Proteolytic Hydrolysis of Muscle Proteins of Harp Seal (*Phoca groenlandica*)

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Protein hydrolysates were prepared from mechanically separated seal meat (MSSM) using commercially available microbial enzymes Alcalase and Neutrase. The enzyme in each case was inactivated by acidification with 4 N HCl to pH 4.0. Heme groups were removed, and the hydrolyzed proteins were bleached with charcoal, neutralized to pH 7.0, and dehydrated. The yield of hydrolysate (YH) and the degree of hydrolysis (DH) using Alcalase, under optimum conditions, were 92.75% and 19.5%, respectively. The YH for Neutrase-assisted hydrolysis of MSSM was 72.85%. The dehydrated seal protein hydrolysate (SPH) contained 77.3% crude proteins, 0.74% lipid, and 20.67% minerals, mainly sodium chloride. The amino acid composition of SPH was similar to that of the original MSSM. The product so obtained was bland in taste and off-white in appearance (Hunter L value of 84.6) and had a solubility of ≥93.5% over the pH range 2.5–10.4.

Keywords: *Harp seal; muscle proteins; hydrolysis; proteolytic enzymes; Alcalase; Neutrase; functional properties; solubility*

INTRODUCTION

Harp seal (*Phoca groenlandica*) is the principal species of seal found in the waters of Newfoundland and Labrador. Although the population of harp seals at the end of 1985 was estimated by the Royal Commission on Seals and Sealing Industry in Canada at approximately 2.0–2.5 million, their present number is revised to 2.6–3.4 million. They feed on a variety of fish species and consume over 5 million metric tons of cod, herring, capelin, and salmonids. The Royal Commission at the time of its reporting expressed concern regarding the possibility of continued increases in the number of seals on the status of fisheries in the Atlantic region. Although Federal regulations allow a total annual catch of 186 000 seals, only 50 000–70 000 animals have been caught in recent years. This provides approximately 1.2–2.1 million kilograms of meat in Newfoundland each year (Synowiecki and Shahidi, 1991). Traditionally, harp seals have been hunted principally for their pelt. Blubber may have been used as a cheap source of margarine ingredient or employed for other industrial applications. The carcasses were then dumped or reduced to low-grade silage. Nonetheless, local food use of seal meat, particularly seal flippers, is well-known to Newfoundlanders.

Seal meat is a rich source of nutritionally valuable proteins with a well-balanced essential amino acid composition (Shahidi et al., 1990; Shahidi and Synowiecki, 1993). However, full utilization of seal meat is limited due to its dark color and intense flavor. Hemoproteins not only affect the color of the meat but also act as prooxidants, causing rapid deterioration of products upon storage (Synowiecki and Shahidi, 1991). Preparation of seal protein hydrolysate (SPH) provides a means for better utilization of seal meat since products so obtained may be used in a variety of applications including dietetic foods, processed meats, and extruded products (Wismar-Pedersen, 1979; Adler-Nissen and Olsen, 1982). The product is soluble in brine and can be incorporated into whole pieces of meat such as ham, by either massaging or injection. The protein hydrolysate, when applied in meat formulations or as a dip for processing of seafoods, may also serve as a water-retaining agent and as an alternative to polyphosphates.

MATERIALS AND METHODS

Raw Material. Harp seals (*P. groenlandica*) from 1 to 4 years of age were caught in the coastal areas of Newfoundland during the month of April, bled, and skinned; the blubber was removed, and the carcasses were eviscerated. The carcasses weighing up to 30 kg, without head and flippers, were placed inside plastic bags and stored on ice for up to 3 days. Each carcass was then washed with a stream of cold water (+10 °C) for approximately 15 s to remove most of the surface blood. Mechanical separation of meat from carcasses of 15 seals was carried out using a Poss deboner (Model PDE500, Poss Limited, Toronto, ON). Small portions of mechanically separated seal meat (MSSM) were vacuum packed in polyethylene pouches and kept frozen at −20 °C until used.

