction. However, a less evident peak is observed for the NR profile. Such decrease in the percent hydrolysis and nitrogen recovery over the higher temperatures is explained by the increasing denaturation of the enzyme. Similar dependence, between enzyme activity and temperature, has been observed for hydrolytic reactions using fungal (Ferreira and Hultin, 1994) and animal proteases (Surowka and Fik, 1994).

The influence of reaction pH on the hydrolysis of shark meat is depicted in Figure 4.2. Both DH and nitrogen recovery should maximum values at pH 8.0. It has been suggested that optimum pH on the hydrolysis of food protein by Alcalase® occurs in the range of 8.0-8.5 (Novo Industri, 1978; Adler-Nissen, 1986).

An increase in the enzyme - substrate ratio resulted in a higher percent hydrolysis and nitrogen recovery after a two-hour reaction (Figure 4.3). It can also be noticed a non-linear trend between E/S ratio and both DH

Figure 4.1 Effect of reaction temperature on the hydrolysis of and nitrogen recovery from the dogfish muscle protein using Alcalase®. Reaction conditions: pH: 8.0, E/S: 2 %, S: 8 %, time of hydrolysis: 120 min. DH: □ ; NR: ■.

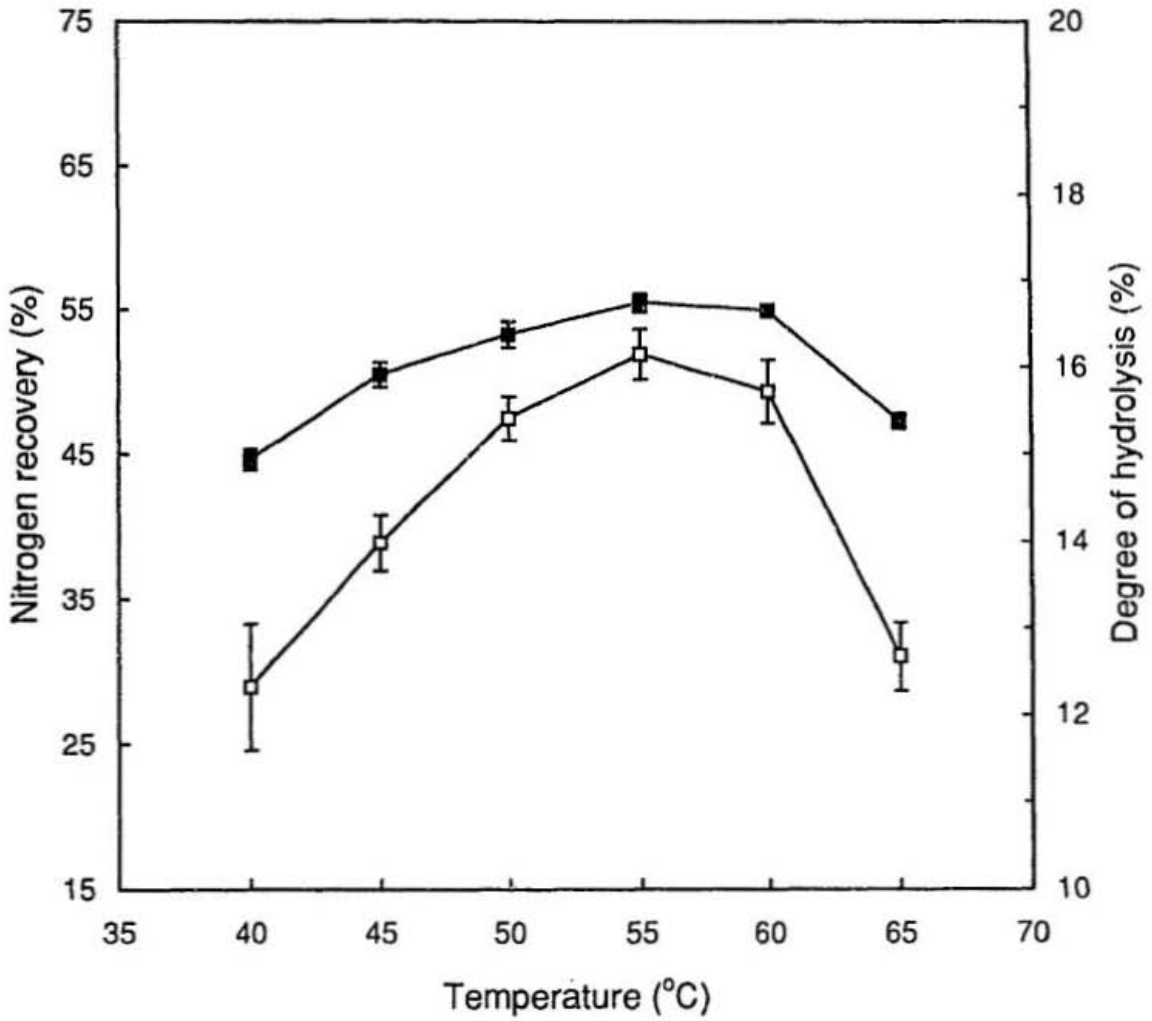

75
65
55
45
35
25
15
Nitrogen recovery (%)
20
18
16
14
12
10
Degree of hydrolysis (%)
35
40
45
50
55
60
65
70
Temperature (°C)

Figure 4.2 Effect of pH on the hydrolysis of and nitrogen recovery from the dogfish muscle protein using Alcalase®. Reaction conditions: T: 55 °C, E/S: 2 %, S: 8 %, time of hydrolysis: 120 min. DH: □ ; NR: ■.

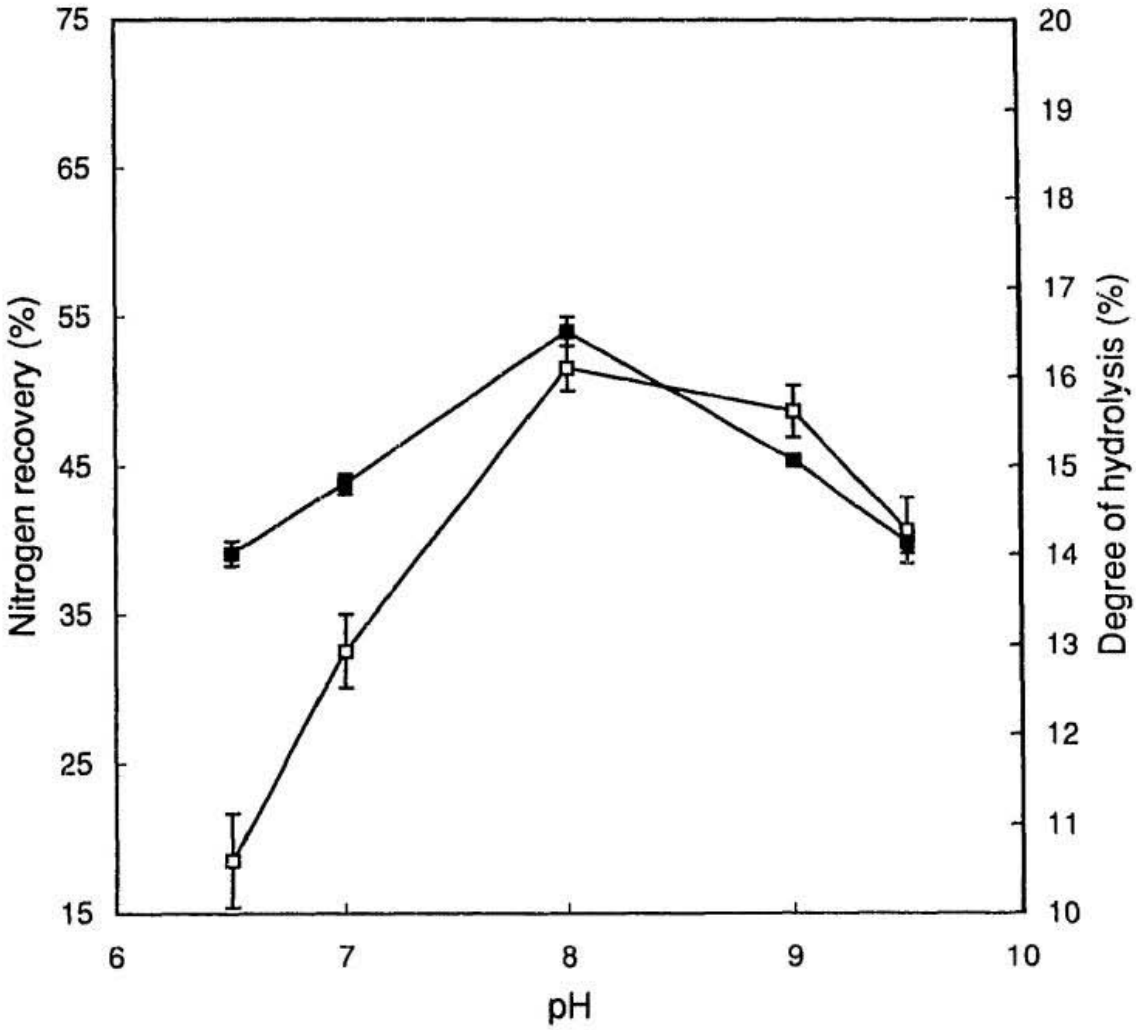
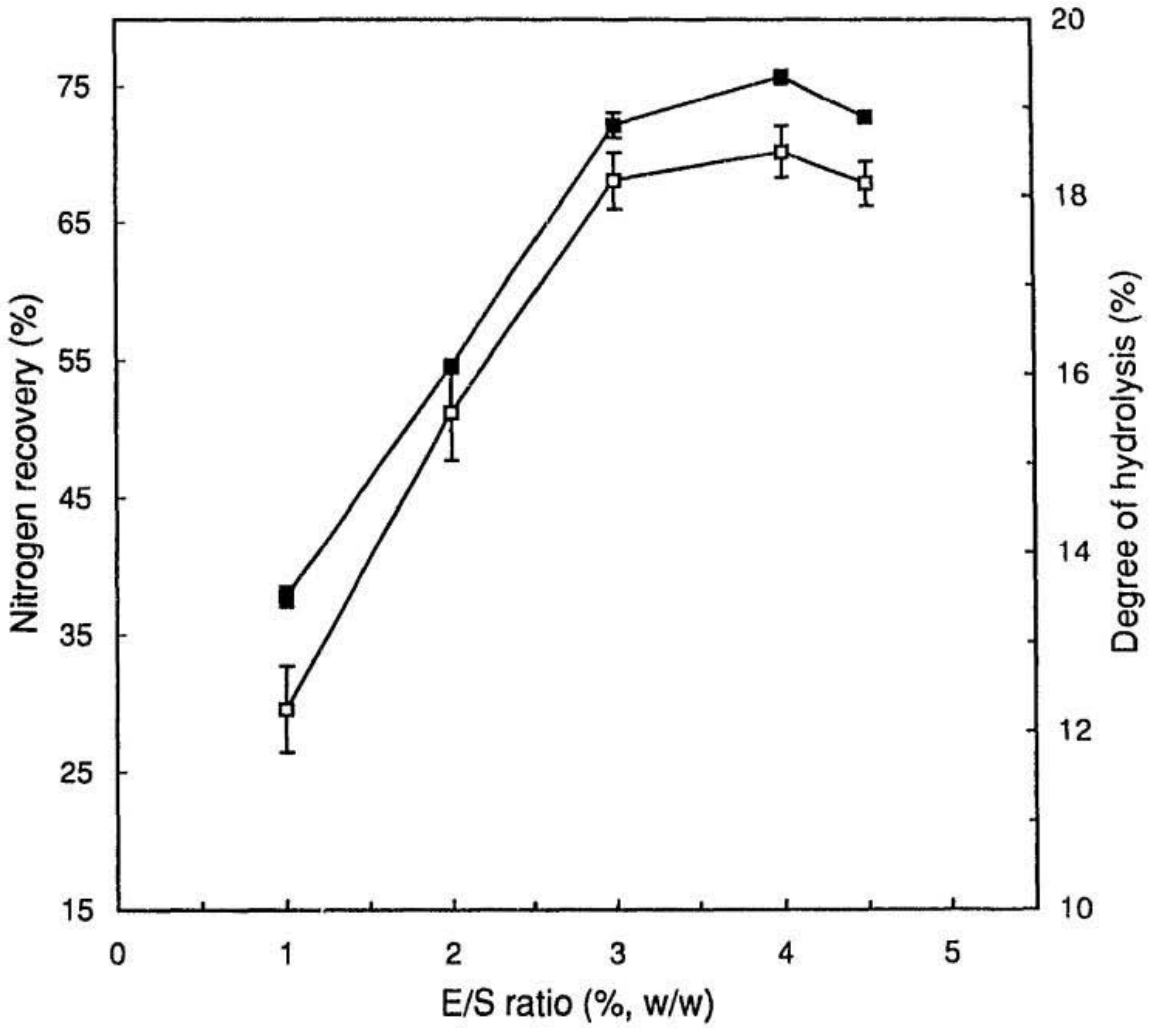

75
65
55
45
35
25
15
Nitrogen recovery (%)
20
19
18
17
16
15
14
13
12
11
10
Degree of hydrolysis (%)
6
7
8
9
10
pH

Figure 4.3 Effect of enzyme - substrate ratio on the hydrolysis of and nitrogen recovery from the dogfish muscle protein using Alcalase®. Reaction conditions: T: 55 °C, pH: 8.0, S: 8 %, time of hydrolysis: 120 min. DH: □ ; NR: ■.

