Available online at www.sciencedirect.com
SCIENCE DIRECT®
Food Chemistry 97 (2006) 50–55
Food Chemistry
www.elsevier.com/locate/foodchem

Partial purification and comparison of precipitation techniques of proteolytic enzymes from trout (*Salmo gairdnerii*) heads

Maria Michail, Maria Vasiliadou, Anastasios Zotos *

Technological Educational Institution of Thessaloniki, School of Food Technology and Nutrition, Department of Food Technology, 57400 Thessaloniki, P.O. Box 141, Greece

Received 20 December 2004; received in revised form 11 March 2005; accepted 11 March 2005

Abstract

Proteolytic enzymes have been detected and partially purified from trout (*Salmo gairdnerii*) heads, which were preserved at −20 °C. Proteolytic enzymes, either in crude extract or in partial purified samples, were stable for 15 days with an optimum temperature of 55 °C. Proteolytic activity was very high in either alkaline or acidic pH regions. A particular ratio of cold acetone to crude extract (1.25:1) was found to be best for the partial purification of proteases, with a 99% recovery, compared with the partial purifications achieved using different cold acetone ratios or ammonium sulphate. This recovery was also confirmed via measurement of the particles (particle size analyser) contained either in crude extract or in the precipitated samples. The existence of mainly Zn–serine and possibly some Zn-acidic proteases was observed.

Keywords: Trout (*Salmo gairdnerii*); Proteolytic enzymes; Partial purification; Acetone precipitation; Ammonium sulphate precipitation

1. Introduction

Proteases constitute the most important group of industrial enzymes used in the world today and have several applications in the food industry (El-Beltagy, El-Adaway, Rahma, & El-Bedawey, 2004). Fish is regarded to be one of the richest sources of proteolytic enzymes and fish processing generates large amounts of solid and liquid wastes, such as heads, tails, skin, bones and intestines (El-Beltagy et al., 2004; Hordur & Rasco, 2000; Regenstein & Regenstein, 1991; Shahidi & Kamil, 2002).

Utilization of these wastes is a major problem for fishermen and the fish industry. Their disposal has a major economic and environmental impact (Hordur & Rasco, 2000; Regenstein & Regenstein, 1991; Shahidi & Kamil, 2002).

Liver is the organ that has been mostly utilized in order to produce fish oil, while bones and intestines are used for the production of fish flour. In some cases, even the skin is used (Regenstein & Regenstein, 1991; Shahidi & Kamil, 2002).

A variety of digestive proteolytic enzymes has been isolated from the internal organs of fish and crustaceans. The fish proteases, found in the pyloric caeca as active forms, have also been purified from different species, such as cod, rainbow trout and salmon (Hernández-Santoyo, Hernández-Arana, Arreguín-Espinosa, & Rodríguez-Romero, 1998).

An intensive effort to utilize fish wastes has been made by isolating and purifying proteolytic enzymes and fatty acids, which can be used in the food or pharmaceutical industry. This effort leads to the isolation, purification and characterization of many proteolytic enzymes, such as serine proteases and many alkaline proteases (Regenstein & Regenstein, 1991).

* Corresponding author. Fax: +30 2310 791360.
E-mail address: zotos@food.teithe.gr (A. Zotos).

0308-8146/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2005.03.022

Thus, whereas the usage of the intestines has been successfully developed (mostly regarding the isolation and purification of proteolytic enzymes) utilization of the rest of wastes (e.g., heads, tails) is not proportionally developed (Regenstein & Regenstein, 1991; Shahidi & Kamil, 2002).

Thus, the aim of this research was to partially purify and identify proteolytic enzymes from the heads of trout (*Salmo gairdnerii)* and to evaluate the purification techniques used, an attempt that might be further used by the food and pharmaceutical industries.

2. Materials and methods

2.1. Rainbow trout heads

Farmed trout (*Salmo gairdnerii*) was purchased from Ioannina Greece. Immediately after arrival, fish samples were stored in ice at 0–1 °C. The average weight of the samples was 900 g to 1 kg. About 20 h after harvesting, they were eviscerated, head removed and stored at −20 °C.