Production of Hydrolysate. Seal protein hydrolysate was prepared according to the flowsheet given in Figure 1. Microbial proteases Alcalase 2.4L and Neutrase 0.5L (Novo Industrie AS, Bagsvaerd, Denmark) with maximum activities at pH 8.5 and 6.5, respectively, were used. During enzymatic hydrolysis of proteins, the pH was maintained by continuous addition of a 4 N NaOH solution to the mixture. The degree of hydrolysis (DH), defined as percentage of the total peptide bonds cleaved, was related to the amount of base used during the course of hydrolysis reaction according to the equation given by Olsen (1983)

$$\mathrm{DH}\ (\%) = (XN/\alpha h M_p) \times 100$$

where X is the volume of NaOH solution (mL) consumed, N is the normality of the NaOH solution, α is the degree of dissociation of the α-NH_2 group expressed as $\alpha = 10^{pH-pK}/(1 + 10^{pH-pK})$, h is the total number of peptide bonds (equivalents

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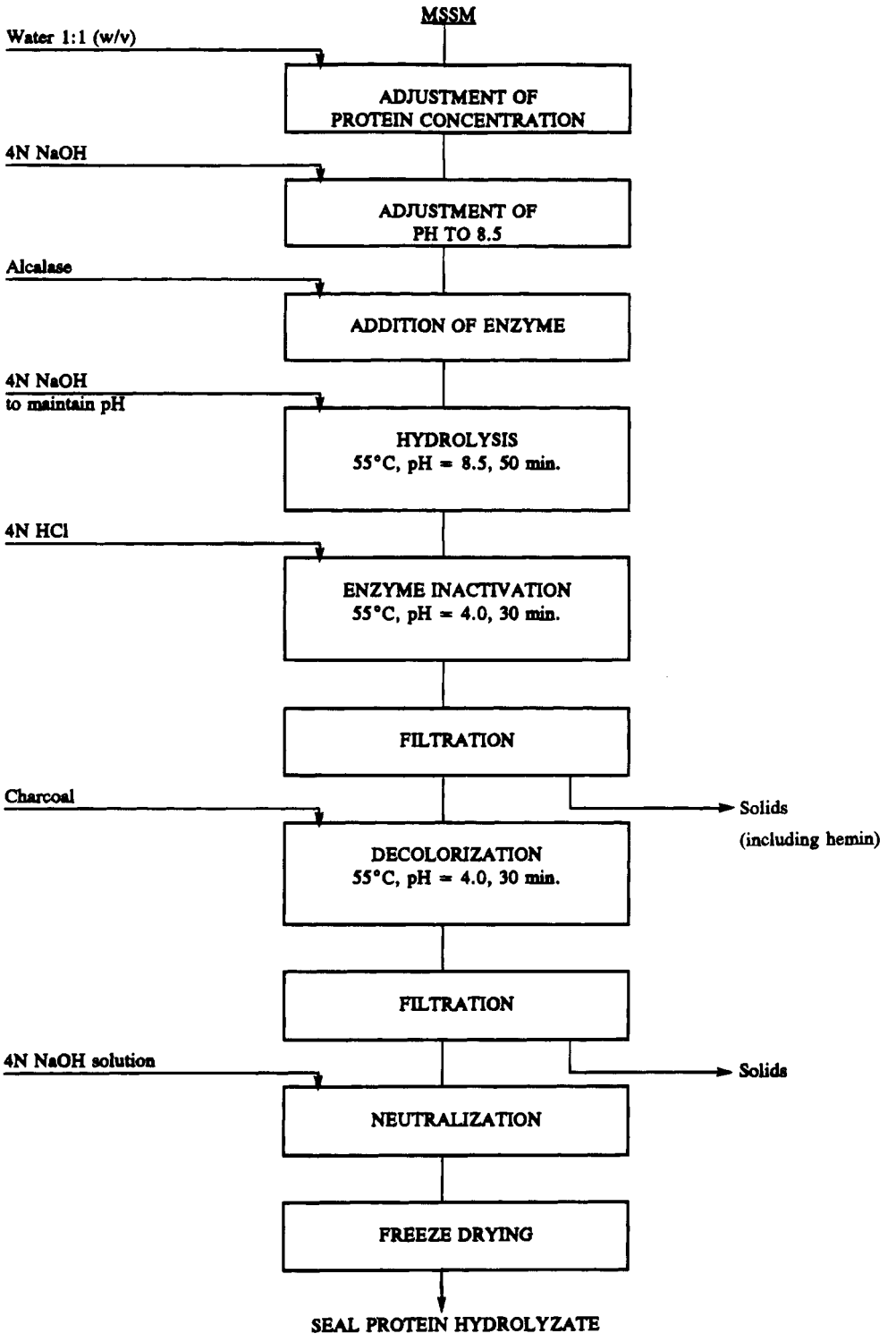

