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Production of Cod (*Gadus morhua*) Muscle Hydrolysates. Influence of Combinations of Commercial Enzyme Preparations on Hydrolysate Peptide Size Range

LAURA GILMARTIN* AND LES JERVIS

Department of Biological Sciences, University of Plymouth, Plymouth PL4 8AA, United Kingdom

The complement of enzyme activities of a selection of commercial protease preparations were determined using fluorogenic substrates. Alcalase was used in combination with other commercial enzyme preparations to produce cod muscle (*Gadus morhua*) hydrolysates. Each muscle hydrolysate was characterized with respect to the percentage degree of hydrolysis (DH %), peptide molecular weight range, and free amino acid content. The enzyme preparations containing predominantly protease or endopeptidase activities achieved high DH % and produced significant amounts of peptides below a molecular weight of 3000. Alcalase combined with exopeptidase-rich preparations produced hydrolysates rich in low-molecular-weight peptides. Selecting combinations of enzyme preparations with complementary activity profiles could be used to manipulate the peptide molecular weight profile of hydrolysates.

KEYWORDS: Atlantic cod; *Gadus morhua*; enzymatic hydrolysis; Alcalase; Flavourzyme

INTRODUCTION

Stabilization of total world fisheries production figures, coupled with a projected growth in demand for fish, and environmental concerns have led to an increased interest in the bio-processing of fish waste (*1*−*3*). Fish processing waste is a rich source of under-utilized protein, where up to 75% (by weight) of round fillet may be discarded during a filleting process. When Shahidi et al. (*2*) compared cod (*Gadus morhua*) fillet and offal protein content, they were found to be 14.3% and 17.8%, of wet weight, respectively.

Traditional methods of fish hydrolysate production, such as fish ensilage, exploit the endogenous enzymes of the viscera to produce hydrolysates of a desired composition (*4*, *5*). Although endogenous fish enzymes possess some favorable properties, there are inherent problems associated with their application. The nature and levels of activities of enzymes depend on the physiological condition of fish, pH, temperature, and acids added during the silage process (*6*). In addition, the process can take days to months to complete; there are residual organic acids and potential spoilage of raw material. Free amino acids may contribute to a lower nutritional value, and degradation of essential amino acids such as tryptophan and histidine may take place.

Exogenous proteases have been employed successfully in the production of protein hydrolysates from sources such as whey, rapeseed, and sunflower protein (*7*−*9*). A number of applications of commercial enzymes in fish hydrolysate production have been described in the literature. Imm and Lee (*10*) compared the abilities of Savorase and Flavourzyme to produce seafood flavor from Red Hake (*Urophycis chuss*). Research of the nutritive role of fish hydrolysates has already found them to possess high protein efficiency ratios and biological values (*11*), and in some instances nutritional advantages over fishmeal. Substituting di- and tripeptides for fish meal protein in sea bass (*Dicentrarchus labrax*) larval diet improved development and enhanced survival rates (*12*, *13*).

The use of exogenous enzymes has significantly increased protein recovery when compared to autolysis. Shahidi et al. (*14*) reported protein recovery to be 22.9% for a capelin (*Mallotus villosus*) hydrolysate produced using endogenous enzymes, and a hydrolysate produced using Alcalase allowed a 70.6% recovery. In addition to the increased efficiency, their use offers the possibility of selecting activities to produce hydrolysates with specific properties. The choice of proteases and peptidases can influence the degree of hydrolysis of a substrate, and the type and abundance of peptides and amino acids in the resulting hydrolysate. Tuna waste treated with Alcalase produced peptides in the range of 6500 Da to dipeptides and amino acids, and proved to be a suitable nitrogenous source in microbial growth media (*15*). The 3000−500 Da peptide fraction of cod muscle hydrolyzed with Neutrase had an immunostimulatory response on Atlantic salmon *Salmo salar* L. head kidney leucocytes (*16*).

Several of these biological properties have been attributed to low-molecular-weight peptides. Producing these low-molecular-weight peptones has typically required a combination of commercial enzyme preparations. Angiotensin converting enzyme (ACE) inhibitory peptides, in the range 1.9−0.9 kDa, were produced from Alaska Pollack (*Theragra chalcogramma*) skin

* To whom correspondence should be addressed at present address: Buck Institute, 8001 Redwood Blvd., Novato, CA 94945. E-mail: lgilmartin@buckinstitute.org. Tel: 00 1 415 209-2081. Fax: 00 1 415 209-2231.