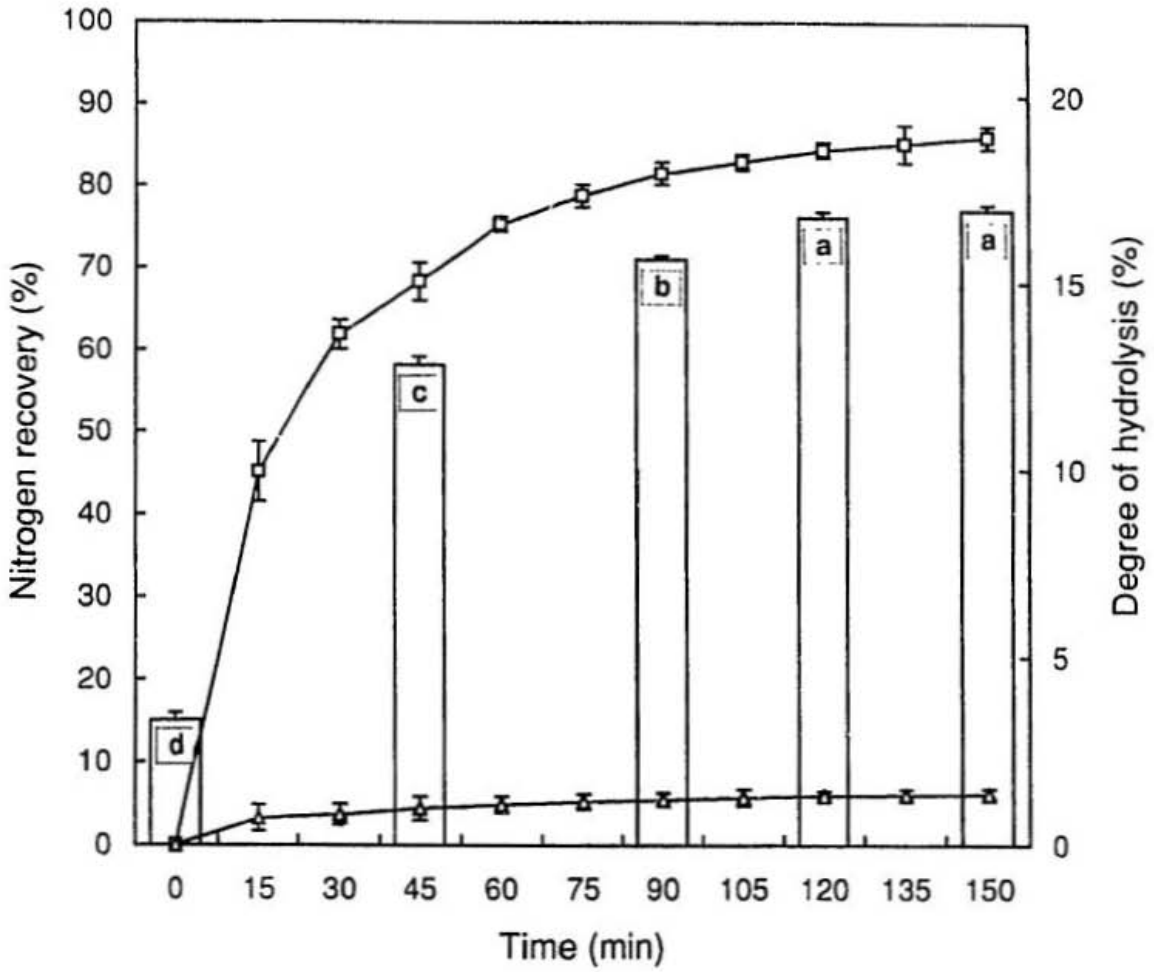

Nitrogen recovery (%)
75
65
55
45
35
25
15
Degree of hydrolysis (%)
20
18
16
14
12
10
0
1
2
3
4
5
E/S ratio (%, w/w)

and NR. Increases for these response variables were observed to E/S up to 3 % (w/w), after which degree of hydrolysis and nitrogen recovery level off. The slight decrease detected after 4 % of E/S might be explained by the auto-digestion of the enzyme. Alcalase® would hydrolyse itself, especially at high dosages and low protein concentration (Cheftel *et al.*, 1971; Ferreira and Hultin, 1994; Pommer, 1996).

Figure 4.4 shows the hydrolysis curves for the proteolysis of the dogfish using Alcalase® and the control. The hydrolysis from the control sample was due to the endogenous enzymes naturally present in the fish muscle. In fact, serine proteinase activity has been demonstrated in the muscle of white croaker (*Micropogon opercularis*) (Busconi *et al.*, 1989), and proteolytic activity by dogfish muscle (Malencik *et al.*, 1975).

The downward curvature of the hydrolysis curve is typical from enzyme-assisted reactions on food proteins, such as casein, gelatin, maize and soy isolates, and wheat gluten (Adler-Nissen, 1977; 1986). The first 30 min of reaction indicated a high hydrolysis rate, which slows down afterwards. It has been pointed out that the loss in enzyme activity, substrate depletion or product inhibition accounts for such decrease in the rate of hydrolysis (Mutilangi *et al.*, 1995). Significant increments on nitrogen recovery were not observed after two hours of reaction ($P > 0.05$), which suggests that the reaction reached a post steady-state phase.

Figure 4.4 Effect of reaction time on the hydrolysis of and nitrogen recovery from the dogfish muscle protein using Alcalase®. Reaction conditions: T: 55 °C, pH: 8.0, E/S: 4 %, S: 8 %. Alcalase®-assisted reaction: □ ; No enzyme addition: Δ . Nitrogen recovery values are represented by columns. [a-d] Values in columns with different letters are significantly different ($P < 0.05$).


Nitrogen recovery (%)
Degree of hydrolysis (%)
Time (min)
0
10
20
30
40
50
60
70
80
90
100
0
5
10
15
20
0
15
30
45
60
75
90
105
120
135
150
a
a
b
c
d

These experiments were conducted in blocks, with all factors held constant except the one to be examined. Therefore, interaction effects among variables were not taken into consideration. In fact, process operations usually account for some sort of interaction within the experimental conditions. The use of RSM to examine the hydrolysis progress would be a more adequate technique, since it would describe the combined effects of the most important reaction variables. Nonetheless, the single factor experiment, also known as single factor at-a-time, is still an important approach, because it presents a general information about the studied process and a more specific range, within each reaction variable, to use in a factorial design in the RSM.

4.3 Combined effects of reaction variables on the degree of hydrolysis

Based on the results of the preliminary experiments, a further investigation of the combined effects of temperature, pH and enzyme - substrate ratio were conducted. It was decided to hold the variable reaction time at two hours, because from that point onwards no significant increments were reached ($P < 0.05$) (Figure 4.4).

The coded and uncoded values of the independent variables (process conditions) and the experimental plan, as well as the experimental hydrolysis procedure, were the same as shown in the Materials and Methods section. Table 4.3 presents the results for the dependent variable DH. The DH values ranged from 11.47 to 17.96 %, at design points '4' and '5', respectively. Both were obtained at coded value zero of E/S ratio, but at different levels of pH and temperature. Such behaviour suggests possible interaction between the independent variables in the hydrolysis of dogfish muscle by Alcalase®.

Analysis of variance (*F*-test) showed that the second order model is well adjusted to the experimental data (Table 4.4). The adjusted coefficient of determination (R^2_{ad}) implies that 98 % of the behaviour variation could be explained by the fitted model. Moreover, the lack-of-fit test, which measures the fitness of the model obtained, did not result in a significant *F*-value, indicating that the model is sufficiently accurate for predicting the degree of hydrolysis for any combination of independent variable values within the ranges studied. Linear and quadratic terms were significant at the 1 % level, and one crossproduct term was significant at the 5 % level.

Table 4.3 Responses of the dependent variable degree of hydrolysis (%) for the Alcalase®-assisted hydrolysis of dogfish muscle

Design point[a]	Independent variables			Dependent variable
	pH X_1	Temperature (°C) X_2	E/S/ ratio (%) X_3	Degree of hydrolysis (%)[b]
1	1	1	0	17.31
2	1	-1	0	14.73
3	-1	1	0	15.33
4	-1	-1	0	11.47
5	0	0	0	17.96
6	1	0	1	17.47
7	1	0	-1	15.41
8	-1	0	1	15.38
9	-1	0	-1	12.02
10	0	0	0	17.92
11	0	1	1	17.85
12	0	1	-1	15.57
13	0	-1	1	16.54
14	0	-1	-1	12.06
15	0	0	0	17.69

[a] Experiments were conducted in a random order.

[b] Average of duplicate determinations from different experiments.

Table 4.4 Analysis of variance for the response of degree of hydrolysis (DH %)

Source	dF*	Sum of squares	Mean square	*F*-ratio
Regression				
Linear	3	48.7573	-	134.900[a]
Quadratic	3	19.5721	-	54.171[a]
Crossproduct	3	2.0421	-	5.652[b]
Total	9	70.3715	-	64.924[a]
Residual				
Lack of fit	3	0.5597	0.1866	8.786
Pure error	2	0.0425	0.0212	-
Total error	5	0.6022	0.1204	-
% variability explained				
$R^2_{adj} = 0.98$				
Factors				
pH	4	27.0384	6.7596	56.127[a]
Temperature	4	24.2556	6.0639	50.351[a]
E/S ratio	4	23.8381	5.9595	49.484[a]

* Degrees of freedom

[a] Significant at 1 % level.

[b] Significant at 5 % level.

Statistical analysis also indicated that within each term all three hydrolysis factors had a strong influence on DH. In fact, Adler-Nissen (1986), investigating the hydrolysis of soy protein by bacterial proteases, pointed out that pH, temperature and enzyme - substrate ratio markedly influenced the peptide bond cleavage in the protein substrate. The *T*-test was performed to determine which regression coefficients were significant, and at what level each was significant (Table 4.5), thereby determining the best explanatory equation.

The best explanatory equation for degree of hydrolysis is given in Table 4.6, and shows that all linear and quadratic terms, and also one crossproduct interaction ($X_2 \cdot X_3$), contribute to the response. The equation indicates that the variables had approximately equal effect on the hydrolysis of dogfish protein, since they had similar slope values.

Canonical analysis revealed a maximum region for DH (Table 4.7), which is demonstrated by the negative eigenvalues obtained. The stationary point had the following critical values: pH = 8.3, temperature = 53.6°C and E/S ratio = 3.6 % (w/w). The predicted DH value for these conditions was 18.77 %, which indicates high proteolytic activity. Alcalase-assisted reactions have been repeatedly reported to exhibit higher degrees of hydrolysis compared to neutral or acid enzymes from plant, animal or microbial origin, such as papain, pepsin and Neutrase, respectively (Quaglia and Orban, 1987b; Rebeca *et al.*, 1991; Sugiyama *et al.*, 1991).

Three-dimensional representations of the combined effects of each pair of variables indicate that in the hydrolysis of dogfish protein an increase in DH is

Table 4.5 Regression coefficients and *t*-values of quadratic response-surface equations for degree of hydrolysis (%)

Coefficient (model term)	Degree of hydrolysis (%)	
	Parameter estimator	*t*-value
β_o (intercept)	17.86	89.123 [a]
β_1 (pH)	1.34	10.921 [a]
β_2 (Temperature)	1.41	11.471 [a]
β_3 (E/S)	1.52	12.409 [a]
β_{11} (pH^2)	- 1.79	- 9.916 [a]
β_{22} ($Temperature^2$)	- 1.36	- 7.507 [a]
β_{33} (E/S^2)	- 1.00	- 5.514 [a]
β_{12} (pH · Temperature)	- 0.32	-1.844
β_{13} (pH · E/S)	- 0.33	-1.873
β_{23} (Temperature · E/S)	- 0.55	- 3.170 [b]

[a] Significant at 1 % level.

[b] Significant at 5 % level.

Table 4.6 Selected prediction equation for the dependent variable degree of hydrolysis (%) of dogfish muscle

Dependent variable	Best explanatory equation	R^2_{ad} [a]	P level [b] (%)
DH (%) [c]	$Y_{(DH)} = 17.86 + 1.34pH + 1.41T + 1.52E/S - 1.79pH^2 - 1.36T^2 - E/S^2 - 0.55T \cdot E/S$	0.98	0.01

[a] Adjusted R^2, according to Hamilton (1992).

[b] Probability, significant at % level.

[c] Degree of hydrolysis.

Table 4.7 The canonical analysis of response surface

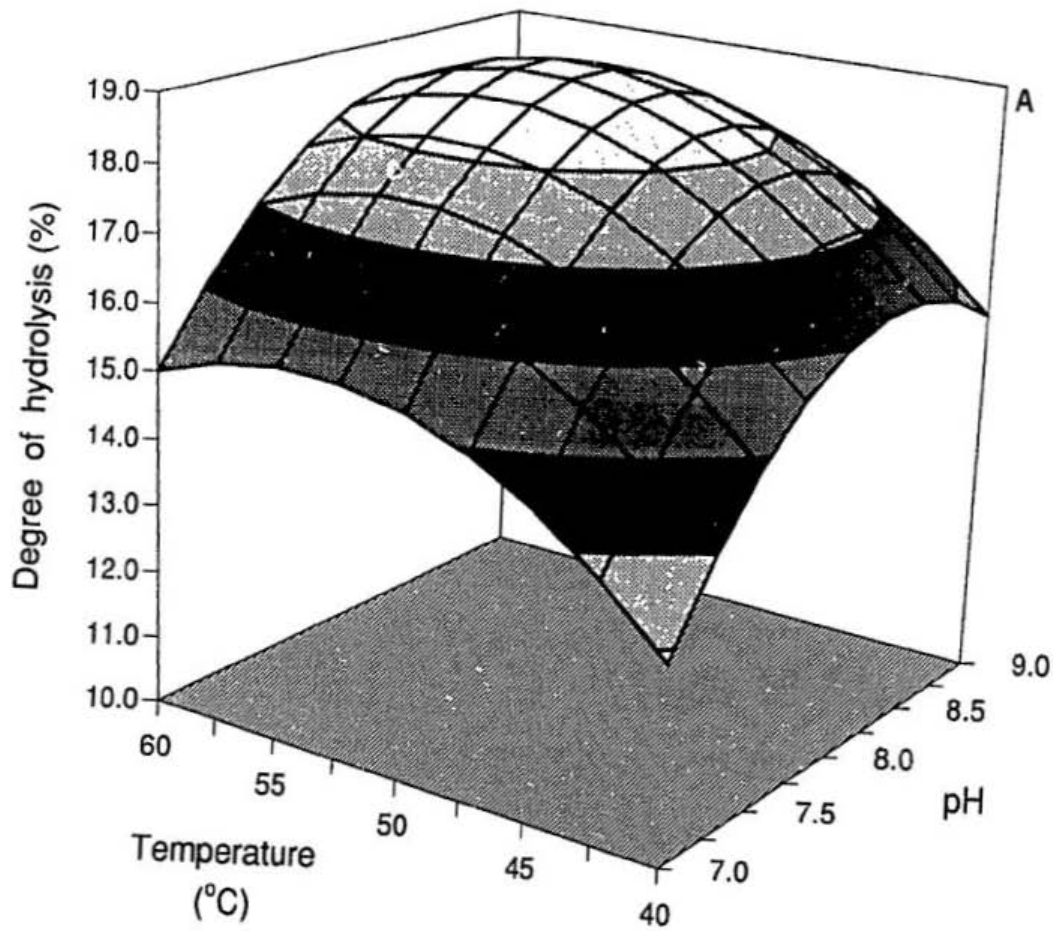
Response variable	Critical value of hydrolysis factor			Predicted value (DH%)	Stationary point
	pH	Temperature (°C)	E/S ratio (%, w/w)		
Degree of hydrolysis (%)	8.3	53.6	3.6	18.77	maximum

achieved by increases in pH, temperature and E/S (Figures 4.5 to 4.7) up to certain levels, beyond which DH decreases. These *3D* graphs also show that there was no linear relation between the addition of enzyme and DH for each particular pH (Figures 4.6) and temperature (Figures 4.7) level. A decrease in DH was observed after E/S of 3.6 % (iso-contour plot). This appears to indicate that, under a high E/S ratio, inhibition of the enzyme occurs, including the possibility that the enzyme hydrolyses itself. Similar reaction behaviour has been noticed in the hydrolysis of capelin, *Mallotus villosus*, using Alcalase® (Martin and Patel, 1992). This is in close agreement with earlier findings on the hydrolysis of other kinds of protein substrate, such as lean beef (O'Meara and Munro, 1984), and soy protein (Constantinides and Adu-Amankwa, 1980).

The accuracy of the model was further tested by conducting hydrolysis experiments using the critical values, obtained from the canonical analysis for maximum DH response. The DH values after the two-hour reaction of the dogfish protein - Alcalase® system ranged from 18.59 to 18.91 % (Figure 4.8). Since this range of results contained the predicted value for the degree of hydrolysis (DH = 18.77 %), it was concluded that the mathematical model was capable of predicting the studied response.

The non-linear relation between enzyme concentration and DH suggests that the hydrolytic reaction depends on the availability of susceptible peptide bonds on which the primary enzymic attack is concentrated, and also on the physical structure of the protein molecule (Clegg and McMillan, 1974; Raghunath, 1993).