Figure 1. Flowsheet for Alcalase-assisted production of hydrolysates.

per kg of protein), which is 7.6 for meat (Olsen, 1983), and M_p is the mass of protein in sample (g).

To establish optimum hydrolysis conditions, the influence of temperature, time, and type of enzyme, as well as concentration of enzyme and substrate on the yield and quality of the product, was investigated. Variables examined were meat to water ratio of 1:1 to 1:3 (w/v), hydrolysis period of up to 3 h, temperature of 50–60 °C, and concentration of enzyme of 13.5–60 Anson units (AU)/kg of substrate protein (Olsen, 1983). These determinations were replicated three times. Recommended conditions were designated as those giving maximum yield by using optimum amounts of reagents and reaction time/temperature. Clarification of hydrolysates was achieved using 1–4% charcoal (Aldrich, Milwaukee, WI), based on the original weight of the meat, and suction filtration.

Analyses. Chemical composition of samples was determined as described below. Moisture content was determined by oven-drying at 105 °C for approximately 12 h to reach a constant weight (AOAC, Method 950.01, 1990). Total nitrogen (protein content = N × 6.25) and ash were assayed according to AOAC (1990) Methods 955.04 and 938.08, respectively. Total lipid was extracted using a chloroform–methanol–water mixture (Bligh and Dyer, 1959). The amino acid composition of hydrolysates was determined after digestion of samples in 6 N HCl at 110 °C (Blackburn, 1978) using a Beckman 121 MB amino acid analyzer (Beckman Instruments Inc., Palo

Alto, CA). The HCl was removed under vacuum, and dried samples were reconstituted using a lithium citrate buffer at pH 2.2 prior to determinations. Cysteine and methionine were determined as cysteic acid and methionine sulfone, respectively, using performic acid oxidation prior to their digestion in 6 N HCl (Blackburn, 1978). Analysis of tryptophan was carried out by hydrolysis of the sample under vacuum in 3 M mercaptoethanesulfonic acid at 110 °C (Penke et al., 1974).

The solubility of seal protein hydrolysate was determined according to the method of Chobert et al. (1988) with minor modifications. Hydrolysates were dispersed in distilled water (1% w/v) and the pH of the solution was adjusted between 2.5 and 10.5 with addition of a 4 N HCl or NaOH solution. After a 30 min equilibration period at room temperature, the solution was centrifuged at 12000*g* using a Sorvall Superspeed RC2-13 automatic refrigerated centrifuge. The solubility was determined by measuring the amount of Kjeldahl nitrogen in the supernatant (AOAC, 1990). The nitrogen solubility index (NSI) was calculated according to the formula

$$\text{NSI (\%)} = \frac{\text{nitrogen content in the supernatant}}{\text{nitrogen content in the hydrolysate}} \times 100$$

The tristimulus Hunter color parameters L (lightness, 100 = white; 0 = black), a (red, +; green, −), and b (yellow, +; blue, −) of the samples were measured using a XL-20 Colorimeter (Gardner Laboratory, Inc., Bethesda, MD). Standard plate No. XL-20-167C with Hunter L value of 92.0, a value of −1.1, and b value of 0.7 was used as a reference.

Statistical Analysis. Analysis of variance and Tukey's studentized range tests (Snedecor and Cochran, 1980) were used to determine differences in mean values of three to four replicates of each measurement. Significance was determined at $p < 0.05$.