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Table 1. Principal Activities Associated with Commercial Enzyme Preparations As Outlined by Manufacturers

enzyme	EC no.	temperature range °C	pH range	source	specificity
Alcalase 2.4 L FG	3.4.21.62	55–70	6.5–8.5	*Bacillus licheniformis*	endoprotease
Neutrase 0.5 L	3.4.24.28	45–55	5.5–7.5	*Bacillus subtilis*	endoprotease
Protamex	3.4.21.62	35–60	5.5–7.5		protease complex
alkaline protease concentrate	3.4.21.14	60	9–10	*Bacillus licheniformis*	endopeptidase
bromelain concentrate		50–60 at pH 5.0	3–9	pineapple stem	proteolytic
papain concentrate		65–80	5–7	*Carica papaya* fruit	
Corolase 7089	3.4.24.4	< 60	5–7.5	*Bacillus subtilis*	endopeptidase
Validase TSP Concentrate II	3.4.21.14	45–55	6.5–8	*Bacillus subtilis*	endopeptidase
Corolase N	3.4.24.4	< 60	5–7.5 at 30 °C	*Bacillus subtilis*	endoprotease
Corolase PN-L	3.4.23.6	<50	5–8	*Aspergillus sojae*	endo and exopeptidase
Corolase LAP	3.4.23.18	< 70	6–9	*Aspergillus sojae*	exopeptidase
Flavourzyme 1000 L	3.4.11.1		7.0	*Aspergillus oryzae*	endoprotease/exopeptidase

using, in a sequential manner, Alcalase, Pronase E, and collagenase (*17*). Treatment of cod frame protein with tuna pyloric caeca extract followed by papain or Pronase was found to improve yields of ACE inhibitory peptide activity (*18*).

Authors recognizing the influence of choice of commercial enzymes on the functionality of their products have undertaken extensive investigations of their properties (*7*, *19*). This study set out to examine the effectiveness of combinations of commercial protease and exopeptidase preparations to produce hydrolysates rich in low-molecular-weight peptides.

The commercial enzyme preparations of this study varied in their origins, principal enzyme activities, pH optima, and temperature optima (**Table 1**). The nature of the activities present in the preparations was investigated with the aid of fluorometrically labeled substrates. Among the substrates chosen were Suc-Leu-Leu-Val-Tyr-AMC, BZ-Arg-AMC, and Suc-Ala-Ala-Ala-pNA, which are principally hydrolyzed by chymotrpsin, trypsin and elastase-like activities, respectively. A selection of substrates to detect peptidase activity such as Arg-AMC and Gly-AMC and dipeptidase activity, such as Gly-Arg-AMC, were also employed. These substrates would aid in the determination of the variety of activities present in the preparation, but did not constitute an exhaustive survey of all the protease and peptidase activities present in each preparation. It was hoped the results of this portion of the study might serve as a useful indicator of the molecular weight range of peptides and amounts of amino acids likely to be produced by a specific preparation or combination of preparations.

The free amino acid concentration of hydrolysates was estimated in each case. Extensively hydrolyzed protein could contain a high percentage of free amino acids. Cod offal hydrolyzed by Alcalase followed by Pancreatine produced 40−45% free amino acids (*11*). The free amino acid content of a hydrolysate can affect the functionality of the product. Hydrolysates produced as nutritional supplements with a high free amino acid composition have been demonstrated to have a low biological value. They are absorbed at a lower rate in the digestive tract, as the osmotic pressure of free amino acids is higher than that of peptides (*9*, *12*, *20*). In addition, the relative abundance of individual amino acids can impart flavor (*17*).

The hydrolysates of this study were evaluated on the basis of degree of hydrolysis (DH), peptide molecular weight distribution, and free amino acid concentration. By combining this information it was hoped to select an appropriate combination of enzymes to maximize the quantity and diversity of medium and small molecular weight peptides produced in a cod muscle hydrolysate.

MATERIALS AND METHODS

Enzymes. Commercial enzyme preparations were received as gifts from manufacturers. Validase FP 60, Validase TSP Concentrate II, and Alkaline Protease Concentrate were gifts from Valley Research Inc., 1145 Northside Blvd., South Bend, IN 46615-3920. Corolase LAP, Corolase N, Corolase PN-L, and Corolase 7089 were gifts from Röhm enzyme GMBH, Kirschenallee 45, D-64293 Darmstadt, Germany. Alcalase, Neutrase, and Flavourzyme were gifts from Novo Nordisk A/S, Novo Allé, DK-2880 Bagsvaerd.

Substrates. Aminomethylcoumarin (AMC) and *p*-nitroanilide (pNA) labeled substrates were obtained from Bachem Feinchemikalien, Bubendorf, Switzerland. Superdex HR peptide 10/30 column was obtained from Pharmacia Fine Chemicals A. B., Uppsala, Sweden.