2.2. Analytical reagents

Inhibitors: Iodoacetamide, phenyl-methyl-sulfonyl fluoride (PMSF), *p*-benzoquinone, silver nitrate, ethylene diamine tetraacetic acid (EDTA), copper sulfate, zinc sulfate and soybean trypsin inhibitor were purchased from Sigma Chemical Co. Ltd., Fancy Road, Poole, Dorset. All reagents used were of analytical grade.

2.3. Preparation of crude extract

Head samples were thawed for about 2 h at room temperature and 60 g of minced head were weighed on an analytical balance and homogenized with 600 ml of 0.1 M phosphate (Na_2HPO_4–NaH_2PO_4) buffer, pH 7.0, for 2 min. The homogenisation was performed in ice in order to avoid increase in temperature. The mixture was centrifuged at 12,100*g* for 30 min at 4 °C. The supernatant was collected and used as crude protease extract.

2.4. Assay of protease activity

The reaction mixture contained 2.5 ml of 0.5 % casein (except where otherwise stated) in 0.1 M phosphate buffer, pH 7.0, and 0.3 ml of the supernatant. The mixture was incubated in a water bath at 55 °C for 40 min. Then, 2.8 ml of 5% (w/v) trichloroacetic acid (TCA) was added to stop the reaction and to precipitate protein. The mixture was allowed to stand for 1 h at room temperature. The precipitate was removed by filtration through Whatman No. 1 filter paper. The absorbance of the supernatant was measured at 280 nm in a 1-cm silica cell. A blank was run by adding the enzyme solution after TCA was added.

HCl–glycine, Na_2HPO_4–NaH_2PO_4, NaOH–glycine and NaOH–Na_2HPO_4 (at final concentration of 0.1 M) buffers were used in the pH ranges of 2.4–3.6, 6.4–7.4, 8.6–10.6 and 11.0, respectively. The 40-min time and 55 °C temperature were chosen after incubation of the mixture at 20, 30, 40, 50 and 55 °C for 20, 25, 30, 35 and 40 min (Zotos & Taylor, 1996). One unit of protease is defined as the amount of enzyme that catalyses the release of 1 μmol of L- tyrosine per minute under the above assay conditions (Alam et al., 2005).

2.5. Acetone precipitation

Acetone, which was precooled to about −15 °C, was slowly added to the crude protease preparations until the ratio between enzyme solutions and acetone was 1:0.75 and the mixture were stirred for 10 min. The precipitates were separated from the supernatant after centrifuging at 12,100*g* (Sorval RC-28S) for 10 min at 4 °C.

Precooled acetone was added to the supernatants until the ratios between enzyme solutions and acetone were 1:0.75 (no further acetone was added), 1:1 and 1:1.25, the solutions were stirred for another 10 min before being centrifuged at 12,100*g* for 10 min at 4 °C. The precipitates collected were dissolved in 40 ml phosphate buffer, pH 7.0. After standing of about 2 h at 4 °C, the protease preparations were centrifuged at 12,100*g* for 15 min at 4 °C to remove inactive residues (Zotos & Taylor, 1996).

2.6. Ammonium sulfate precipitation

Ammonium sulfate solutions (30% and 50%) were slowly added to the crude protease preparations until the ratio between enzyme solutions and ammonium sulfate was 1:0.75 and the mixtures were stirred for 10 min. The precipitates were separated from the supernatant after centrifuging at 12,100*g* (Sorval RC-28S) for 10 min at 4 °C.

Ammonium sulfate solutions (30 and 50%) were further added to the supernatant until the ratios between enzyme solution and ammonium sulfate were 1:1.25, the solutions were stirred for another 10 min before being centrifuged at 12,100*g* for 10 min at 4 °C. The precipitates collected were dissolved in 40 ml of 0.2 M phosphate buffer, pH 7.0. After standing for about 2 h at 4 °C, the protease preparations were centrifuged at 12,100*g* for 15 min at 4 °C to remove inactive residues.

2.7. Inhibition of proteases

The effect of inhibitors on protease activity was determined by preincubating the protease preparation with

the inhibitor at room temperature for 30 min before assay and the residual activity was estimated by the standard protease assay at pH 7.0. $CoCl_2$,$MnCl_2$, PMSF, *p*-benzoquinone, $AgNO_3$, iodoacetamide, EDTA, $CuSO_4$, and $ZnSO_4$ were dissolved in 0.1 M phosphate buffer, pH 7.0, at a concentration of 2 mM, whilst the trypsin inhibitor was in a concentration of 0.5 mg/ml. A control was run by pre-incubating the protease preparation with 0.1 M phosphate buffer, pH 7.0 (Zotos & Taylor, 1996).