Table 1. Hydrolysate Yield (Percent) of MSSM after 2 h of Hydrolysis at Different Temperatures and Enzyme Concentrations[a]

enzyme	enzyme concn (AU/kg of protein)	temp 50 °C	temp 55 °C	temp 60 °C
Alcalase	13.5	68.58 ± 0.52^{ax}	76.89 ± 0.36^{bx}	79.16 ± 0.38^{cx}
	30.0	78.10 ± 0.94^{ay}	81.59 ± 0.25^{by}	82.60 ± 0.25^{cy}
	60.0	84.05 ± 0.76^{az}	92.75 ± 1.05^{bz}	89.42 ± 1.02^{cz}
Neutrase	16.2	51.40 ± 0.22^{ax}	59.81 ± 0.48^{bx}	51.19 ± 0.93^{ax}
	24.2	58.14 ± 0.59^{ay}	67.76 ± 0.82^{by}	59.48 ± 0.62^{ay}
	30.0	63.35 ± 0.78^{az}	72.85 ± 0.44^{bz}	66.52 ± 0.60^{cz}

[a] Results are mean values of three replicates ± standard deviation. Values in each column and row with the same superscript are not significantly ($p > 0.05$) different from one another. Yields of hydrolysis were calculated as percentage ratio of the content of nitrogen in the hydrolysate to that in the original slurry containing MSSM.

Table 2. Coefficients of the Binomial Equation at 55 °C Depending on Enzyme Concentration[a]

enzyme	enzyme concn (AU/kg of protein)	YH = $a + b[X] + c[X]^2$ a	b	c
Alcalase	13.5	−346.72	14.35	−0.12
	30.0	−93.20	5.91	−0.05
	60.0	−664.60	27.00	−0.24
Neutrase	16.2	−969.37	37.45	−0.34
	24.2	−1022.55	39.51	−0.36
	30.0	−902.28	35.14	−0.32

[a] YH = yield of hydrolysis; [X] = concentration of enzyme.

RESULTS AND DISCUSSION

Figure 1 summarizes unit operations for production of SPH from MSSM. The content of protein in the original MSSM was 23.21 ± 0.13%. The high content of hemoproteins (5.93%) in this material was responsible for its dark color, as reflected in its Hunter L value of 16.6 ± 0.2 (Synowiecki and Shahidi, 1991). The main steps in the preparation of SPH were enzymatic hydrolysis at a constant pH adjusted by addition of base, inactivation of enzyme under acidic conditions, separation of heme residues, and decolorization by charcoal followed by neutralization and dehydration (Figure 1). The lower pH optimum of Neutrase (6.5) as compared with that of Alcalase (8.5) made it difficult to control the degree of hydrolysis as only small amounts of base were required for pH maintenance (55.6 g of NaOH/kg of protein for Alcalase and 7.5 g of NaOH/kg of protein for Neutrase).

For proper mixing of the substrate with NaOH solution and maintenance of the pH during the hydrolysis it was necessary to add a minimum amount of water to MSSM. Preliminary studies showed that proper regulation of pH was possible when a meat to water ratio of 1:1 (w/v) was used. This corresponds to 11.6% protein in the mixture. At higher amounts of meat, the slurry did not mix properly and some problems for regulation of pH during the hydrolysis were also encountered.

The influences of temperature and enzyme concentration on the yield of SPH are shown in Table 1. The best yields were 92.75% and 72.85% using Alcalase and Neutrase, respectively. These yields were obtained after a 2 h reaction at 55 °C using an enzyme concentration of 60 or 30 AU/kg of protein, respectively. The dependence of the yield of hydrolysate (YH) on the concentration of enzyme ([X]) at each temperature was best described by a quadratic polynomial equation YH = $a + b[X] + c[X]^2$, where a, b, and c are constants. Using the least-squares errors method, constants a, b, and c were determined (Table 2). At a given temperature, the reaction yield increased as enzyme concentration was increased. The calculated maximum yield of hydrolysis was obtained at 55 °C at an Alcalase concentration of 60 AU/kg of protein. However, for Neutrase, the best yield was obtained at 55 °C at an enzyme concentration of 30 AU/kg of protein. This is in good agreement with the experimental data given in Table 1. The yield and characteristics of protein hydrolysate depended on the degree of hydrolysis (DH) of the product (Tybor et al., 1975).