Hydrolysis of Cod Muscle. Fresh, gutted, Atlantic cod was headed, and filleted by hand. The fillets were then heated to 95 °C in a microwave, held at 95° for 30 min in a waterbath with occasional manual agitation, and cooled and stored at −20 °C until required. A sample of this muscle (50 g) was suspended in 450 mL of water with constant agitation. The reaction was started by addition of the appropriate concentration of enzyme relative to substrate under conditions outlined in **Tables 2** and **3**. Conditions were controlled and monitored using a bioreactor (Adaptive Biosystems Ltd.) over 2 h with constant agitation. The pH was adjusted using 0.4 N NaOH. The reaction was terminated by placing the samples in a water bath at 95 °C and holding at this temperature for 30 min with occasional agitation. The pH was then adjusted to 7.0 with 6 N HCl. Hydrolysates were cooled, freeze-dried, or stored at −20 °C until analysis.

Calculation of DH %. The degree of hydrolysis (DH %) was estimated using the Adler−Nissen method (*21*). This is the percent of peptide bonds cleaved (*h*) relative to the total number of bonds per unit weight (h_{tot}) (7.51 mequiv/g). Using this method the amount of base consumed (*B*) (mL) to maintain a pH is proportional to the hydrolysis equivalents *h* (mequiv g^{-1} of protein) at near neutral or alkaline conditions.

$$\text{DH \%} = \frac{h}{h_{\text{tot}}} \times 100$$

$$h = B \times N_{\text{b}} \times \alpha^{-1}(\text{MP}^{-1})$$

$$\alpha = \frac{10^{(\text{pH} - \text{p}K)}}{1 + 10^{(\text{pH} - \text{p}K)}}$$

$$\text{p}K = 7.8 + \frac{(298 - T)}{298 \times T} \times 2400$$

where N_b = normality of alkali, *M*p = mass of protein in grams (% N × 6.25),α = average degree of dissociation of α − NH_2, and *T* = temperature in Kelvin.

Table 2. Synthetic Substrates Hydrolyzed by Commercial Enzyme Preparations[a]

	Alcalase 2.4 L	Neutrase 0.5 L	Protamex	alkaline protease concentrate	bromelain concentrate	papain concentrate	Corolase 7089	Validase TSP Concentrate II	Validase FP 60	Corolase N	Corolase PN-L	Corolase LAP	Flavourzyme 1000 L
Suc-Leu-Leu-Val-Tyr-AMC	4370	47.99	942.9	1373	118.7	315.7	786.28	16547	0	3597	345.6	142.7	326.8
Suc-Ala-Ala-Ala-pNA	259.5	0	179.9	259.4	5.47	134.46	1.45	35.28	143	1.59	159.3	0	40.06
BZ-Pro-Phe-pNA	1.29	0	0.59	0.43	0.15	0.71	0.53	0.57	731.46	1.21	1.64	0	1.78
Bz-Arg-AMC	1.79	0	0	0	16.89	112.2	0	0	55.93	0	0	0	1.1
Gly-Pro-AMC	0	0	0	0	0.17	0	0	0	0	0	393.39	4.24	7.05
Leu-AMC	69.44	12.01	6.94	32.23	37.33	6.53	23.7	3.65	0	39	730.31	2397	9268
Arg-AMC	4.69	1.29	2.36	8.85	3.12	1.1	10.6	1.06	0.65	12.66	58.43	1259	326.8
Ala-AMC	1.85	0	0	0	1.32	0	0	0	0	0	1.72	36.39	13.51
Gly-AMC	0	0	0	0	0	0	0	0.67	1.29	0	0	0	1.17
Pro-AMC	0	0	0	0	0	0	0	0	0	0	0	4.2	8.64
Asp-AMC	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] Activity expressed as nmol of AMC or μmol of PNA released per min per mg of protein in 0.1 M Tris, pH 7.5, at 40 °C.

Table 3. Degree of Hydrolysis of Cod Muscle Achieved Using Commercial Enzyme Preparations Containing Endoprotease and Endopeptidase Activities under Conditions Suggested by Suppliers (**Table 1**)

enzyme preparation	concentration %	temp °C	pH	DH %
Alcalase	1	40	8	16.33
Neutrase	5	45	7.5	12.62
Protamex	1	40	6.5	ND
alkaline protease concentrate	1	50	7.8	18.99
bromelain	0.1	55	7.7	10.78
papain	0.1	65	7.7	19.93
Corolase 7089	1	45	7.5	9.86
Validase TSP Concentrate II	0.5	50	7.5	14.69
Validase FP 60	0.2	50	3	ND

[a] ND, not done.