2.8. Protein content

Protein concentration was determined using the method of Lowry, Rosebrough, Farr, and Randall (1951).

2.9. Particle size measurement using the Mastersizer 2000 particle size analyser

Fifty millilitre of crude extracts, partial by purified with cold acetone 1:0.75, 1:1 and 1:1.25 and partially purified with 30% and 50% ammonium sulfate samples were diluted to 1 l with 0.1 M phosphate buffer, pH 7.0, and the size of their contained particles was measured using the Using Mastersizer 2000 particle analyser. Care was taken to avoid protein aggregation and thus the samples were agitated before their measurement. The instrument's capability has a range from 20 to 2000 μm. This measurement was conducted in order to estimate the suitability of the applied precipitation methods. An analysis of variance (ANOVA) was applied to detect any differences between the subtraction of the particles in the 5 precipitated samples (cold acetone: crude extract 1.25:1, 1:1, 0.75:1 and ammonium sulfate (30% and 50%): crude extract 1.25:1) from the crude extract.

3. Results and discussion

3.1. Optimum temperature, incubation time and stability

It can be observed from Fig. 1 that the protease activity at pH 7.0 was independent of the incubation time between 25 and 40 min. It can also be seen that higher protease activities were obtained at 55 and 50 °C. These results are similar to those presented by Cao, Osatomi, Hara, and Ishihara (2000) and Hernández-Santoyo et al. (1998). However, optimum temperatures of 35–40 °C have also been reported (El-Beltagy et al., 2004; Nielsen & Nielsen, 2001). It can also be seen, from Fig. 2, that protease activity was almost linearly increased by increasing temperature, except from the samples incubated for 20 min, in which uneven results were obtained. The stability of the proteases at room temperature was also checked and it was found that their activity remained stable even after 15 days, indicating that these proteases might be used by the industry in mild heating processes (data not shown).

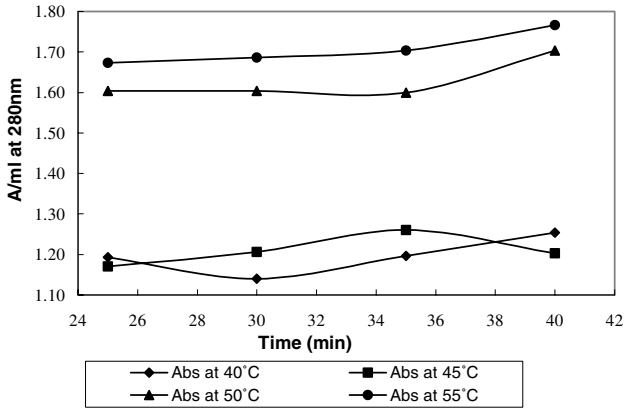

Fig. 1. Effect of incubation time on protease activity.

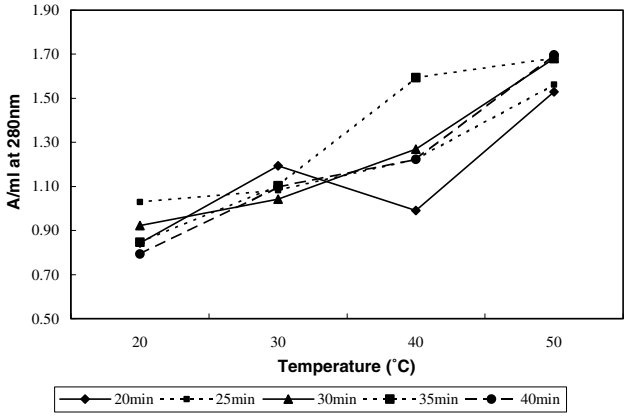

Fig. 2. Effect of temperature on protease activity.

3.2. Optimum pH

The activity of proteolytic enzymes was determined at different pH values (2.4–3.6, 6.4–7.4, 8.6–11.0). In the 2.8–3.6 pH range, casein was diluted to a concentration of 0.2% (w/v) instead of 0.5%, due to the fact that casein is nearly insoluble at this pH because of its isoelectric point (~4.6).