The DH at a fixed substrate concentration may be influenced by both the time and temperature of hydrolysis (Figure 2) as well as the type and concentration of enzyme employed (Figure 3). The DH reached after a 3 h period using 30 AU of Alcalase/kg of protein was 21.42%. However, the DH obtained by Neutrase was lower than that for Alcalase and did not exceed 16.19% under identical reaction conditions. The YH was correlated ($r = 0.99$) with DH according to the regression equation YH = 24.96 + 2.644(DH). The YH obtained at DH values of 9.6%, 12.1%, 15.0%, and 19.0% were 51.60%, 54.96%, 65.12%, and 75.45%, respectively. Products with higher DH values may possess a slight bitter taste, as has been reported for hydrolysates of meat and fish and as judged by the experimenters (Olsen, 1983). Therefore, a DH of about 19.5% is recommended for preparation of SPH to obtain products with a near-bland taste. Olsen (1983) has reported that the bitterness of protein hydrolysates is completely masked after their incorporation into meat products such as ham, sausages, and pastes.

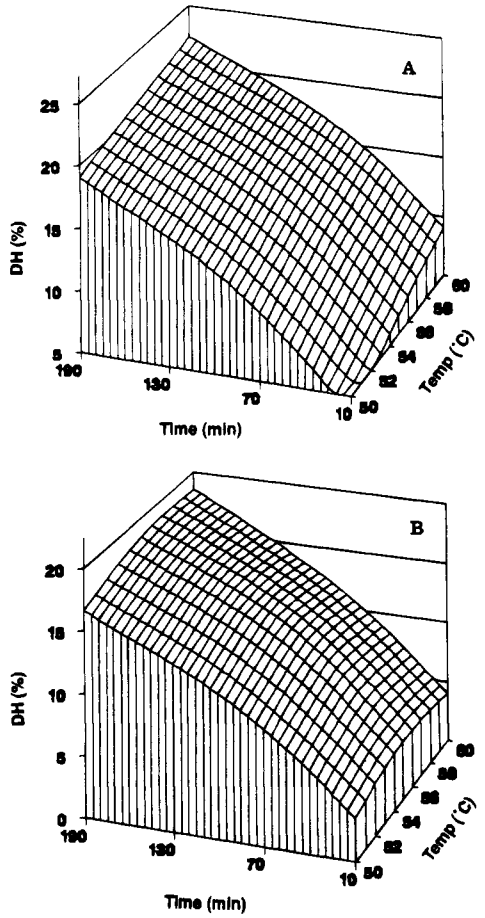

Figure 2. Three-dimensional representation of the effects of temperature and reaction time on the degree of (A) Alcalase-assisted and (B) Neutrase-assisted hydrolysis (DH%) of mechanically separated seal meat (MSSM).

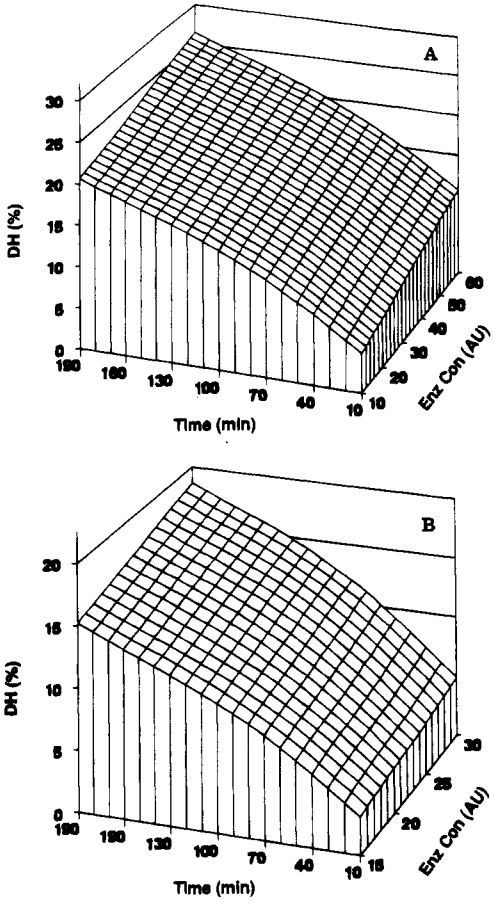

Figure 3. Three-dimensional representation of the effects of enzyme concentration [Anson unit (AU) per kilogram of substrate protein] and reaction time on the degree of (A) Alcalase-assisted and (B) Neutrase-assisted hydrolysis (DH%) of mechanically separated seal meat (MSSM).