Preparation of Commercial Protease Samples. Enzyme preparations were prepared by dilution in distilled water to give 0.01% (w/v) solutions. Aliquots were removed, diluted as necessary, and assayed for protease and peptidase activity using pNA and AMC labeled substrates. The protein concentration of each preparation was determined using the Hartree−Lowry method (*22*). Absorbance was read at 680 nm in a Perkin-Elmer Lambda Bio 20 spectrophotometer.

Determination of Hydrolysis of AMC Labeled Substrates. The enzyme preparation (50 μL) was added to 150 μL of water and 200 μL of 0.1 M Tris−HCl, pH 7.5. The assay mixture was preincubated at 40 °C for 5 min. The reaction was started by the addition of 100 μL of the appropriate substrate in water to give a final concentration of 0.1 mM. After 15 min, the reaction was terminated by the addition of 1 mL of 1.5 M acetic acid. The liberated 7-amino-4-methylcoumarin was determined fluorometrically (Perkin-Elmer LS 50 B) using excitation and emission wavelengths of 370 and 440 nm, respectively, and quantified using a standard curve in the rangeof 0−15 nmol. Results were expressed as nmol AMC produced/mg protein.

Determination of Hydrolysis of pNA Labeled Substrates. Enzyme preparation (50 μL) was added to 150 μL of water and 200 μL of 0.1 M Tris−HCl, pH 7.5. The assay mixture was preincubated at 40 °C for 5 min. The reaction was started by the addition of 100 μL of the appropriate substrate in water to give a final concentration of 4 mM in the case of Suc-Ala-Ala-Ala-pNA and 1 mM in the case of BZ-Pro-Phe-pNA. After 15 min, the reaction was terminated by the addition of 1 mL of 1.5 M acetic acid. The resulting absorbance was measured at 410 nm. Blanks were prepared by substituting the appropriate buffer for sample in each case. The liberated pNA was quantified using a standard curve constructed using pNA in the concentration range 0 to 100 μM. Results were expressed as μmol pNA produced/mg protein.

Measurement of Free Amino Acid Content. Free amino acid concentration was determined by treating 500 μL of an appropriate dilution of enzyme preparation with 500 μL of 20% trichloroacetic acid. Samples were centrifuged at 5000*g* for 15 min. The resulting supernatant was then assayed for free amino acids by a modification of the Doi method (*23*). Samples of hydrolysate (100 μL) were incubated at 85 °C for 5 min with 700 μL of ninhydrin reagent (0.8 g of ninhydrin in 80 mL of ethanol and 10 mL of acetic acid to which 1 g of $CdCl_2$, dissolved in 1 mL of water, was added). Samples were then cooled and 100 μL of distilled water was added to each; then the samples were centrifuged at 5000*g* for 5 min and their absorbance was measured at 504 nm in a spectrophotometer. Blanks were prepared by substituting sample with distilled water. A standard curve was prepared using 0−60 nmol of arginine.

SDS−PAGE Electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) was performed as described by Schagger and von Jagow (*24*). Separating gel was prepared with 48% acrylamide and 1.5% bisacrylamide. Stacking gel was prepared with 46.5% acrylamide and 3.0% bisacrylamide. The molecular weight of the protein band was calculated by reference to the migration of SDS−PAGE molecular weight standards (Novex Electrophoresis, GmbH, Germany).

Fractionation of Peptides on Superdex HR 10/30. The relative molecular mass range of samples was estimated by gel filtration on a calibrated Superdex HR peptide 10/30 column with a fractionation range of 7000−100 Da. Samples were eluted in 30% acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 0.25 mL/min. Absorbance was measured at 280 nm continuously by a UV-1 monitor, Pharmacia, Sweden. The column was calibrated with apoprotinin 6500 Da, insulin chain B 3495 Da, bradykinin 1060 Da, and tyrosine 181 Da. The void volume (V_o) was determined using cytochrome C 12 400 Da.

RESULTS AND DISCUSSION

With the aid of fluorogenic and chromogenic substrates, the broad specificity of each enzyme preparation was investigated at 40 °C, pH 7.5, over 2 h. A profile of the types of activities present in each of each enzyme preparations was obtained (**Table 2**). By selecting a temperature at the lower end of the manufacturers indicated range of temperatures and a pH at the midpoint of the suggested range it was hoped to detect the broadest range of activities. Under varying pH and temperature conditions, different levels of enzyme activity would most likely be detected. Smyth and Fitzgerald (*19*) recorded significant differences in rates of hydrolysis when they assayed protease activity in a number of commercial preparations using a range of substrates, at both pH 7 and 8, and at temperatures of 37 and 50 °C.