Figure 4.5 Three-dimensional (A) and contour (B) surface plots of the combined effect of pH and temperature on the two-hour hydrolysis of dogfish muscle with Alcalase® at optimum enzyme / substrate ratio.


Degree of hydrolysis (%)
19.0
18.0
17.0
16.0
15.0
14.0
13.0
12.0
11.0
10.0
A
60
55
50
45
40
Temperature
(°C)
9.0
8.5
8.0
7.5
7.0
pH

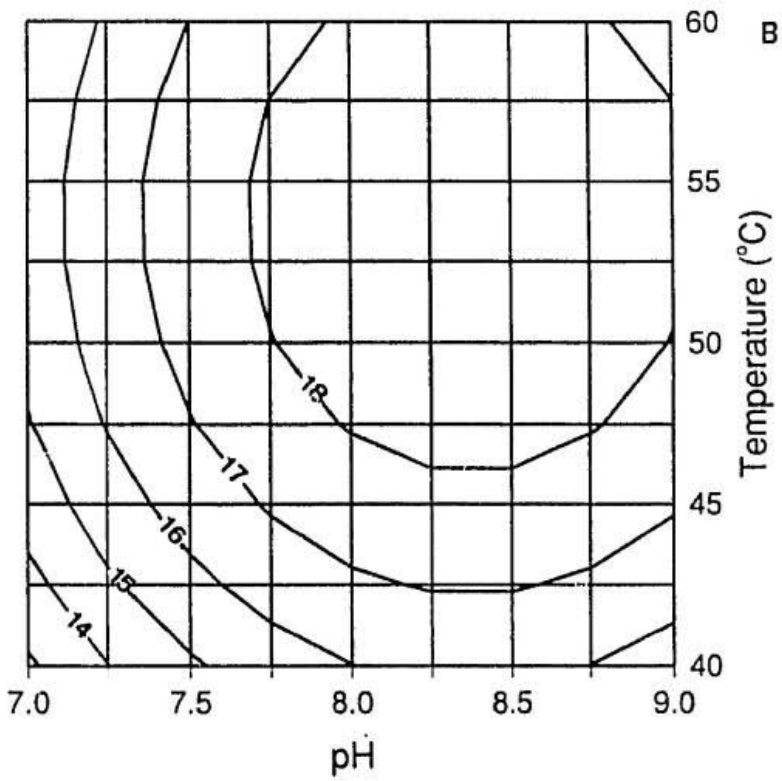
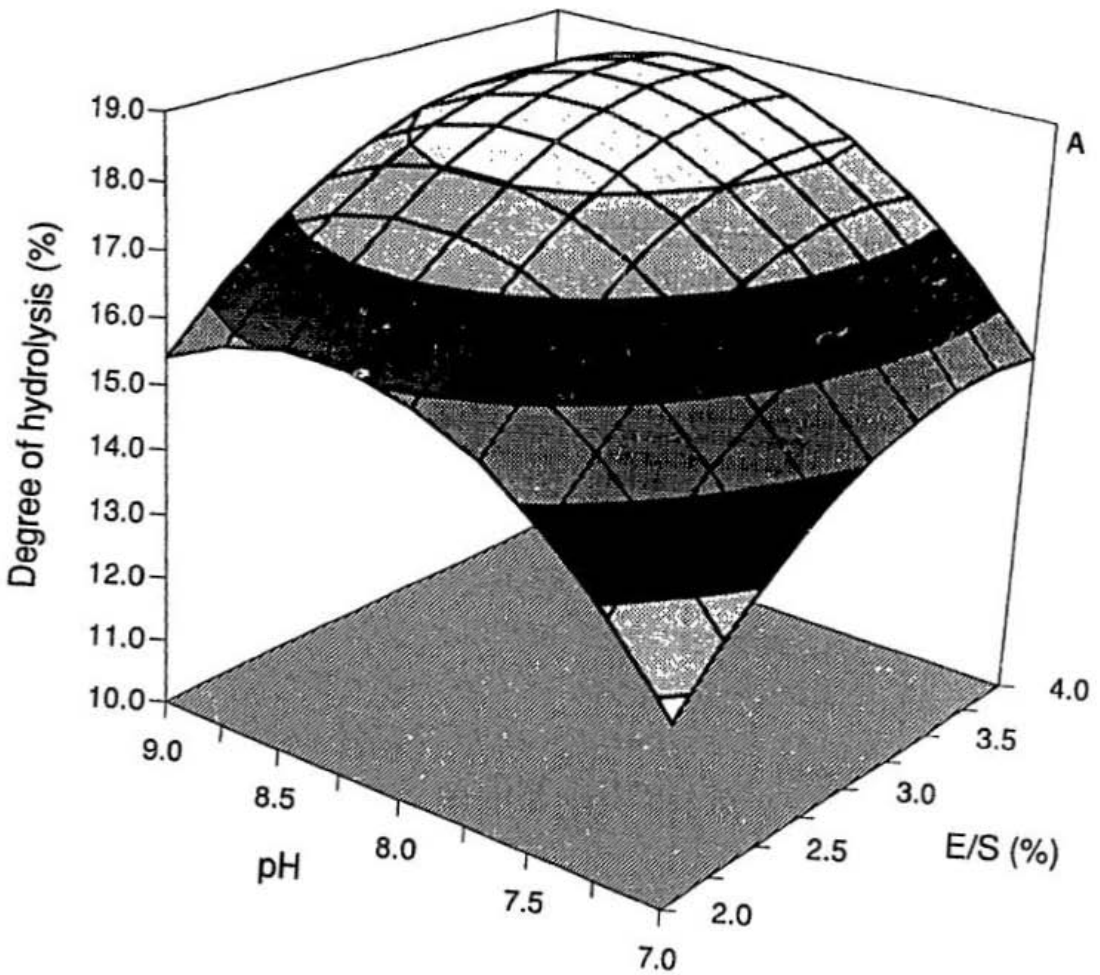

B
60
55
50
45
40
Temperature (°C)
18
17
16
15
14
7.0
7.5
8.0
8.5
9.0
pH

Figure 4.6 Three-dimensional (A) and contour (B) surface plots of the combined effect of enzyme / substrate ratio and pH on the two-hour hydrolysis of dogfish muscle with Alcalase® at optimum temperature.


Degree of hydrolysis (%)
19.0
18.0
17.0
16.0
15.0
14.0
13.0
12.0
11.0
10.0
A
9.0
8.5
8.0
7.5
7.0
pH
4.0
3.5
3.0
2.5
2.0
E/S (%)

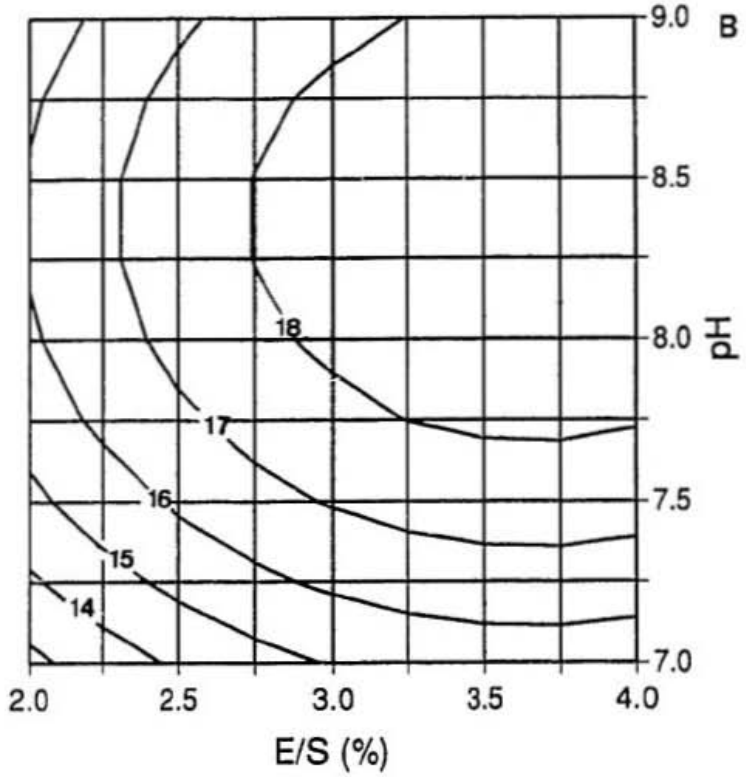
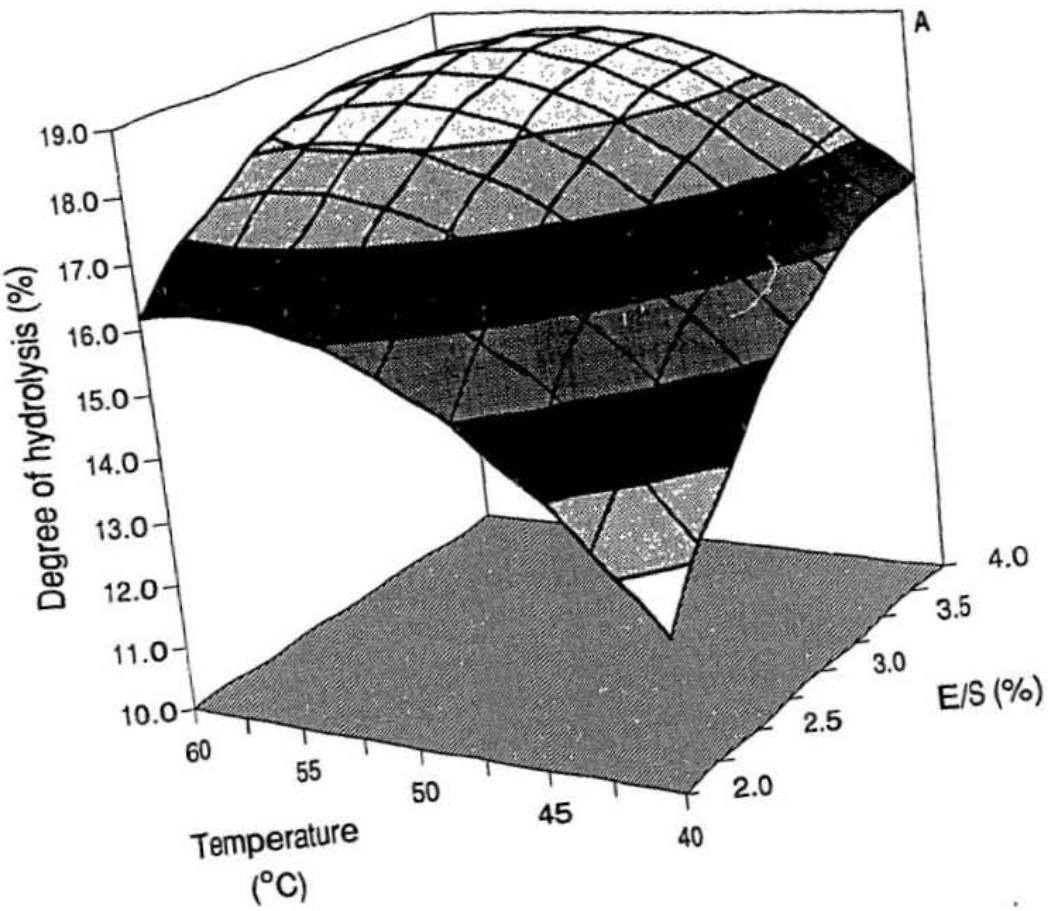

9.0
8.5
8.0
7.5
7.0
pH
B
18
17
16
15
14
2.0
2.5
3.0
3.5
4.0
E/S (%)

Figure 4.7 Three-dimensional (A) and contour (B) surface plots of the combined effect of enzyme / substrate ratio and temperature on the two-hour hydrolysis of dogfish muscle with Alcalase® at optimum pH value.


A
19.0
18.0
17.0
16.0
15.0
14.0
13.0
12.0
11.0
10.0
Degree of hydrolysis (%)
60
55
50
45
40
Temperature
(°C)
2.0
2.5
3.0
3.5
4.0
E/S (%)

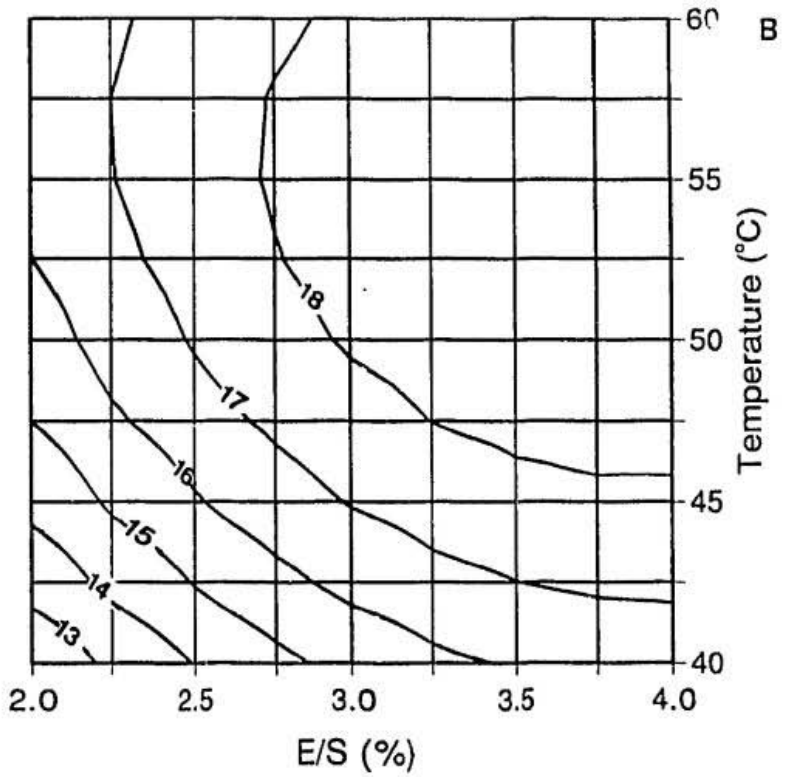
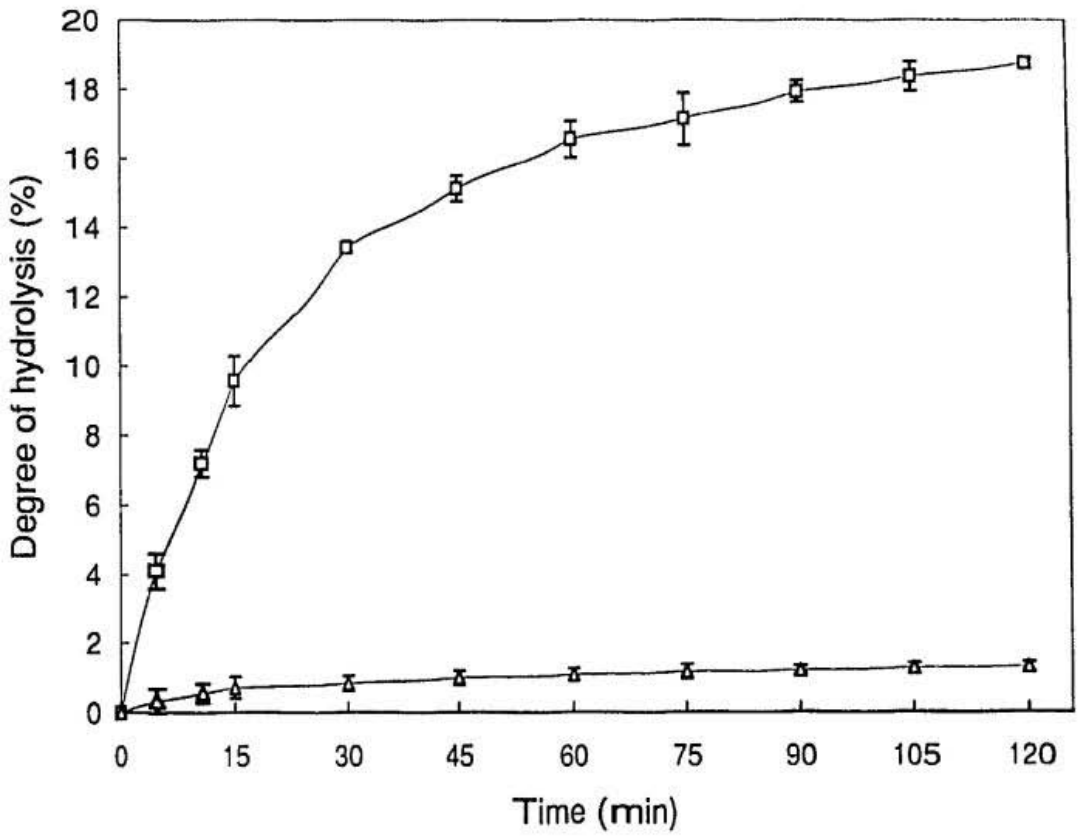

B
60
55
50
45
40
Temperature (°C)
18
17
16
15
14
13
2.0
2.5
3.0
3.5
4.0
E/S (%)

Figure 4.8 Hydrolysis of dogfish muscle protein with Alcalase® by the pH-stat method under the critical conditions obtained from canonical analysis. Enzyme / substrate ratio (3.6 %, w/w): □. No enzyme addition: Δ . Each value represents the mean of three determinations ± standard deviation.