As shown in Fig. 3, both acidic and alkaline proteases were observed in the crude extract, indicating that trout heads may be a promising source of proteolytic enzymes.

These results are in agreement with those reported in the literature. Proteolytic enzymes with optimum pHs of 2.4 and 3.5 have been reported (Chong, Hashim, Chow-Yang, & Ali, 2002; Wilhite, Elden, Brzin, & Smigocki, 2000). Besides, other authors have reported optimum pHs of 7.0 and 10.0 (Giménez, Studdert, Sánchez, & De Castro, 2000; Wang, Hsiao, & Ghang, 2002), confirming the results found in this work.

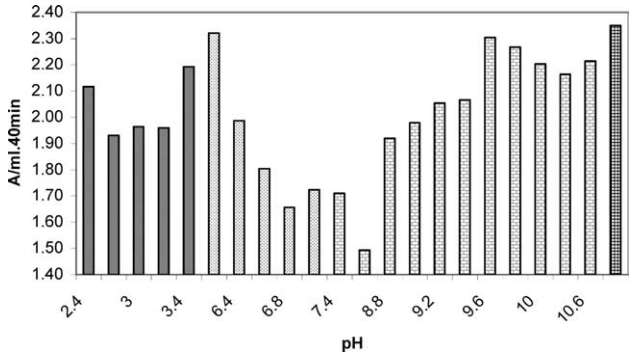

Fig. 3. Effect of pH on enzymic activity.

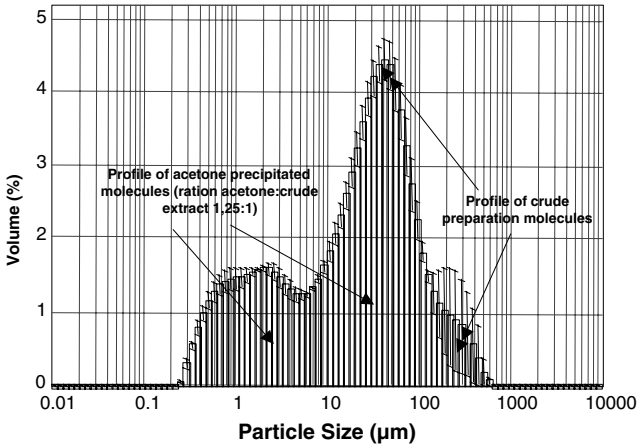

Fig. 4. Comparison of the particles between crude preparation and acetone-precipitated molecules (acetone: crude extract 1.25:1).

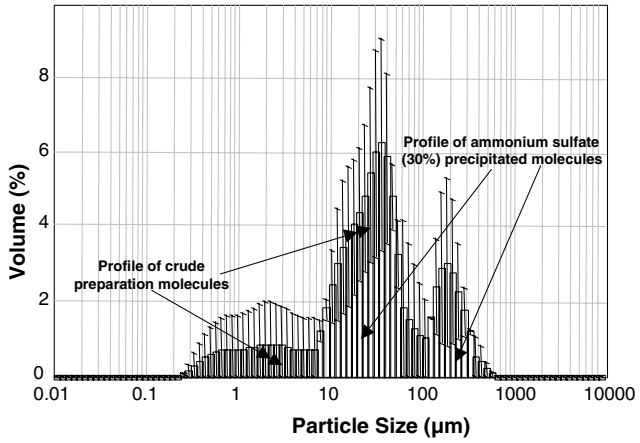

Fig. 5. Comparison of the particles between crude preparation and ammonium sulfate precipitated molecules (ammonium sulfate 30%:crude extract 1.25:1).

3.3. Partial purification of the proteolytic enzymes

The results of the two applied purification methods (cold acetone and ammonium sulfate precipitation) and the recoveries obtained are shown in Table 1.

As can be seen from Table 1, the precipitation of proteolytic enzymes, achieved using cold acetone (−15 °C) in a ratio of acetone to crude extract of 1.25:1 was much higher than any other ratio of acetone:crude extract used. Besides, all acetone ratios were also much higher than the precipitation obtained using the 30% or 50% ammonium sulfate technique. It should be noted that the recovery of protease activity using the ratio of acetone to crude extract of 1.25:1 was 99% (Table 1). These results indicate that cold acetone is probably then most effective agent for the initial step of protease purification.