Subtilisin type activity, detected by hydrolysis of Suc-Ala-Ala-Ala-pNA, was recorded at significant levels in Alcalase, Protamex, papain, alkaline protease concentrate, and Corolase PNL preparations. These preparations are reported by manufacturers to contain protease and/or endopeptidase activities. Among these protease preparations, Neutrase alone did not hydrolyze this substrate. Chymotrypsin-like activity, detected

as hydrolysis of the flourometrically labeled substrate Suc-Leu-Leu-Val-Tyr-AMC, was present at significant levels in all preparations, with Alcalase and Validase TSP Concentrate hydrolyzing at 4370 and 16 547 nmol/min/mg protein, respectively. Exopeptidase-type activities exhibited by these endoprotease/peptidase enzyme preparations, such as Corolase N and Neutrase, were in general limited to hydrolysis of Leu-AMC and Arg-AMC.

As expected, the exopeptidase rich preparations (Corolase PNL, Corolase LAP, and Flavourzyme) were found to hydrolyze Leu-AMC and Arg-AMC at a greater rate than any of the protease and endopeptidase preparations. Flavourzyme, under the conditions of this assay, also hydrolyzed the chymotrypsin substrate Suc-Leu-Leu-Val-Tyr-AMC, Suc-Ala-Ala-Ala-*p*NA, and the dipeptidase substrates of Gly-Arg-AMC and Gly-Pro-AMC. Hydrolysis of these substrates may be attributable to the combined action of endopeptidase and exopeptidase activities.

However, from the data presented here, it is apparent that the protease and endopeptidase preparations had limited activity against di- and aminopeptidase substrates. To produce a variety of peptides in the low-molecular-weight range these protease preparations would be aided by supplementation with an exopeptidase rich preparation. This approach to improving the functionality and yield of biologically active peptides from hydrolysates has been successfully employed in a number of studies. Rapeseed protein was sequentially hydrolyzed by Alcalase and Flavourzyme to increase solubility (*8*). The combined action of Flavourzyme and Savorase have been used to increase the amounts and types of free amino acids in the production of seafood flavor from red hake *(Urophycis chuss*) (*10*). Pepsin and trypsin increased the yield of ACE inhibitory peptides, opioid peptides, and immunostimulating peptides in a hydrolysate, of UHT bovine milk, fermented by *Lactobacillus* GG (*25*).

To determine the DH % achieved by each of the protease preparations cod muscle was heat treated to inactivate endogenous enzymes before hydrolysis. The synergistic action of endogenous enzymes and exogenous activities could have resulted in erroneously elevated DH % values. A 4% DH difference after 6 h between heat-treated and untreated red hake *(Urophycis chuss*) frame mince, hydrolyzed by endogenous enzymes, was reported by Imm and Lee (*10*). Heat treatment prior to hydrolysis has been employed in other studies (*26*−*28*). The DH % achieved by those preparations containing protease and/or endopeptidase activities are presented in **Table 3**. Among the proteolytic preparations papain achieved the highest degree of hydrolysis (19.93%) followed by alkaline protease concentrate (19.9%) and then Alcalase (16.33%). The lowest DH % recorded were for the preparations Neutrase (12.6%) and Corolase 7089 (9.9%). Both these preparations were found to lack significant Suc-Ala-Ala-Ala-pNA activity (**Table 2**). The DH % values achieved with this heat-treated substrate are comparable to those reported for raw substrates. A DH % of 17.9% was reported for Alcalase hydrolyzed dogfish muscle at 3% enzyme-to-substrate ratio, pH 8.0, and 50 °C (*29*).

Alcalase has been employed in previous fish hydrolysis studies because of the high degrees of hydrolysis that can be achieved in a relatively short time under moderate conditions (*29*, *30*). In this study, Alcalase was found to possess broad specificity in addition to achieving a high DH %. Consequently, it was selected as the protease component of a protease−peptidase combination of activities to extensively hydrolyze cod muscle. In addition, Alcalase activity has been optimized for a number of fish substrates. Optimum activity has been reported in the pH range 7.0−9.0, at 50−60 °C and an enzyme-to-substrate ratio of 0.2−3.7% (*29*−*31*). Ideally, a peptidase preparation chosen to act in combination with Alcalase would be active under similar hydrolysis conditions. For this reason the exopeptidase type activities of this study were assayed at pH 8.3 and 53 °C and enzyme concentration of 3.3% (v/v). The DH % values obtained under these conditions were in the range 2.71−4.79% (**Table 4**). When these enzymes were combined with Alcalase, DH % values were in the range 32.7−39.9% (**Table 5**). The greatest DH % achieved was in the presence of Alcalase and Flavourzyme (39.9%), while the combination of Alcalase and Validase TSP achieved the smallest increase in DH %, when compared to the DH % achieved by Alcalase alone. These enzyme preparations shared similar activity profiles (**Table 2**).