Degree of hydrolysis (%)
20
18
16
14
12
10
8
6
4
2
0
0
15
30
45
60
75
90
105
120
Time (min)

In general, high degree of hydrolysis values were obtained over a pH range of 8 to 8.5 and a temperature range of 50 to 60 °C. These results agreed closely with the manufacturer's suggestions for maximum proteolytic activity using Alcalase® (Novo Industri, 1988).

The graphic representations indicate the conditions necessary to obtain a maximum DH. Contour plots were generated by the predictive model to locate the critical points and the influence of each factor on the surrounding regions. From these representations it can be concluded that the optimum degree of hydrolysis can be obtained using the critical values of the conditions described in this study. Furthermore, *2D*- and *3D*-graphs assist the selection of specific combinations of hydrolysis variables in order to attain any desired level of hydrolysis, which in turn will influence the application in food formulations of this new source of protein, since DH is the prime factor on variations of the protein functionality.

4.4 Optimization of nitrogen recovery from the hydrolysis of dogfish muscle protein

Optimization of the hydrolysis process was based on the maximization of the nitrogen recovery from the original substrate, dogfish muscle. According to the experimental design the values obtained for NR by the hydrolysis of dogfish muscle are depicted in Table 4.8. Nitrogen recovery ranged from 42.55 to 75.45 % at design points '9' and '11', respectively. These results indicate that enzyme concentration had a strong influence on the recovery of nitrogen from the substrate studied, since the design points for minimum and maximum NR corresponded to the minimum and maximum levels for E/S ratio, respectively.

Analysis of variance showed that the second order model for NR (%) is also well adjusted to the experimental data (Table 4.9). The high coefficient of determination (R^2_{ad}) indicates that the model can explain 95 % of the behaviour variation. The non-significant *F*-value for the fitness test indicates a high degree of accuracy for predicting nitrogen recovery from any combination of values of the independent variables within the experimental range. Linear and quadratic terms were significant at the 1 % level, with one crossproduct term significant at the 10 % level. Statistical analysis revealed that within each equation term the three hydrolysis factors had a significant influence on the response studied. Of these, however, as shown by the higher *F*-value, E/S plays the most important role in the process, on the basis of nitrogen recovery.

Table 4.8 Responses of the dependent variable nitrogen recovery (%) from the Alcalase®-assisted hydrolysis of dogfish muscle

Design point[a]	Independent variables			Dependent variable
	pH X_1	Temperature (°C) X_2	E/S/ ratio (%) X_3	Nitrogen recovery (%)[b]
1	1	1	0	65.24
2	1	-1	0	58.96
3	-1	1	0	60.64
4	-1	-1	0	52.37
5	0	0	0	73.74
6	1	0	1	74.05
7	1	0	-1	45.15
8	-1	0	1	59.51
9	-1	0	-1	42.55
10	0	0	0	69.96
11	0	1	1	75.45
12	0	1	-1	58.53
13	0	-1	1	64.96
14	0	-1	-1	43.26
15	0	0	0	72.76

[a] Average of triplicate determinations on duplicate experiments

[b] Experiment design points were conducted in random order.

Table 4.9 Analysis of variance for the regression model, adjusted coefficient of determination (R^2_{ad}) and main effects of hydrolysis variables on nitrogen recovery from dogfish muscle using Alcalase®

Source	dF*	Sum of squares	Mean square	*F* - ratio
Regression				
Linear	3	1195.5444	-	57.954[a]
Quadratic	3	514.5010	-	24.941[a]
Crossproduct	3	42.3430	-	2.053
Total	9	1752.3884	-	28.316[a]
Residual				
Lack of fit	3	26.6855	8.8952	2.312
Pure error	2	7.6963	3.8481	-
Total error	5	34.3818	6.8764	-
% variability explained				
R^2_{ad}** = 0.95				
Factors				
pH	4	438.8906	109.7226	15.957[a]
Temperature, °C	4	263.3534	65.8384	9.575[b]
E/S ratio, %	4	1157.8379	289.4595	42.095[a]

* Degrees of freedom.

** Adjusted R^2, according to Hamilton (1992)

[a] Significant at 1 % level.

[b] Significant at 5 % level.

Table 4.10 shows the results of the test of significance on the parameter estimators from the regression analysis. Linear and quadratic terms, and the crossproduct interaction between $X_1 \cdot X_2$, which are pH and E/S ratio, respectively, should comprise the mathematical equation for predicting NR. Therefore, according to the polynomial model presented in Equation 8, the second order equation becomes:

$$Y_{(NR)} = 72.15 + 3.54\boldsymbol{pH} + 5.04\boldsymbol{T} + 10.56\boldsymbol{E/S} - 9.04\boldsymbol{pH}^2 - 3.81\boldsymbol{T}^2 - 7.79\boldsymbol{E/S}^2 + 2.99\boldsymbol{pH} \cdot \boldsymbol{E/S} \qquad \text{Eqn [14]}$$

In the best explanatory equation, the highest slope is that of the E/S ratio, indicating that this variable strongly affected NR. Rebeca *et al.* (1991) observed that enzyme concentration also had a strong influence on nitrogen yield in the pH-controlled proteolysis of eviscerated mullet (*Mugil cephalus*) using an alkaline protease, Pescalase 560, which is similar to Alcalase®. Canonical analysis revealed a maximum point for the following critical values: pH = 8.3, temperature = 55.3°C and E/S ratio = 3.7 % (w/w). The predicted NR value for these conditions was 77.68 % (Table 4.11).

Quaglia and Orban (1987b) achieved an NR of 70 to 75% when hydrolysing sardine (*Sardina pilchardus*) using Alcalase® at E/S ratios between 3.5 and 4 %. Rebeca *et al.* (1991) reported NR values ranging from 69.5 to 75.7 % after two hours of proteolysis using alkaline proteases on eviscerated mullet (*M. cephalus*)

Table 4.10 Model coefficients and test of significance (*t*-test) for the second-order response equation for nitrogen recovery from the hydrolysis of dogfish muscle using Alcalase®

Coefficient	Nitrogen recovery (%)	
(model term)	Parameter estimator	*t*-value
β_0 (intercept)	72.15	47.658 [a]
β_1 (pH)	3.54	3.820 [b]
β_2 (Temperature)	5.04	5.435 [a]
β_3 (E/S)	10.56	11.390 [a]
β_{11} (pH^2)	- 9.04	- 6.626 [a]
β_{22} ($Temperature^2$)	- 3.81	- 2.790 [b]
β_{33} (E/S^2)	- 7.79	- 5.712 [a]
β_{12} (pH · Temperature)	- 0.50	- 0.379
β_{13} (pH · E/S)	2.99	2.277 [c]
β_{23} (Temperature · E/S)	- 1.20	- 0.911

[a] Significant at 1 % level.

[b] Significant at 5 % level.

[c] Significant at 10 % level.

Table 4.11 Canonical analysis of the response surface (NR %) for the hydrolysis of dogfish muscle protein using Alcalase®

Response variable	Critical value of hydrolysis factor			Predicted value (NR%)	Stationary point
	pH	Temperature (°C)	E/S ratio (%, w/w)		
Nitrogen recovery (%)	8.3	55.3	3.7	77.68	maximum

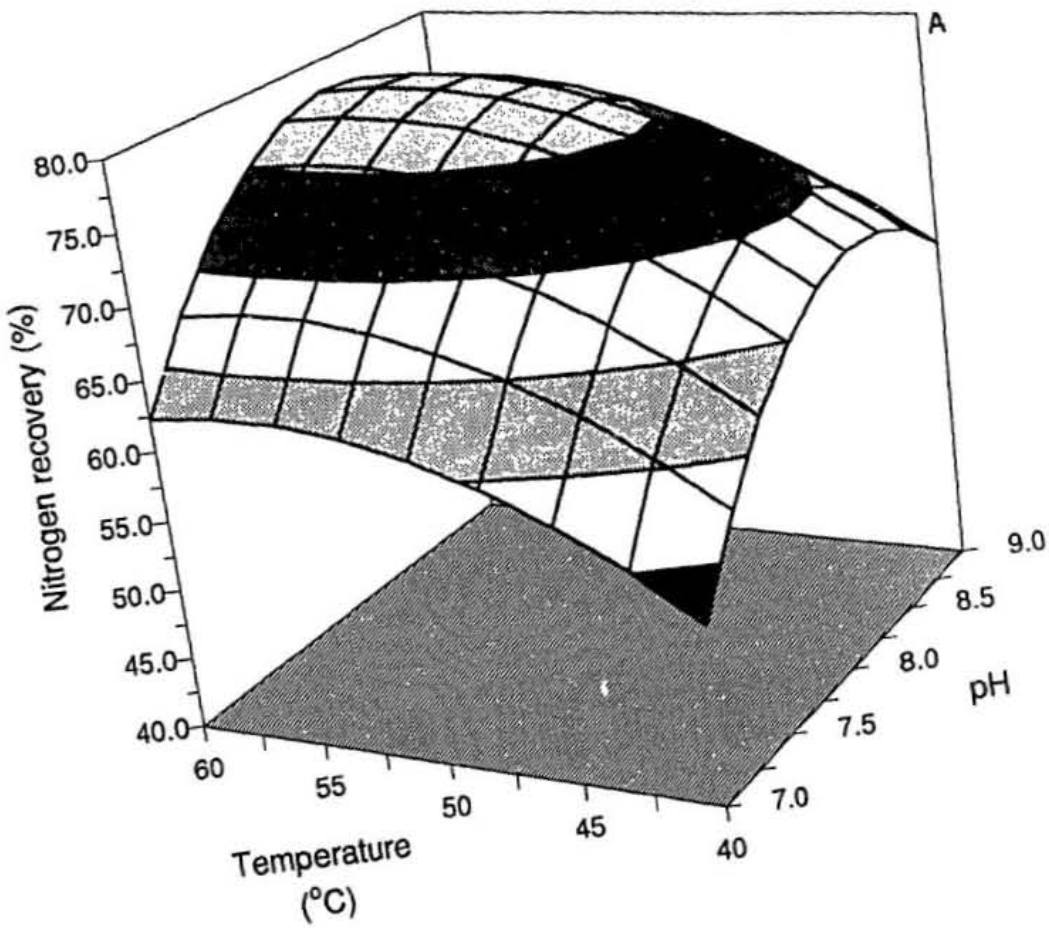
in pH controlled reactions. Mackie (1994) stated that around 20 % of the nitrogen remains insoluble, even with further hydrolysis. These findings are in agreement with the value of 77.68 % for nitrogen recovery at the stationary point in this work.

Three-dimensional graphs showing the influence of each variable on NR are presented in Figures 4.9 to 4.11. These graphs emphasize the strong influence of enzyme concentration upon nitrogen recovery. Contour plots, each showing the response of NR to two independent variables, locate the point of maximum recovery.

The accuracy of the model was further tested by conducting a set of experiments using the critical values for optimum NR. Under these conditions, nitrogen recovery after two-hour proteolyses ranged from 77.10 to 77.75 % (Figure 4.12). Since these results contained the predicted value for NR, 77.68 %, it was concluded that the model was suitable for predicting the studied response. Degree of hydrolysis was used to monitor the hydrolytic reaction.

The liquid shark protein hydrolysate was spray dried at conditions described in Material and Methods section. The average yield of dry powder from spray drying was 14.6 ± 0.15 %, on the basis of the initial weight of fish muscle used.

Figure 4.9 Three-dimensional (A) and contour (B) plots of the effects of pH and temperature on nitrogen recovery (%) at optimum E/S ratio in an Alcalase®-assisted hydrolytic reaction of dogfish muscle protein.