The effectiveness of cold acetone as a purification agent for proteolyctic enzymes was reported by Popova and Pishtiyski (2001). Also, insufficient partial purification by ammonium sulfate was observed by Wang et al. (2002). Maehashi et al. (2002) and Olivas-Burrola et al. (2001) also reported that cold acetone was a much better purification agent.

The purification results were further confirmed through the measurement of the particles contained in all samples (crude extract and precipitated) using the particle size analyser (Mastersizer 2000). The one-way analysis of variance (ANOVA) of the samples obtained by the subtraction of the particles in the 5 precipitated samples (cold acetone: crude extract 1.25:1, 1:1, 0.75:1 and ammonium sulfate (30% and 50%): crude extract 1.25:1) from the crude extract, revealed that the sample precipitated using cold acetone in the ratio 1.25:1 was statistically different ($p = 0.000$) from all others. The mean subtraction value of the samples precipitated with cold acetone in the ratio 1.25:1 was 0.288 ± 0.25, and 1.30 ± 1.05, 1.29 ± 1.07, 1.26 ± 1.14 and 1.55 ± 1.02 for the samples precipitated with cold acetone in a ratio of 1:1, 0.75:1 and ammonium sulfate 50%, 30%, respectively. The distribution of the 1.25:1 cold acetone: crude extract and

Table 1
Partial purification of proteases with cold acetone and ammonium sulfate ($(NH_4)_2SO_4$)

Fraction	Total protease activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)
Crude extract	31426 (830)	660	47.6	100
Acetone:crude extract 1:0.75	17195 (490)	12	1433	55
Acetone:crude extract 1:1	15507 (289)	16	969	49
Acetone:crude extract 1:1.25	31177 (295)	20	1559	99
$(NH_4)_2SO_4$ 30% 1:1.25	2489 (176)	1.6	1556	8
$(NH_4)_2SO_4$ 50% 1:1.25	814.5 (95)	1.2	679	3

Activity was measured at pH 7.0 with casein as substrate. Data are means of sixfold determinations. Standard deviations are shown in parentheses.

30% ammonium sulfate precipitated particles in comparison with the particles identified in crude extract are representatively shown in Figs. 4 and 5. It can be seen from the figures that successful precipitation was achieved with cold acetone in the above ratio, which is in line with the 99% recovery obtained and the high difference in the distribution of the particles observed between crude extract and 30% ammonium sulfate precipitation. Similar were the distribution of the particles in all other precipitated samples.

3.4. Effect of protease inhibitors

The effect of a range of inhibitors on crude extract and on the sample wherein better precipitation was achieved (1.25:1 cold acetone: crude extract) was investigated (Table 2). It was found that proteases, particularly in the precipitated sample, were strongly inhibited by PMSF, trypsin inhibitor, Ag^{+}, Co^{2+} and strongly activated by Zn^{2+}, clearly indicating the existence of Zn–serine proteases. Similar results were reported by Chong et al. (2002) and Barata et al. (2002), confirming the existence of serine proteases. Besides, the inhibition achieved by EDTA, a classic metalloprotease inhibitor, may indicate the existence of further metalloproteases in the head samples of trout and, due to the action observed at low pHs (Fig. 3), they might be acidic metalloproteases, probably Zn-acidic proteases. These results are in agreement with those reported by Capiralla, Hiroi, Hirokawa, and Maeda (2002), Munilla-Morán and Sadorido-Rey (1996), Barata, Andrade, Dias Rodrigues, and Castro (2002).

The activation observed due to the action of iodoacetamide and *p*-benzoquinone possibly excludes the existence of thiol proteases either in crude extract or in the acetone precipitated samples.