The Hunter color parameters of SPH depended on the degree of hydrolysis (Table 3). Increasing DH from 9.6% to 15.0% resulted in a decrease in Hunter a (redness) values from 2.7 to 1.1. Better release of heme from hemoproteins present in seal muscle tissues at high DH values may be contemplated. Similarly, the products with higher DH values were generally lighter in color as reflected in their Hunter L values.

The treatment of SPH with charcoal for a period of 30 min at 50 °C and pH 4.0 allowed the removal of all colored impurities and compounds responsible for off-flavor in the product. Therefore, Hunter L and a values changed from 74.2 and 1.1 to 84.6 and −1.0, respectively, upon charcoal decolorization. Nonetheless, results in Table 4 indicate that products were nearly colorless even without any charcoal treatment.

The chemical composition of a typical SPH prepared by Alcalase-assisted hydrolysis is given in Table 4. The total nitrogen content in the product, on a dry basis, remained essentially constant after hydrolysis of MSSM. Removal of lipid and heme residues from the resultant product might have been compensated by the presence of salt in it. Furthermore, addition of a proton and a hydroxyl anion to proteins during the hydrolysis process may affect the results by approximately 3%. Decolorization with charcoal did not influence the total nitrogen content of the product (Table 4). Therefore, compounds responsible for off-flavor and off-color which were removed from the hydrolyzed products by charcoal treatment did not contain any appreciable nitrogen. The amount of minerals in SPH, on a dry weight basis, was

Table 3. Influence of Degree of Hydrolysis on Hunter Color Parameters of Hydrolysates Prepared from MSSM Using Alcalase[a]

Hunter color parameters	degree of hydrolysis			
	9.6%	12.1%	15.0%	19.0%
L	73.3 ± 0.2^a	77.1 ± 0.3^b	77.7 ± 0.1^c	74.2 ± 0.1^d
a	2.7 ± 0.1^a	2.0 ± 0.1^b	1.1 ± 0.1^c	1.1 ± 0.1^c
b	15.8 ± 0.4^a	14.4 ± 0.2^b	11.9 ± 0.7^c	13.3 ± 0.3^d
hue	80.4 ± 0.3^a	82.2 ± 0.3^b	84.6 ± 0.2^c	85.2 ± 0.2^d
chroma	16.0 ± 0.4^a	14.6 ± 0.2^b	12.0 ± 0.6^c	13.4 ± 0.3^d

[a] Results are mean values of four color measurements ± standard deviation. Values in each row with the same superscript are not significantly ($p > 0.05$) different from one another.

20.02–20.67% which is considerably higher than 6.75% found in MSSM. However, the main source of minerals in MSSM is small particles of bones. The mineral fraction in the hydrolysate is composed mostly of sodium chloride produced during the neutralization of the excess NaOH used for adjusting the pH during hydrolysis and enzyme inactivation.

The amino acid composition of SPH was similar to that of the original MSSM (Table V). The hydrolysate contained somewhat higher amounts of glycine and lysine and smaller amounts of isoleucine, phenylalanine, threonine, and valine than those present in the starting material. These differences may possibly originate from existing differences between the unhydrolyzed protein residues and the solubilized hydrolysates and the original meat. Nonetheless, the results given in Table 5 show that the product is well balanced in its content

Table 4. Proximate Composition and Hunter Color Parameters of Hydrolyzed MSSM at DH = 19% Using Alcalase[a]

specification	MSSM	hydrolysate	
		unbleached	charcoal bleached
moisture (%)	70.84 ± 0.11[a]	6.15 ± 0.05[b]	6.07 ± 0.11[b]
nitrogen (%, db)[b]	12.72 ± 0.30[a]	12.47 ± 0.23[b]	12.37 ± 0.49[b]
minerals (%, db)	6.75 ± 0.07[a]	20.02 ± 0.10[b]	20.67 ± 0.05[c]
lipids (%, db)	12.65 ± 0.08[a]	0.78 ± 0.05[b]	0.74 ± 0.02[c]
Hunter color parameters			
L	16.6 ± 0.2[a]	74.2 ± 0.1[b]	84.6 ± 0.1[c]
a	4.3 ± 0.3[a]	1.1 ± 0.1[b]	−1.0 ± 0.0[c]
b	2.1 ± 0.2[a]	13.3 ± 0.3[b]	11.0 ± 0.2[c]
hue	26.1 ± 2.3[a]	85.2 ± 0.2[b]	−84.8 ± 0.1[c]
chroma	4.8 ± 0.3[a]	13.4 ± 0.3[b]	11.1 ± 0.1[c]