A
80.0
75.0
70.0
65.0
60.0
55.0
50.0
45.0
40.0
Nitrogen recovery (%)
60
55
50
45
40
Temperature
(°C)
9.0
8.5
8.0
7.5
7.0
pH

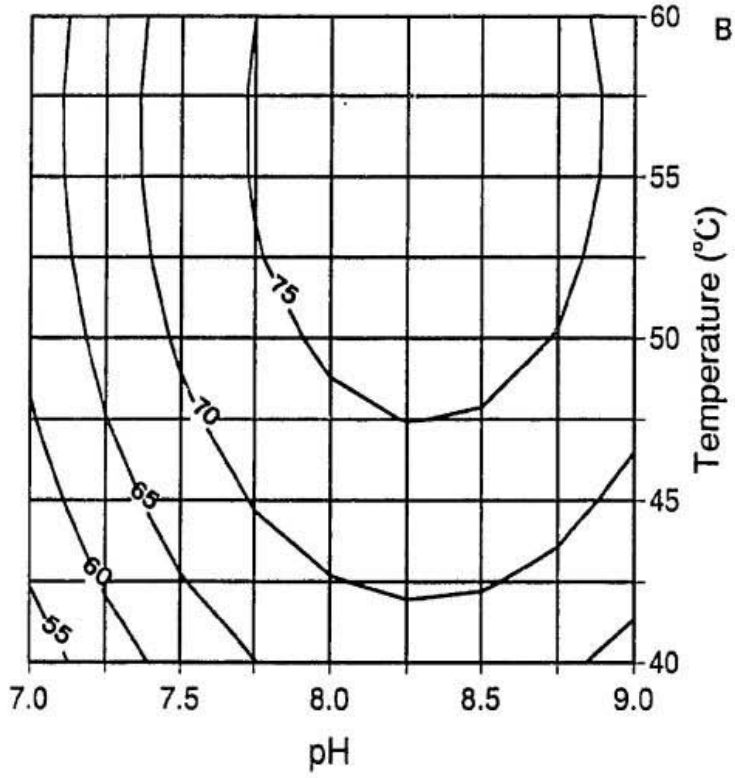
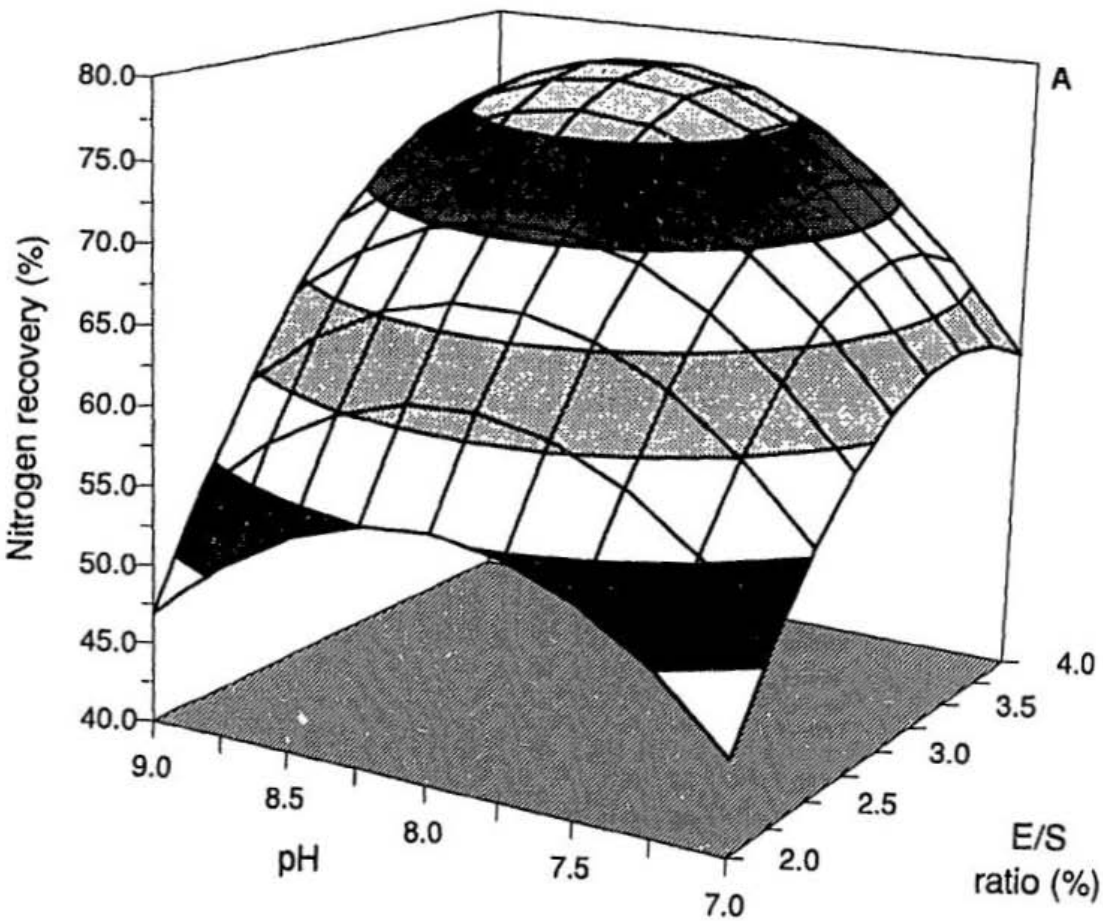

B
60
55
50
45
40
Temperature (°C)
75
70
65
60
55
7.0
7.5
8.0
8.5
9.0
pH

Figure 4.10 Three-dimensional (A) and contour (B) plots of the effects of E/S ratio and pH on nitrogen recovery (%) at optimum temperature in an Alcalase®-assisted hydrolytic reaction of dogfish muscle protein.


A
Nitrogen recovery (%)
80.0
75.0
70.0
65.0
60.0
55.0
50.0
45.0
40.0
9.0
8.5
8.0
7.5
7.0
pH
4.0
3.5
3.0
2.5
2.0
E/S
ratio (%)

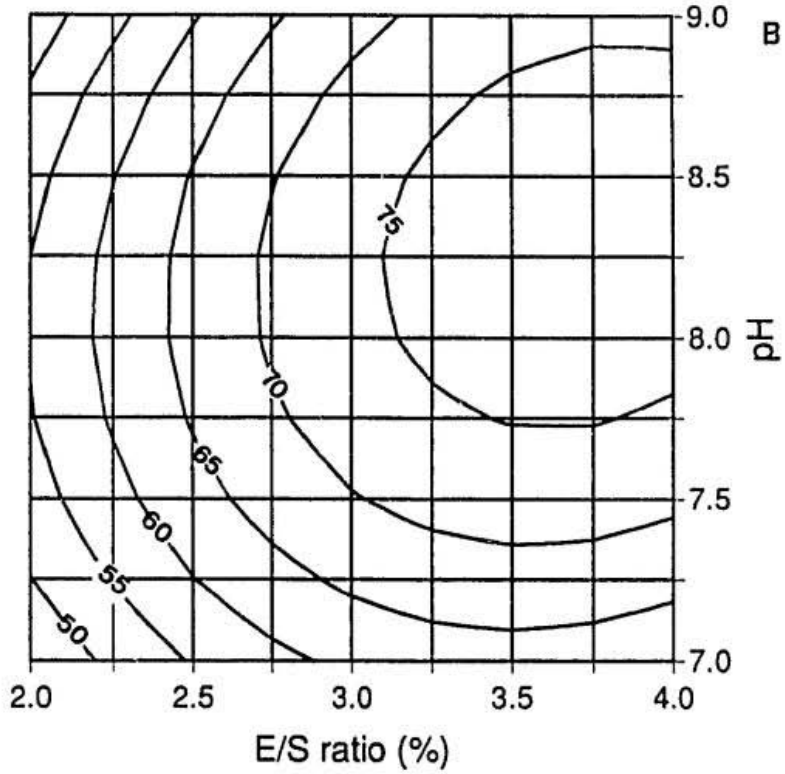
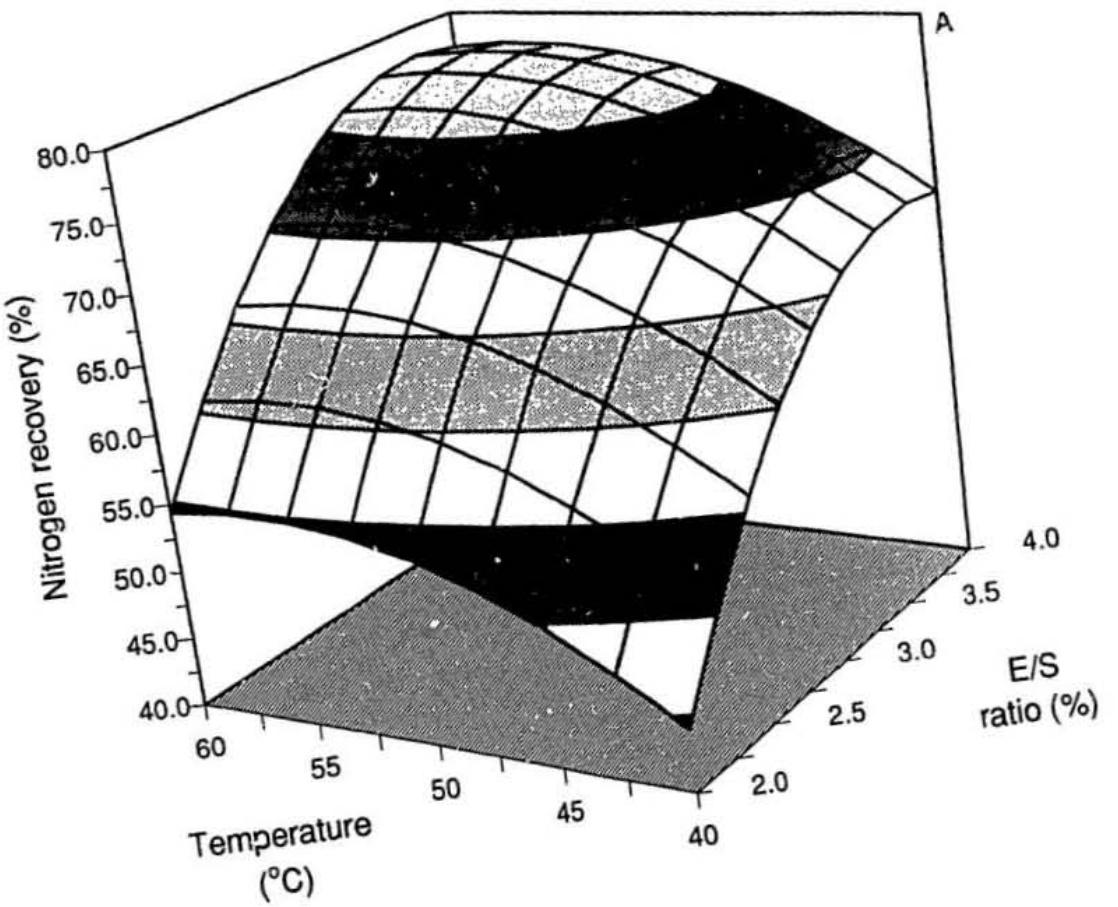

9.0
8.5
8.0
7.5
7.0
pH
B
75
70
65
60
55
50
2.0
2.5
3.0
3.5
4.0
E/S ratio (%)

Figure 4.11 Three-dimensional (A) and contour (B) plots for the effects of E/S ratio and temperature on nitrogen recovery (%) at optimum pH in an Alcalase®-assisted hydrolytic reaction of dogfish muscle protein.


A
80.0
75.0
70.0
65.0
60.0
55.0
50.0
45.0
40.0
Nitrogen recovery (%)
60
55
50
45
40
Temperature
(°C)
2.0
2.5
3.0
3.5
4.0
E/S
ratio (%)

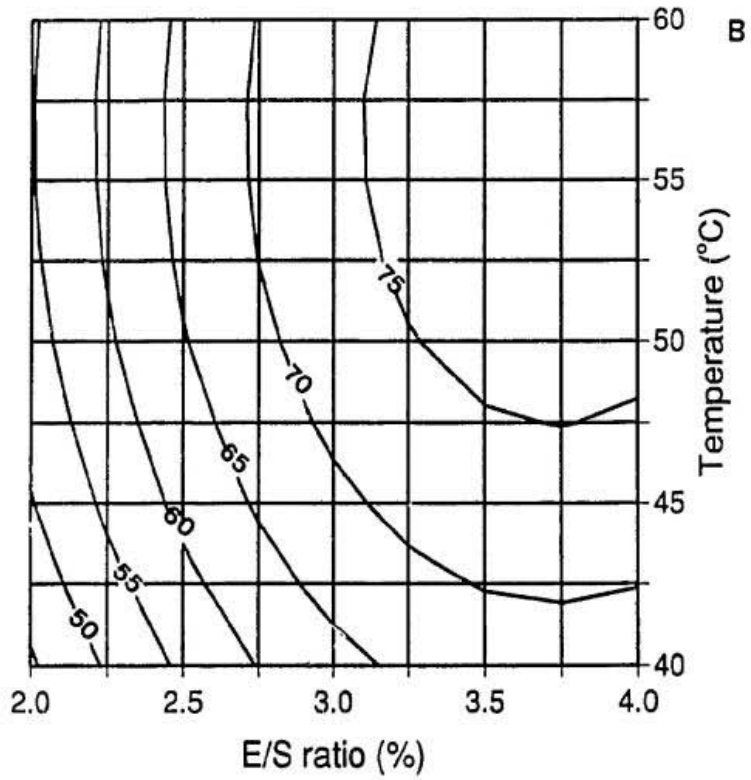
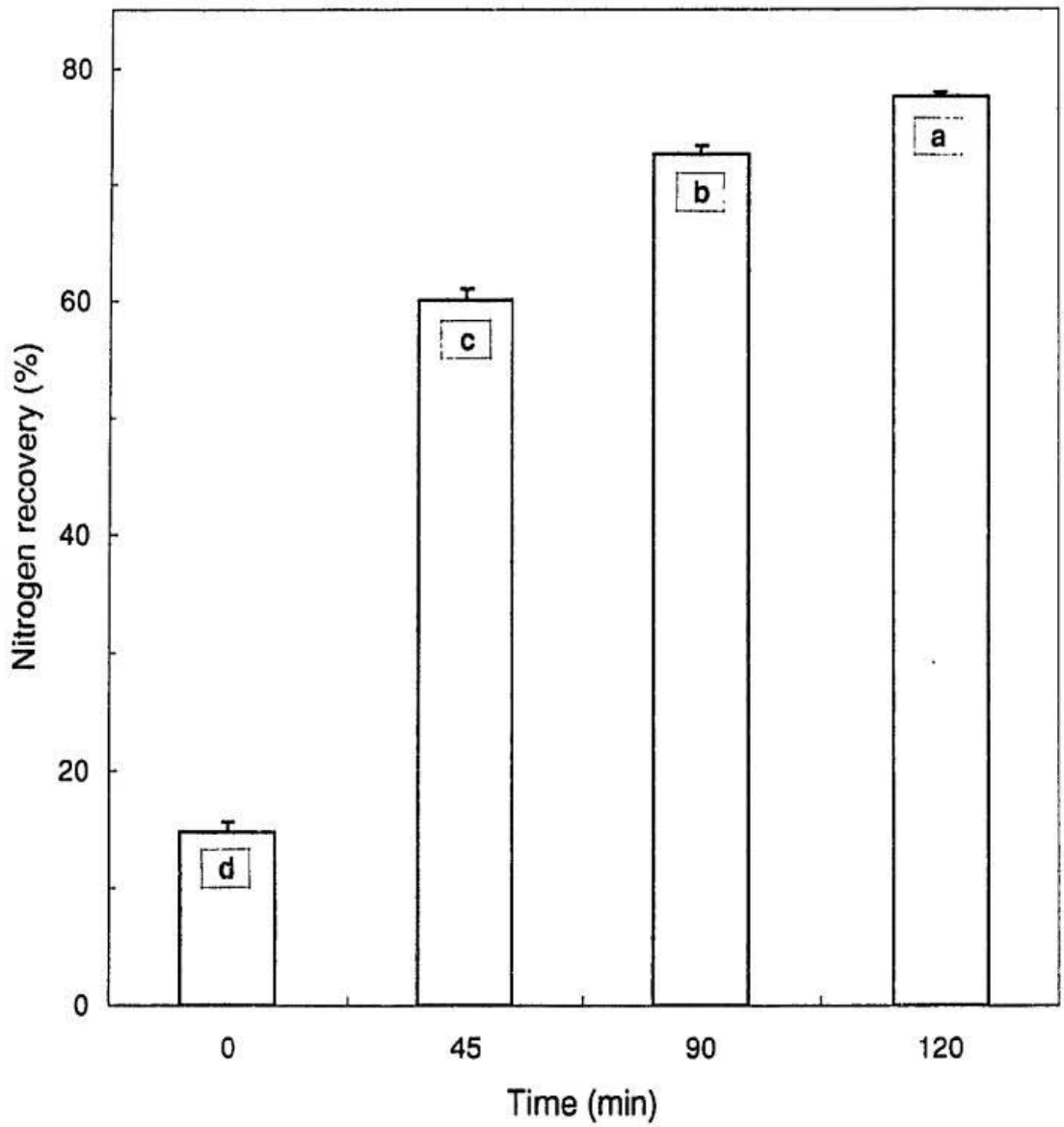

B
60
55
50
45
40
Temperature (°C)
75
70
65
60
55
50
2.0
2.5
3.0
3.5
4.0
E/S ratio (%)

Figure 4.12 Effect of time on the percent nitrogen recovery from the hydrolysis of dogfish muscle protein using Alcalase® under optimum conditions. Each value represents the mean of three determinations ± standard deviation. [a-d] Values in columns with different letters are significantly different ($P < 0.05$).