Table 4. Degree of Hydrolysis of Cod Muscle Achieved Using Commercial Enzyme Preparations under Conditions Optimal for Alcalase Activity (3.3% Relative to Substrate) at 53 °C, pH 8.3 for 2 h

enzyme preparation	concentration %	temp °C	pH	DH %
Corolase N	0.5	53	8.3	4.48
Corolase PNL	0.5	53	8.3	4.79
Corolase LAP	0.25	53	8.3	4.23
Flavourzyme	1	53	8.3	2.71

Table 5. Degree of Hydrolysis of Cod Muscle Obtained by Enzyme Preparations in the Presence of Alcalase (3.3% Relative to Substrate) at 53 °C, pH 8.3 for 2 h

enzyme	enzyme concentration (%)	DH %
Alcalase alone	3.3	32.59
Protamex	1	32.66
alkaline protease concentrate	1	32.46
Corolase 7089	0.5	35.02
Validase TSP	0.1	35.19
Validase FP 60	0.1	34.88
Corolase N	0.5	34.40
Corolase PNL	0.5	35.77
Corolase LAP	0.5	37.27
Flavourzyme	1	39.86

The peptide molecular weight distributions of hydrolysates were studied using SDS−PAGE and size exclusion chromatography. Using SDS−PAGE it was possible to visualize the molecular weight range of peptide fragments over the range 100 000−1000 (**Figure 1**). Hydrolysates prepared with the preparations Corolase N, Corolase PNL, Protamex, Corolase 7089, and alkaline protease all contained proteins in the range 17 000−4000. Validase FP 60 hydrolysate was found to possess peptides that stretched over the broadest molecular weight range of 208 000−4000. The hydrolysate produced with the aid of the Flavourzyme, a preparation that achieved a DH of 2.71%, possessed proteins detected in the range 53 000−4000. Despite the modest increases in DH %, when Alcalase was combined with any of the exopeptidase preparations (**Table 5**), significant alteration of the peptide molecular weight profile was recorded. SDS−PAGE analysis of hydrolysates produced by combining Alcalase with each of the protease preparations of this study revealed peptides of less than 13 000 dominated each hydrolysate. The most marked reduction in molecular weight of detected peptide was in the case of Flavourzyme (**Figure 1**, lanes 18 and 19).

Fractionation of cod muscle hydrolysates on a Superdex HR peptide 10/30 column provided a more detailed view of the effect of combinations of enzyme preparations on peptides in