[a] Results are mean values of four replicates ± standard deviation. Values in each row with the same superscript are not significantly ($p > 0.05$) different from one another. [b] db, on a dry, moisture-free basis.

Table 5. Amino Acid Composition (Percent) of Seal Protein Hydrolysate (SPH) at DH = 19% As Compared with MSSM[a]

amino acid	MSSM	SPH	
		Alcalase	Neutrase
alanine	5.88 ± 0.09	5.80 ± 0.06	6.28 ± 0.34
arginine	6.21 ± 0.08	6.02 ± 0.01	6.50 ± 0.20
aspartic acid	8.23 ± 0.02	8.90 ± 0.01	8.15 ± 0.32
cysteine	0.87 ± 0.01	1.01 ± 0.01	0.50 ± 0.16
glutamic acid	11.46 ± 0.39	12.53 ± 0.05	11.85 ± 0.38
glycine	4.47 ± 0.05	5.58 ± 0.01	6.02 ± 0.17
histidine	5.01 ± 0.06	5.25 ± 0.06	5.82 ± 0.28
hydroxylysine	0.04 ± 0.01	0.10 ± 0.02	0.20 ± 0.05
hydroxyproline	0.75 ± 0.01	0.85 ± 0.02	1.65 ± 0.04
isoleucine	4.58 ± 0.04	3.92 ± 0.10	3.50 ± 0.11
leucine	7.44 ± 0.03	8.50 ± 0.13	5.44 ± 0.35
lysine	8.72 ± 0.06	9.44 ± 0.03	9.83 ± 0.30
methionine	1.64 ± 0.08	1.62 ± 0.03	0.82 ± 0.26
phenylalanine	4.57 ± 0.05	3.96 ± 0.06	2.91 ± 0.10
proline	3.89 ± 0.04	3.86 ± 0.01	4.40 ± 0.18
serine	3.98 ± 0.02	3.60 ± 0.01	3.52 ± 0.33
threonine	4.53 ± 0.06	3.87 ± 0.04	3.82 ± 0.29
tryptophan	1.20 ± 0.01	1.19 ± 0.02	1.08 ± 0.01
tyrosine	2.85 ± 0.01	2.46 ± 0.02	1.83 ± 0.06
valine	5.80 ± 0.07	4.62 ± 0.09	4.42 ± 0.20

[a] Results are mean values of three replicates ± standard deviation.

Table 6. Effect of pH on Solubility of Seal Protein Hydrolysate[a]

pH	NSI[b] (%)	pH	NSI[b] (%)
2.50	93.45 ± 0.55	7.20	97.21 ± 0.11
4.00	94.43 ± 0.15	7.40	98.05 ± 0.01
5.50	95.96 ± 0.01	9.00	97.91 ± 0.05
6.40	96.93 ± 0.00	10.40	98.05 ± 0.12

[a] Results are mean values of three replicates ± standard deviation. [b] NSI, nitrogen solubility index.

of essential amino acids. The NSI of seal protein hydrolysate determined in the pH range 2.5–10.5 attained a minimum value of 93.45% at pH 2.5 (Table 6). Therefore, the product had good solubility characteristics over a wide pH range.

In conclusion, the optimum hydrolysis yield of 92.75% was reached for MSSM proteins using Alcalase at 60 AU/kg of proteins at 55 °C. The optimum reaction time was 50 min (Figure 2A), which gave a DH of 19.5%. Use of Neutrase in the process gave lower product yields. The SPH obtained under optimum reaction conditions had a near-bland taste, an off-white color, and a solubility in water of >93%. Potential use of SPH in a variety of foods and processing applications is being examined.

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