80
60
40
20
0
Nitrogen recovery (%)
d
c
b
a
0
45
90
120
Time (min)

4.5 Proximate composition and nutritional value of the shark protein hydrolysate

The composition of the spiny dogfish protein hydrolysate optimized by the RSM is presented in Table 4.12. The spray dried hydrolysate had a decreased fat content as a consequence of removing the unhydrolysed material after the proteolytic reaction and the lipid layer after centrifugation. The low lipid content in the protein hydrolysate might significantly contribute to minimizing the rate of lipid oxidation, thereby extending the storage stability and sensory qualities longer (Mackie, 1982). In contrast, the ash content increased, which might be explained by the addition of NaOH during the hydrolytic reaction to keep the pH constant. Such an increase in ash content has been observed in other controlled hydrolytic reactions using alkaline proteases (Shahidi *et al.*, 1994; Yu and Fazidah, 1994; Mutilangi *et al.*, 1995).

The increase in the total nitrogen content, on dry weight basis, exhibited in the spray dried hydrolysate is a consequence of the concomitant decrease in the lipids content after the centrifugation step, in which a great deal of fat was removed.

The amino acid composition of the spray dried shark protein hydrolysate is shown in Table 4.13. All essential amino acids were present. Comparing this data with that for the amino acid profile of the unhydrolysed dogfish muscle (Table 4.2), no consistent changes in the levels of total amino acids are noted, demonstrating that the amino acid pattern was unaffected by the enzymatic reaction. An indication that the shark hydrolysate has high nutritional value is the fact that its

Table 4.12 Proximate composition of shark (*Squalus acanthias*) protein hydrolysate produced under optimum conditions[a].

Components	Composition (g/100g)
Ash	13.27 ± 0.24 (13.78)[b]
Lipids	0.84 ± 0.05 (0.87)
Moisture	3.70 ± 0.31
Total nitrogen	13.15 ± 0.07 (13.66)
Non-protein nitrogen	1.01 ± 0.16 (1.05)

[a] Mean values of three determinations of three replicate samples ± standard deviations.

[b] Values in parenthesis represent proximate composition on dry weight basis.

Table 4.13 Amino acid composition (g/100 g crude protein) of the protein hydrolysate prepared with Alcalase® at optimum conditions

Amino acid[a]	SPH	Suggested requirement[b]		Reference[c] pattern	Amino acid score (%)
		Child	Adult		
Acid side chain					
Asp + Asn	9.22				
Glu + Gln	13.53				
Large hydrophobic side chain					
Valine *	4.23	3.5	1.3	5.0	84.6
Phenylalanine*	3.61	6.3[d]	1.9[d]	6.0[d]	108.2[d]
Tyrosine	2.88				
Tryptophan *	0.85	1.1	0.5	1.0	85.0
Leucine *	7.55	6.6	1.9	7.0	107.9
Isoleucine *	4.39	2.8	1.3	4.0	109.8
Proline	4.46				
S-containing side chain					
Methionine *	2.24*	2.5[f]	1.7[f]	3.5[f]	82.6[f]
Cystine	0.66*				
Basic side chain					
Lysine *	8.41	5.8	1.6	5.5	152.7
Arginine	5.67				
Histidine	1.95				
Small neutral side chain					
Glycine	6.63				
Alanine	5.29				
Serine	4.03				
Threonine *	4.56	3.4	0.9	4.0	114.0

* Essential amino acids; [a] Categorized after Adler-Nissen (1986); [b] FAO/WHO (1991); [c] FAO/WHO (1973); [d] Phe + Tyr; * Limiting amino acids; [f] Met + Cys.

amino acid composition meets the requirements for adults and, with the exception of tryptophan, for pre-school children (FAO/WHO, 1991). Moreover, the amino acid score for most essential amino acids is above 100 %, with the exceptions of tryptophan (85 %), valine (84.6 %) and Met + Cys (82.6 %), these last acting as the limiting amino acids. The amino acid score of 152.7 % for lysine is not unexpected, since this essential amino acid is present in large amounts in shark muscle (Geiger & Borgstrom, 1962; Morris, 1975). In addition to lysine, threonine exhibited a higher value compared to the protein standard of FAO/WHO (1973). This endorses the use of shark protein hydrolysate in lieu of vegetable proteins, which usually contain low levels of Lys and Thr, as a protein supplement in food systems.

A mean reduction of only 3.2 % in PER from the shark muscle to that of its hydrolysate was observed (Table 4.14), indicating that the enzymatic hydrolysis process, although attained a relatively high DH value, did not compromise the nutritional quality of the spray dried product. The high PER values for shark protein hydrolysate were another evidence of its good nutritional value.

Although the presence of all essential amino acids, and high PER values for the protein hydrolysate, indicate high biological value, digestibility studies should be carried out to confirm its applicability in food systems.

Table 4.14 Predicted protein efficiency ratio (PER) for shark (*Squalus acanthias*) muscle (SM) and shark protein hydrolysate (SPH)

Regression equation	PER[a]		
	SM	SPH	% loss
$Y = -0.684 + 0.456[Leu] - 0.047[Pro]$	3.04	2.90	4.61
$Y = -0.468 + 0.454[Leu] - 0.105[Tyr]$	3.07	2.99	2.61
$Y = -1.816 + 0.435[Met] + 0.780[Leu] + 0.211[His] - 0.944[Tyr]$	3.23	3.14	2.79
$Y = 0.08084\ X_7 - 0.1094$	3.13	3.04	2.88
$Y = 0.06320\ X_{10} - 0.1539$	3.02	2.92	3.31

[a] Calculated from amino acid data in grams of amino acid residue in the protein sample per 100 g of total amino acid residue.

Y : PER

X_7 : Thr + Val + Met + Ile + Leu + Phe + Lys

X_{10}: X_7 + His + Arg + Trp

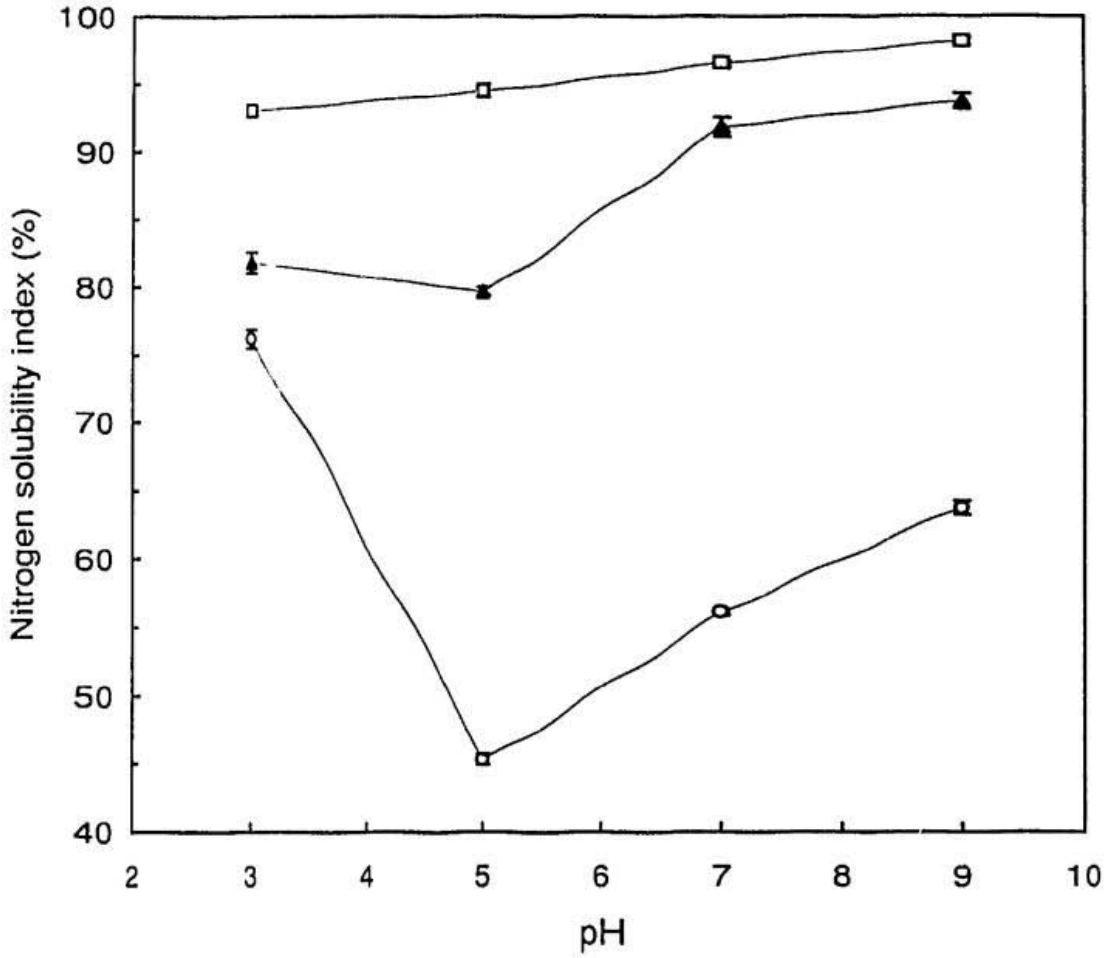
4.5 Functional properties of the optimized protein hydrolysate

The enzymatic hydrolysis considerably increased the nitrogen solubility index (NSI) over the pH range studied (Figure 4.13). It has been suggested that an increase in the solubility of protein hydrolysates over that of the original protein is due to the degradation of its secondary structure, and also due to the enzymatic release of smaller polypeptide units from the protein (Adler-Nissen, 1986; Quaglia and Orban, 1987a; Chobert *et al.*, 1988). The increase in DH, from the use of the commercial enzyme, Alcalase®, over the autolytic process, by the endogenous enzymes, resulted in significantly higher NSI values ($P > 0.05$).

In fact, Alcalase® assisted proteolysis has been reported to produce highly soluble protein hydrolysates from fish (Quaglia and Orban, 1987a) and seal protein (Shahidi *et al.*, 1994).

The solubility of the hydrolysate obtained with endogenous enzymes was also increased in comparison to the control, even though in a smaller magnitude. At pH 5.0, near the isoelectric point at which the net charge of the original protein is minimized and consequently more protein - protein interactions and fewer protein - water interactions occur (Chobert *et al.*, 1988; Adler-Nissen, 1976), there was a large increase in solubility, which was accounted from 45.33 to 79.66 %. The fact that the autolysate consistently exhibited a lower NSI, in comparison to the Alcalase®-assisted hydrolysate, is presumably due to some aggregation of the larger polypeptide units released by the reaction, the extent depending on pH (Turgeon *et al.*, 1992). Furthermore, the denaturation of larger peptide units by the

Figure 4.13 Effect of pH on nitrogen solubility of shark protein hydrolysate. Means ± standard deviation. Control, o ; Autolysis, ▲ ; and SPH, □.


Nitrogen solubility index (%)
100
90
80
70
60
50
40
2
3
4
5
6
7
8
9
10
pH

inactivation step after the reaction might have lowered the NSI (Surowka and Fik, 1992). On the whole, Alcalase® increased the solubility more efficiently than endogenous enzymes.

Dispersibility was notably enhanced to 91.88 % for the hydrolysed sample (*SPH*), compared to 14.45 % for the control (Figure 4.14). Both the autolysed and hydrolysed (*SPH*) samples exhibit high values for dispersibility. However, the *SPH* showed statistically higher dispersiblity ($P < 0.05$) than the autolysed sample. The control had significantly lower dispersibility than all the others. In addition to the effect of proteolytic degradation on the dispersibility, spray drying may have enhanced dispersibility by means of the large surface area of the small particles obtained from this operation.

Water-holding and oil-holding capacities (WHC and OHC, respectively) of the Alcalase®-hydrolysed sample decreased compared to that of the control (Figure 4.15). The control, representing the original protein substrate, had significantly higher values of WHC than the other samples, presumably due to the hydrophillic polar side chains of the original protein. According to Bernardi Don *et al.* (1991), protein is in the form of a network, which is degraded by hydrolysis with consequent diminishing of the sample's absorption capacity. The decrease from the OHC of the control to that of the autolysed sample (0.34 to 0.24 mL / 0.05 g of sample), and the more pronounced decrease in WHC (14.61 to 8.30 mL / g), might have been due to the hydrolytic degradation of the protein structure. It has been pointed out that physical entrapment plays an important role in the oil absorption of

Figure 4.14 Effect of the enzymatic hydrolyisis on the dispersibility (%) of the protein samples. Means ± standard deviation. [a-c] Values in columns with different letters are significantly different ($P < 0.05$).