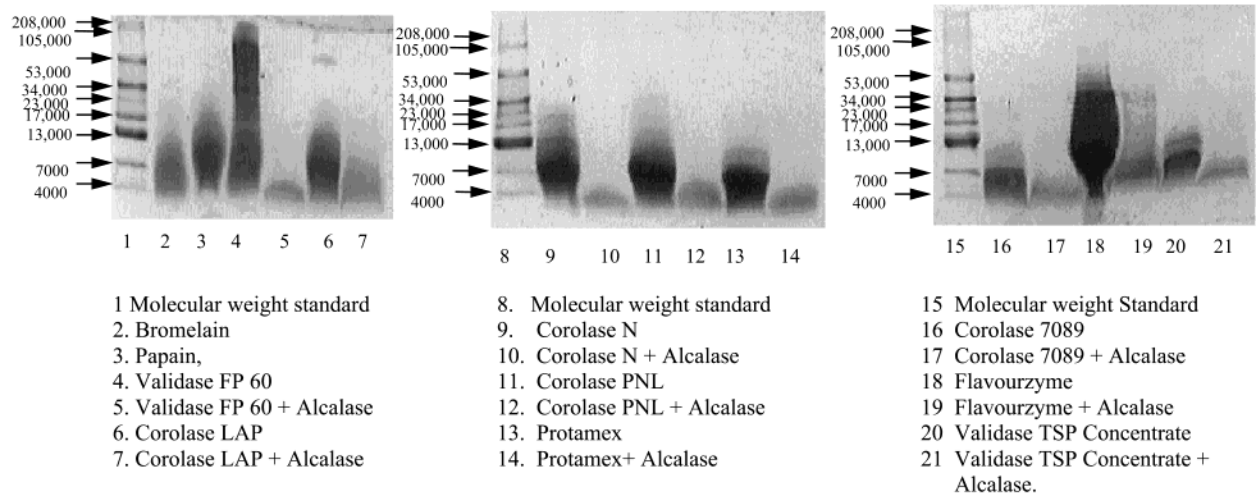

Figure 1. Coomassie blue stained SDS–polyacrylamide gel of hydrolysates of cod muscle (sample loaded 10 μL). Lanes 1, 8, and 15: Low molecular mass standards. Myosin 208 000 DA, phosphorylase B 105 000 Da, glutamic dehydrogenase 53 000 Da, carbonic anhydrase 34 000 Da, myoglobin 23 000 Da, myoglobin 17 000 Da, lysozyme 13 000 Da, aprotinin 6000 Da, and insulin 4000 Da.

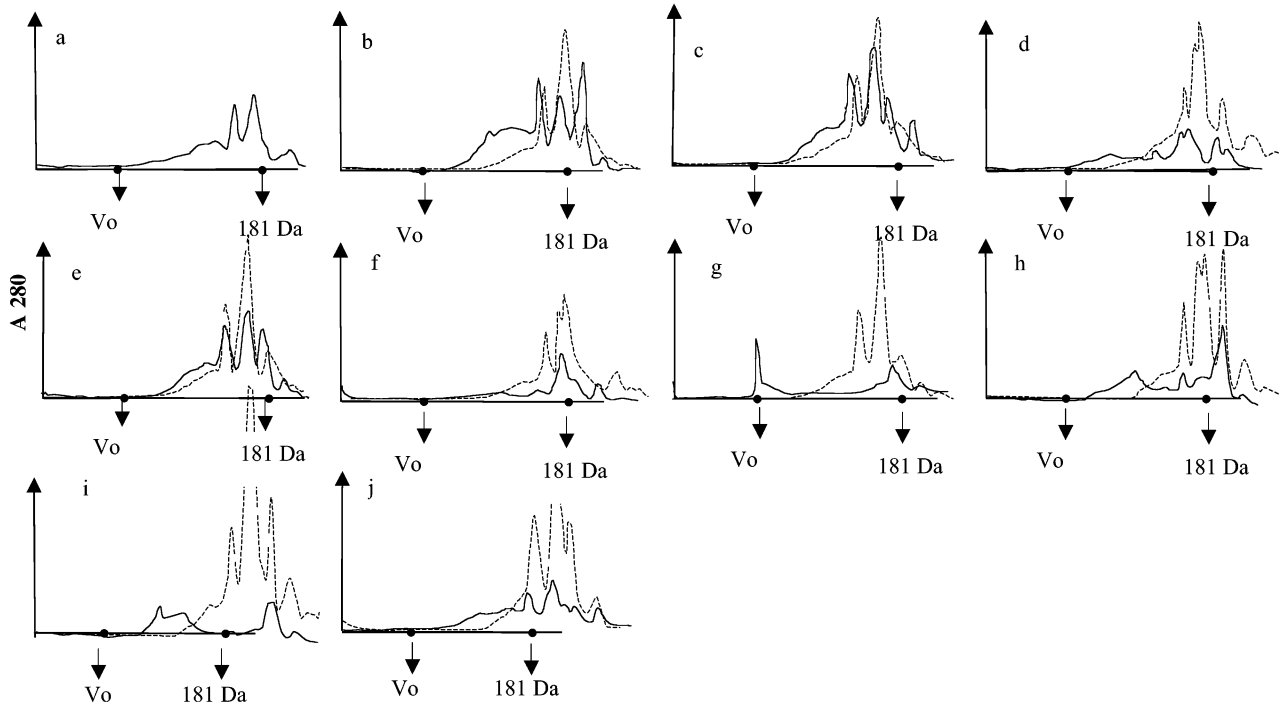

Figure 2. Elution of 50 μL of hydrolysate, prepared as described in **Table 1** from a Superdex HR peptide 10/30 column, eluted in 30% acetonitrile containing 1% triflouroacetic acid, at a flow rate of 0.25 mL/min. Peptides detected at A280. Hydrolysates prepared using enzyme preparation alone (——) and enzyme preparation with Alcalase (-------). Enzyme preparations: (**a**) Alcalase 2.4 L; (**b**) Protamex; (**c**) alkaline protease concentrate; (**d**) Corolase 7089; (**e**) Validase TSP II; (**f**) Validase FP 60; (**g**) Corolase N; (**h**) Corolase PNL; (**i**) Corolase LAP; (**j**) Flavourzyme.