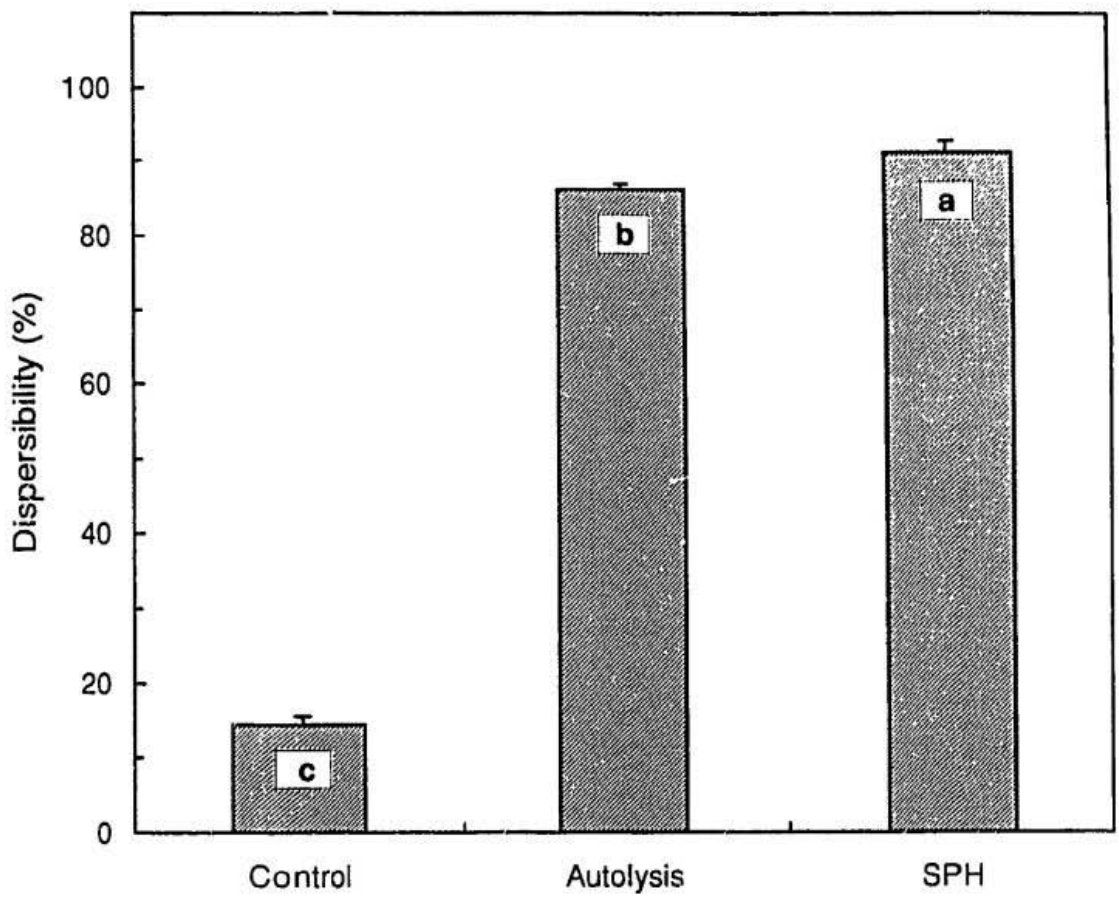
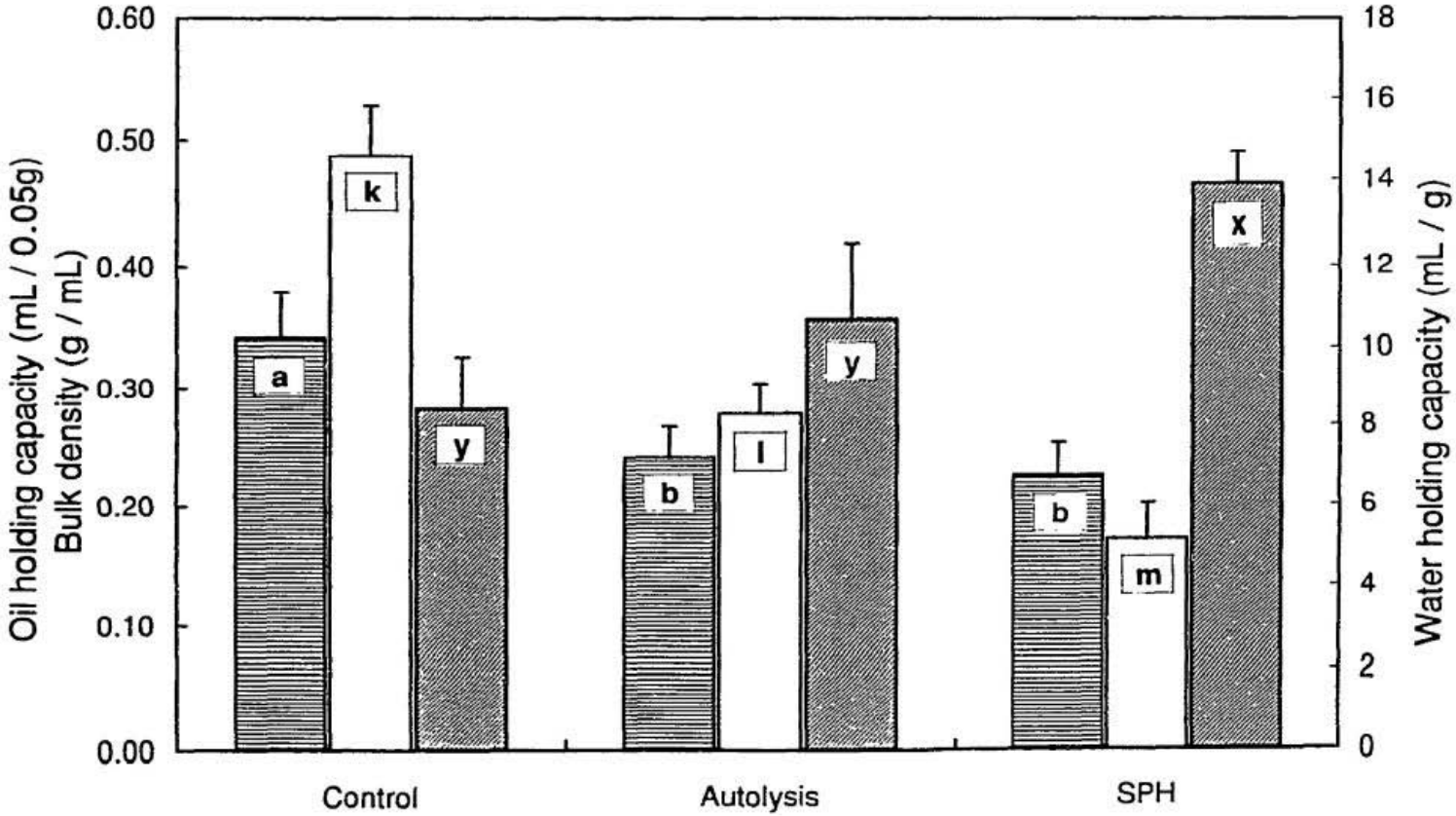

Dispersibility (%)
100
80
60
40
20
0
c
b
a
Control
Autolysis
SPH

Figure 4.15 Effect of the extent of enzymatic modification on the water absorption and oil absorption capacities, and on bulk density. Means ± standard deviation.

▤ : Oil holding capacity (OHC) in mL of oil held / 0.05 g of sample. $^{a-b}$ Values in columns with different letters are significantly different ($P < 0.05$).

□ : Water holding capacity (WHC) in mL of H_2O / g of sample. $^{k-m}$ Values in columns with different letters are significantly different ($P < 0.05$).

▨ : Bulk density (g/mL) of sample. $^{x-y}$ Values in columns with different letters are significantly different ($P < 0.05$).


Oil holding capacity (mL / 0.05g)
Bulk density (g / mL)
Water holding capacity (mL / g)
0.60
0.50
0.40
0.30
0.20
0.10
0.00
18
16
14
12
10
8
6
4
2
0
a
k
y
b
l
y
b
m
x
Control
Autolysis
SPH

the original protein (Dench *et al.*, 1981). Wang and Kinsella (1976) reported a similar observation regarding fat absorption, and indicated that the same holds for water absorption as well. These investigators indicated a negative relationship between bulk density and the water and fat absorption capacities, when studying alfalfa leaf protein. Dev and Quensel (1986) suggested similar relationship for oil absorption and bulk density.

The fact that the capacity of the powdered hydrolysate to absorb and retain water was negatively influenced by extensive hydrolysis might restrict this product's application as a texture enhancer of comminuted meats and baked dough, even though this high-protein ingredient could increase their nutritional quality.

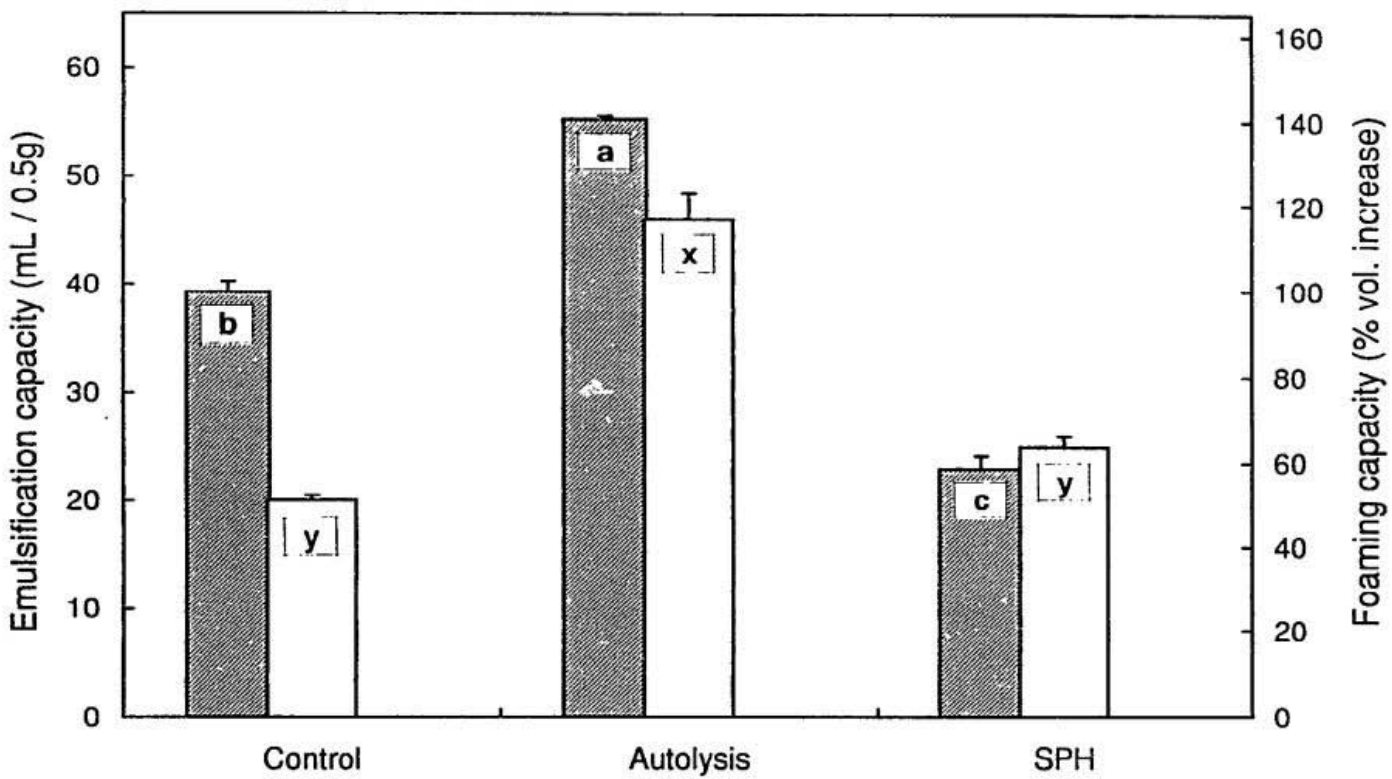
In Figures 4.13 and 4.15, an inverse correlation can be observed between solubility and water- and oil-holding capacities. This is in agreement with Orban *et al.* (1992), who observed that high solubility decreased the WHC of fish protein. Moreover, Dev and Quensel (1986) reported that vegetable protein exhibits a similar tendency.

The emulsifying capacity of the Alcalase®-assisted hydrolysate was significantly decreased compared to the autolysis sample, as seen in Figure 4.16. For example, the emulsification value of the SPH sample (23.09 mL of oil emulsified / 0.5 g of protein powder) was relatively small in comparison to reported values for fish isolates obtained from whole or gutted fish, 51.2 and 48.0 mL oil / 0.5 g, respectively (Rakesh and Metz, 1973). This agrees with Quaglia & Orban (1990) and Surowka and Fik (1994), who observed that degree of protein

Figure 4.16 Effect of the extent of enzymatic modification on the emulsification and foaming capacities. Means ± standard deviation.

▨ : Emulsification capacity, in mL of oil emulsified / 0.5 g of sample. $^{a-c}$ Values in columns with different letters are significantly different ($P < 0.05$).

□ : Foaming capacity (FC) in % of volume increase from initial volume sample. $^{x-y}$ Values in columns with different letters are significantly different ($P < 0.05$).

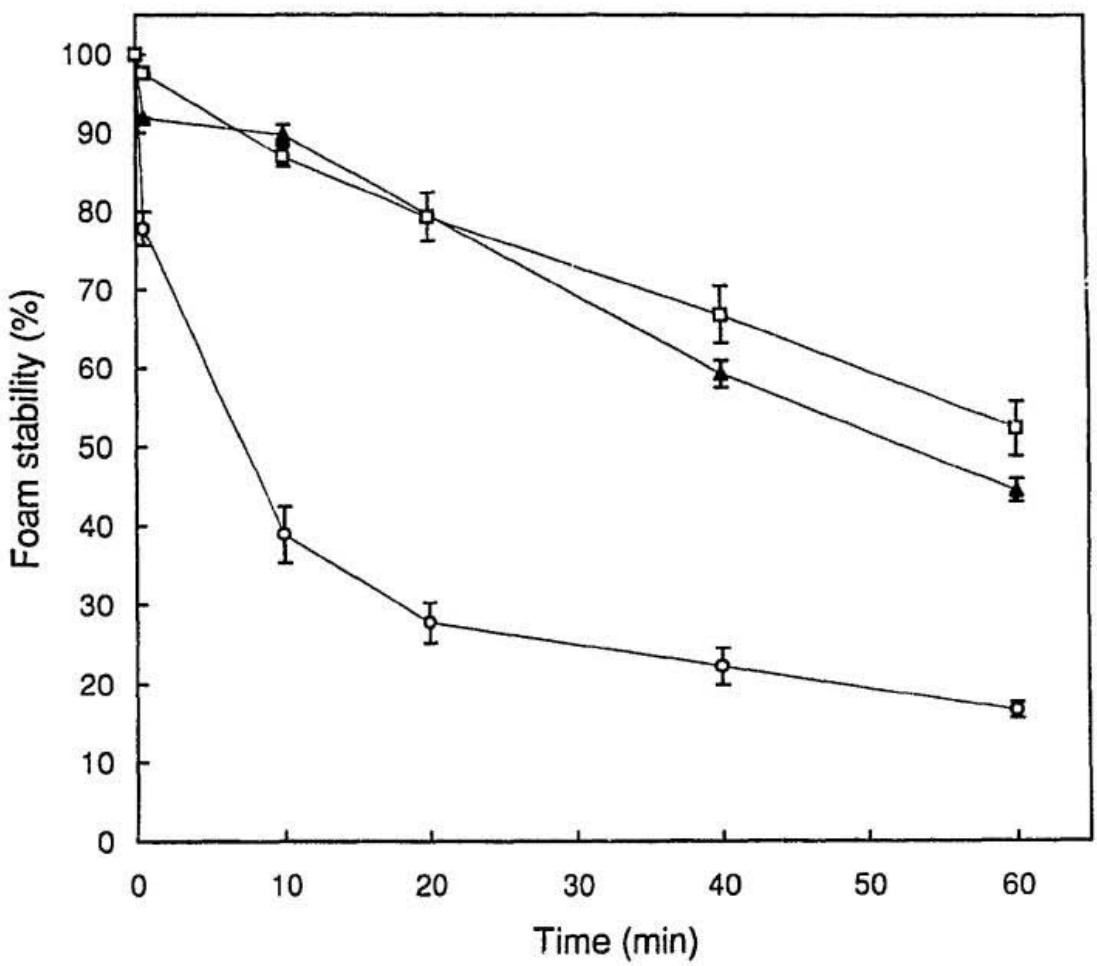

Emulsification capacity (mL / 0.5g)
Foaming capacity (% vol. increase)
0
10
20
30
40
50
60
0
20
40
60
80
100
120
140
160
b
y
a
x
c
y
Control
Autolysis
SPH

hydrolysis holds an inverse relationship to emulsifying capacity. This relationship is presumably due to the formation of nonamphiphillic peptides (Chobert *et al.*, 1988). Nonetheless, hydrolysis of shark muscle protein with Alcalase® resulted in a better emulsifying capacity than that produced by the hydrolysis of lobster (*Panulirus* spp.) processing waste with a fungal protease (Vieira *et al.*, 1995).

The higher emulsification capacity value obtained for the autolysed sample than for the control protein can be attributed to the low level of degradation of the protein molecules by the endogenous enzymes. This increased the availability of large peptide units to the oil-water interface, resulting in larger surface area and, consequently, greater emulsion formation (Puski, 1975). Kim *et al.* (1990) observed that reduced levels of disruption contributed to enhancing emulsion formation in enzyme-modified soy protein isolates.