the fractionation range of 7000 to 100 Da. Detection was by measuring eluate absorbance at 280 nm (**Figure 2**). Some peaks were detected after the total column volume and were found to coelute with tryptophan standard. An investigation of this phenomenon under the same conditions of this study was reported by Tossavainen et al (*32*). They suggested that smaller peptides and amino acids interacted with the column matrix. As suggested by the DH % data (**Table 3**), hydrolysates prepared using the enzyme preparations Validase TSP II or alkaline protease concentrate appeared to produce the greatest amounts of peptides over the broadest molecular mass range (**Figure 2**, panels e and c). Treatment of cod muscle with Flavourzyme and Corolase LAP resulted in hydrolysates with low amounts of peptide detected in this fractionation range (**Figure 2**, panels i and j). Combining Alcalase with each enzyme preparation of this study increased the detected material eluting below 200 Da in every case, but the hydrolysates produced using Corolase LAP or Flavourzyme displayed the greatest alteration of detected peak sizes at the lower end of the fractionation range (**Figure 2**, panels i and j).

Combinations of endo- and exoproteases have been reported to result in a significant proportion of the protein being converted to amino acids, thus reducing the peptide yield. In the production of Pacific whiting (*Merluccius productus*) silage, 40% of the nitrogen was reported to be in the form of amino acids (*20*). In the case of cod offal hydrolyzed by Alcalase, 30% of the total nitrogen was reported to be in the form of free amino acids, and this increased to 47% after further hydrolysis with Pancre-

Table 6. Free Amino Acid Content of Hydrolysates Produced Using Enzyme Preparations Alone and in Combination with Alcalase under Conditions Outlined in **Tables 2** and **3**

enzyme preparation	amino acid concentration (μmol free amino acid/mL)	
	hydrolysate	hydrolysate produced in the presence of Alcalase
substrate alone	1.105	
Alcalase	35.89	
Protamex	46.96	101.6
alkaline protease concentrate	125.57	185.92
Corolase 7089	58.50	140.24
Validase TSP Concentrate II	90.9	117.36
Validase FP 60	13.41	129.84
Corolase N	13.38	120.08
Corolase PNL	34.87	108.24
Corolase LAP	11.54	298.64
Flavourzyme 1000 L	63.23	254.0

atin (*11*). It would be advantageous to minimize the free amino acid concentration for applications such as fish nutrition (*12*, *20*).

Moderate concentrations of free amino acids were found in hydrolysates prepared using the endopeptidase preparations Corolase PNL (34.87 μmol/mL), Corolase 7089 (58.5 μmol/mL), Validase TSP (90.9 μmol/mL), and Flavourzyme (35−90 μmol/mL) (**Table 6**). The low free amino acid levels detected in the hydrolysate prepared using Corolase LAP (11.54 μmol/mL) could be attributed to the mainly exopeptidase nature of its activities which were unable to hydrolyze the intact proteins in the absence of an endoprotease. In contrast, alkaline protease activity liberated the greatest concentration of amino acids (125.57 μM/mL). A relatively low concentration of amino acids was released by Validase FP 60 (13.41 μmol/mL). This is may be due to hydrolysis being carried out under conditions that were not optimal for any of the enzymes of that preparation (**Table 1**). In this study, free amino acid content increased in every instance where hydrolysis was carried out with a peptidase rich preparation in combination with Alcalase (**Table 6**). The largest increases were measured in the cases of Corolase LAP (287.1 μmol/mL) and Flavourzyme (190.77 μmol/mL) agreeing with the size exclusion chromatography data (**Figure 2**, panels i and j). In the absence of amino acid profile information, the relative contributions of the peptidase activities to the generation of free amino acids cannot be determined. A similar study performed by Mullally et al. (*7*) noted that the aminopeptidase activities of commercial enzyme preparations, detected using fluorogenic substrates, did not reflect the free amino acid profile of the hydrolysates.

Imm and Lee (*10*) reported that a more efficient hydrolysis and a greater DH % could be achieved with Flavourzyme by allowing the pH to drift. This suggests that initial hydrolysis by Alcalase activity under its optimum conditions followed by hydrolysis with Flavourzyme allowing the pH to drift down to its pH optimum of 7.0 would be more effective. When Vioque et al. (*8*) employed this approach to the hydrolysis of rapeseed protein, they obtained 60% DH.

The selection of two enzyme preparations based on enzyme activity, resulting hydrolysate peptide molecular weight range, and amino acid content allowed the production of a hydrolysate rich in low-molecular-weight peptides. The selection of combinations of commercial enzyme preparations on the basis of their activity profiles could be used to manipulate peptide molecular weight distributions and free amino acid content, and increase yields of specific types of biologically active peptides.

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