Proteins in dispersions cause a lowering of the surface tension at the water-air interface, thus creating foam formation (Surowka and Fik, 1992). Figure 4.15 shows a significant increase in the foaming capacity for the autolysed sample (116.69 % volume increase) compared to the control (50.97 % volume increase). This result suggests an increase in surface activity, presumably because hydrophobic groups have become available from the degree of hydrolysis attained with the autolytic enzymes. An improvement in foaming capacity for enzymatically-modified food proteins is reported by Adler-Nissen (1986). The decrease in foaming of the more extensively-hydrolysed sample, *SPH* (63.57 % volume increase), indicates a negative effect on the surface activity. The most stable foam

Figure 4.17 Foam stability of protein samples as a function of time elapsed. Means ± standard deviation. Control, o ; Autolysis, ▲ ; and SPH, □.


Foam stability (%)
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
Time (min)

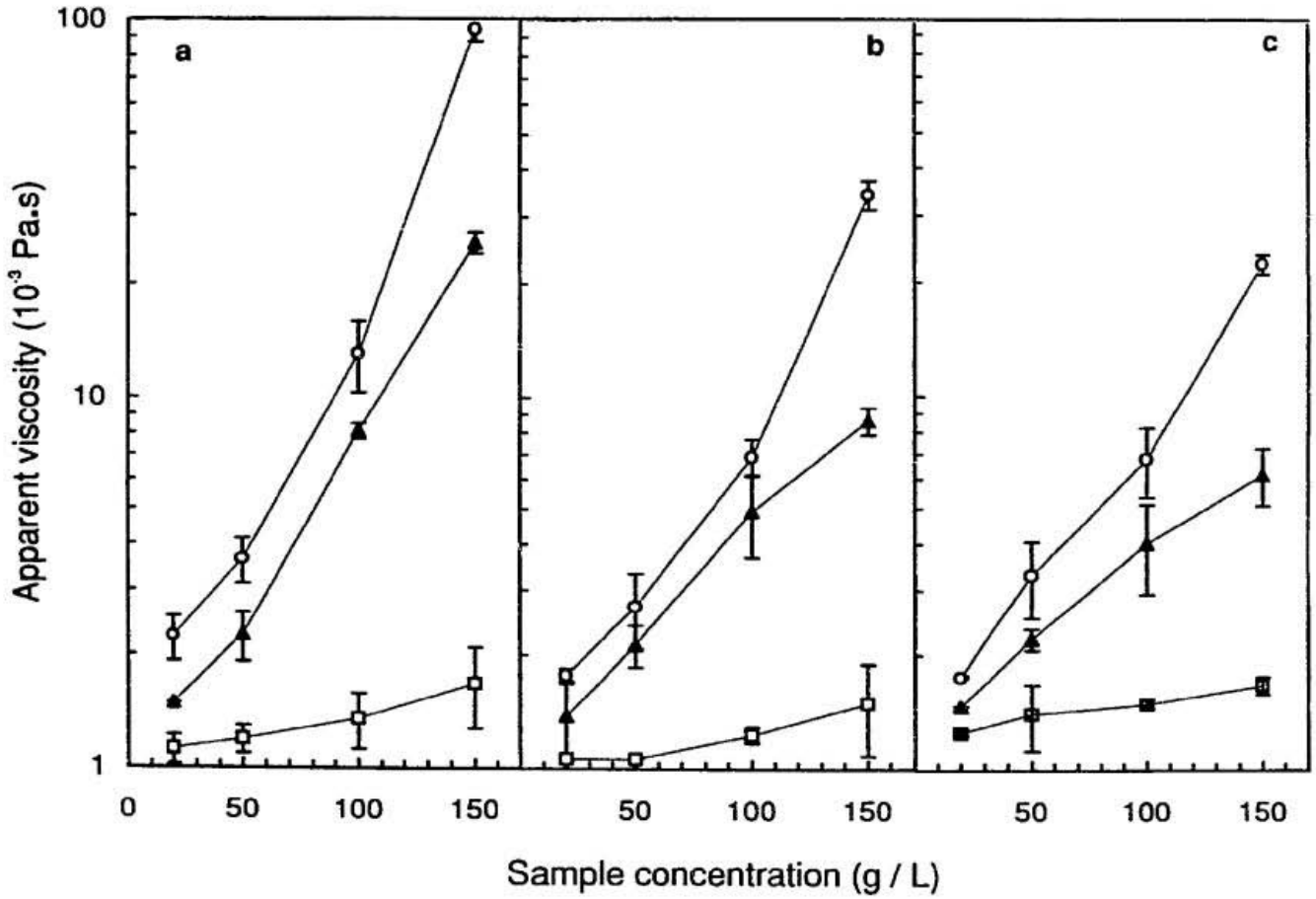
was obtained with the *SPH* sample, which retained 52.31 % of the initial foam after the 60 min quiescent period, far beyond the stability of the control sample, which retained 16.67 % (Figure 4.17).

Measurements of apparent viscosity against sample concentration at shear rates of 150, 750 and 1500 s^{-1} produced similar viscosity profiles (Figure 4.18). The suspension of the enzyme-hydrolysed sample was very fluid even at the highest powder concentration, 150 g / L. Nevertheless, a slight increase in viscosity with increased concentration was observed for this sample. A greater increase in viscosity with increased sample concentration was noticed for the control and autolysed samples at all shear rates.

A viscosity value of 3.5 mPa·s was reported by Surowka and Fik (1992) for a 100 g / L concentration of protein hydrolysate obtained from chicken heads digested by Neutrase®, a bacterial endoprotease with less hydrolytic action than Alcalase®. In Figure 4.18, the Alcalase®-hydrolysed sample presented a maximum value of 1.49 mPa·s at 1500 s^{-1}, and a minimum value of 1.23 mPa·s at 750 s^{-1} at a concentration of 100 g / L. Surowka and Fik (1994) reported a viscosity value of 1.7 mPa·s for a similar protein substrate hydrolysed by pepsin, which exhibited higher proteolytic activity than Neutrase®. These results indicate that proteolytic cleavage in the hydrolysis process reduces the viscosity of the protein dispersions.

The protein - protein interactions in dispersions of the original protein are stronger than the peptides - solvent (water) interactions in the hydrolysed substrates, so hydrolysing the proteins to produce polypeptides results in

Figure 4.18 Effect of sample concentration (g / L) on the apparent viscosity of aqueous dispersions at 20 °C. Means ± standard deviation. Shear rates: **a)** 150 s^{-1}; **b)** 750 s^{-1}; and **c)** 1500 s^{-1}. Control, o ; Autolysis, ▲ ; and SPH, □ .


100
10
1
Apparent viscosity (10^{-3} Pa.s)
a
b
c
0
50
100
150
50
100
150
50
100
150
Sample concentration (g / L)

decreased viscosity. Therefore, an increase in the extent of proteolysis could be expected to reduce viscosity.

The results obtained indicate that the hydrolysed dogfish protein, *SPH*, could find applications in the protein enrichment of liquid food without compromising its mouthfeel. The autolysed product, with higher viscosity and emulsifying capacity, could be a good functional additive for sausages.

The enzymatic hydrolysis of shark muscle produced a protein powder with Hunter colour values closer to the reference plate than the control sample, except the *b* value (Table 4.15). The *b* values of all samples exhibited a large positive shift. Spray drying might have contributed to the increased lightness values (*L*) obtained in the hydrolysed products. The *SPH* and autolysed samples had significantly ($P < 0.05$) higher lightness than the control. The total colour difference (ΔE), which indicates the overall colour deviation from the standard reference, was significantly ($P < 0.05$) reduced for the same samples. In general, the sensory characteristics of the shark protein hydrolysate were favourable. All powdered hydrolysates, except the autolysed sample, had negligible fishy odour. Bitter taste was absent as well. In fact, Alcalase®-assisted hydrolysis of fish protein is consistently reported to produce weak or no bitterness (Adler-Nissen, 1976; Hoyle and Merritt, 1994).

The results of these protein functionality assessments indicate that the dogfish protein hydrolysed under the conditions used in this work has a potential application as an ingredient in food systems. The results indicate that the extent of

Table 4.15 Hunter color parameter values of freeze dried muscle (control) and the enzymatically hydrolysed dogfish muscle *.

Sample	Hunter colour parameters			
	L	*a*	*b*	*ΔE*
Control	73.6 ± 0.7[b]	2.3 ± 0.6[a]	12.4 ± 0.9[b]	22.1 ± 0.1[a]
Autolysis	85.8 ± 0.9[a]	-0.7 ± 0.3[b]	15.1 ± 0.7[a]	15.7 ± 1.0[b]
SPH	86.4 ± 1.3[a]	-0.5 ± 0.1[b]	10.8 ± 0.6[c]	11.6 ± 0.7[c]

* Values represent means of three determinations ± standard deviations.

[a-c] Values within columns with different superscripts are significantly different ($P < 0.05$).

the enzymatic modification should be determined by the intended application of the new food ingredient, since enzyme treatment can cause substantial changes in functionality.

CHAPTER V

CONCLUSIONS

The use of response surface methodology to describe the combined effects of the process variables on the enzymatic hydrolysis of dogfish (*Squalus acanthias*) muscle, as well as to optimize these factors regarding the maximum recovery of proteinaceous substances from the dogfish muscle has proven to be successful. The high coefficients of determination from the regression analysis of both mathematical models, the non-significant lack-of-fit values and the positive results from the confirmatory tests support this evidence. Therefore, these polynomial models could be relied on and used to a great extent in research and industrial production of protein hydrolysates.

The degree of hydrolysis studies provided information on the factors and levels required to obtain different DH values. It was indicated that the degree of hydrolysis was significantly affected ($P < 0.01$) by all linear process variables. The quadratic effects of pH, temperature and enzyme - substrate ratio seem to influence the hydrolytic reaction to a similar extent ($P < 0.01$). The effect of the temperature and enzyme - substrate ratio interaction was also significant, but to a lesser degree ($P < 0.05$). From the graphical representations it can be concluded that a maximum degree of hydrolysis can be obtained using the critical values of the conditions described in this study. Furthermore, *2D*- and *3D*-graphs can be used to assist the selection of specific combinations of

hydrolysis variables in order to attain any desired level of hydrolysis, which in turn will influence the application in food formulations of this new source of protein (Adler-Nissen, 1986).

Regarding the optimization of nitrogen recovery from the dogfish muscle by the enzymatic treatment, several conclusions can be drawn:

1. Nitrogen recovery was significantly affected ($P < 0.01$) by all linear and squared hydrolysis variables, whereas the hydrolysis pH and enzyme - substrate ratio interaction was significant at 10 % probability level.
2. It was indicated by the explanatory equation that the enzyme - substrate ratio, which had the greatest slope, was the most significant linear variable affecting the nitrogen recovery.
3. The Alcalase®-assisted hydrolysis gave a maximum recovery of soluble nitrogen (77.7 % from the original substrate) under the following conditions: pH: 8.3, temperature: 55.3 °C, enzyme - substrate ratio: 3.7 %, after two-hour hydrolysis.

Considering the amino acid spectrum and the PER values of the shark protein hydrolysate obtained at conditions of maximum nitrogen recovery, which was similar to that of the fish muscle, it has been shown that the enzymatic process did not affect the original protein quality of the dogfish muscle. Although a high biological value was indicated by the presence of all essential amino acids and high PER values of the protein hydrolysate, digestibility studies should be carried out to confirm this possibility.

The enzymatic hydrolysis markedly improved several functional properties, resulting in the following qualities: solubility, ranging from 93 to 98 % and dispersibility of 91 %. Viscosity was low even for highly concentrated aqueous dispersions. These characteristics suggest that the optimized shark protein hydrolysate, SPH, has a potential application as a protein supplement in protein-enriched drinks. Moreover, the SPH could also be incorporated as an ingredient in vegetable-based formulations. Nonetheless, its use as a milk replacer for calves and piglets, as well as highly concentrated nitrogen source for microbial growth should not be overlooked.

This study also leads to the conclusion that further investigation should be conducted in the following areas:

1. Possibilities of commercial exploitation of this new source protein, focusing on processing costs and capital investment (scale up the process to pilot plant level). This study will provide information to judge the suitability of the production process.
2. Evaluation of the changes in the nutritional, organoleptic and functional properties of the SPH during long term storage.
3. Potential applications for the unhydrolysed residue, separated in the centrifugation step, with special attention to the production of feed or food.
4. Optimization of the dehydration process aiming to decrease spray drying costs.
5. Possibilities of maximization of nitrogen recovery by step wise addition of

the enzyme.

6. Application of enzyme immobilization techniques to reuse the exogenous enzyme allowing continuous operation.
7. Direct application of proteolytic microorganisms to hydrolyse the fish muscle substrate to produce protein hydrolysates.
8. Potential applications for the protein hydrolysate produced under the optimized hydrolytic conditions.

On the whole, it has been demonstrated that empirical mathematical models can be used to describe adequately the influence of the process variables on the enzymatic hydrolysis of dogfish, and predict the maximum recovery of proteinaceous substances from the original protein substrate over the range of independent variables studied. The protein ingredient obtained under these conditions had high nutritional value and good functional properties, indicating that the dogfish (*Squalus acanthias*) muscle can be converted to a value-added protein hydrolysate with potential applications in food systems.

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APPENDICES

Appendix 1. Taxonomy of the spiny dogfish (*Squalus acanthias*).

Phylum Chordata

Subphylum Vertebrata

Superclass Pisces (Fishes)

Class Chondrichthyes (cartilaginous fishes)

Subclass Elasmobranchii (elasmobranchs)

Order Selachii

Family Squalidae

Genus *Squalus*

Species *Squalus acanthias*

Appendix 2. SAS program for regression analysis of the response variable DH.

```
/*Alcalase 2.4L*/
options ls=72;
data alcalase;
     input y x1-x3 @@;
     label y="DH (%)"
     x1="pH"
     x2="Temperature"
     x3="E/S ratio";
     cards;
17.31   1  1  0
14.73   1 -1  0
15.33 - 1  1  0
11.47  -1 -1  0
17.96   0  0  0
17.47   1  0  1
15.41   1  0 -1
15.38  -1  0  1
12.02  -1  0 -1
17.92   0  0  0
17.85   0  1  1
15.57   0  1 -1
16.54   0 -1  1
12.06   0 -1 -1
17.69   0  0  0
;
proc sort;
     by x1-x3;
proc print;

proc rsreg;
     model y=x1-x3 / lackfit;
run;
```

Appendix 3. SAS program for regression analysis of the response variable NR.

```
/*Alcalase 2.4L*/
options ls=72;
data alcalase;
     input y x1-x3 @@;
     label y="NR (%)"
     x1="pH"
     x2="Temperature"
     x3="E/S ratio";
     cards;
65.24   1  1  0
58.96   1 -1  0
60.64  -1  1  0
52.37  -1 -1  0
73.74   0  0  0
74.05   1  0  1
45.15   1  0 -1
59.51  -1  0  1
42.55  -1  0 -1
69.96   0  0  0
75.45   0  1  1
58.53   0  1 -1
64.96   0 -1  1
43.26   0 -1 -1
72.76   0  0  0
;
proc sort;
     by x1-x3;
proc print;

proc rsreg;
     model y=x1-x3 / lackfit;
run;
```