Table 2
Effect of some inhibitors on proteases in crude extract and 1.25:1 cold acetone:crude extract partial by purified samples

	Final concentration (mM)	Activity retained (%)	
		Crude extract	Partial purified
Control	–	100	100
PMSF	2	105 (2.60)	59 (1.45)
Trypsin inhibitor	0.5 mg/ml	89 (3.25)	75 (2.38)
$ZnCl_2$	2	100 (1.24)	238 (2.27)
EDTA	2	108 (2.32)	83 (2.34)
Iodoacetamide	2	123 (3.43)	157 (3.41)
p-Benzoquinone	2	136 (3.28)	358 (4.53)
$AgNO_3$	2	64 (1.45)	38 (0.93)
$CoCl_2$	2	60 (3.34)	53 (1.74)
$CuSO_4$	2	97 (2.10)	70 (1.24)
$MnCl_2$	2	89 (1.51)	114 (2.49)

Activity was measured at pH 7.0 with casein as substrate. Data are means of sixfold determinations. Standard deviations are shown in parentheses.

4. Conclusions

Whole heads (waste) from trout (*Salmo gairdnerii*) could be considered as a good source of proteolytic enzymes, mainly Zn–serine proteases and Zn-acidic proteases, which have shown optimum temperature at 55 °C and sufficient stability.

Cold acetone precipitation in two steps (1:0.75 and 1:1.25 crude extract:acetone) was indicated to be the best precipitation method, with a 99% recovery. This effectual precipitation technique was confirmed through measurement of the particles obtained in the different precipitated samples in comparison with the crude extract.

References

Alam, S. I., Dube, S., Reddy, G. S. N., Bhattacharya, B. K., Shivaji, S., & Singh, L. (2005). Purification and characterisation of extracellular protease produced by *Clostridium* sp. from Schirmacher oasis, Antarctica. *Enzyme and Microbial Technology, 36*, 824–831.

Barata, R. A., Andrade, M. H. G., Dias Rodrigues, R., & Castro, I. M. (2002). Purification and characterization of an extracellular trypsin-like protease of *Fusarium oxysporum var. lini*. *Journal of Bioscience and Bioengineering, 94*(4), 304–308.

Cao, M. J., Osatomi, K., Hara, K., & Ishihara, T. (2000). Identification of a myofibril-bound serine proteinase (MBSP) in the skeletal muscle of lizard fish *Saurida waniesco* which specifically cleaves the arginine site. *Comparative Biochemistry and Physiology B, 25*, 255–264.

Capiralla, H., Hiroi, T., Hirokawa, T., & Maeda, S. (2002). Purification and characterisation of a hydrophobic amino acid-specific endopeptidase from *Halobacterium halobium* S9 with potential application in debittering of protein hydrolysates. *Process Biochemistry, 38*, 571–579.

Chong, A. S. C., Hashim, R., Chow-Yang, L., & Ali, B. A. (2002). Partial characterization and activities of proteases from the digestive tract of discus fish (*Symphysodon aequifasciata*). *Aquaculture, 203*, 321–333.

El-Beltagy, A. E., El-Adaway, T. A., Rahma, E. H., & El-Bedawey, A. A. (2004). Purification and characterization of an acidic protease from the viscera of bolti fish (*Tilapia nilotica*). *Food Chemistry, 86*, 33–39.

Giménez, M. I., Studdert, C. A., Sánchez, J. J., & De Castro, R. E. (2000). Extracellular protease of *Natrialba magadii*: purification and biochemical characterization. *Extremophiles, 4*, 181–188.

Hernández-Santoyo, A., Hernández-Arana, A., Arreguín-Espinosa, R., & Rodríguez-Romero, A. (1998). Purification and characterization of several digestive proteases from the blue abalone *Haliotis fulgens*. *Aquaculture, 159*, 203–216.

Hordur, K. G., & Rasco, B. A. (2000). Biochemical and functional properties of atlantic salmon (*salmo salar*) muscle proteins hydrolyzed with various alkaline proteases. *Journal of Agricultural Food Chemistry, 48*, 657–666.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, K. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry, 50*, 265–275.

Maehashi, K., Abe, T., Yasuhara, T., Yamasato, K., Yamamoto, Y., & Udaka, S. (2002). Purification and characterization of a novel glutamil aminopeptidase from chicken meat. *Meat Science, 64*, 163–168.

Munilla-Morán, R., & Sadorido-Rey, F. (1996). Digestive enzymes in marine species. I. Proteinase activities in gut from redfish (*Sebastes*

mentella), Seabream (*Sparus aurata*) and Turbot (*Scopthalmus maximus*). *Comparative Biochemistry and Physiology B, 113*(2), 395–402.

Nielsen, B. L., & Nielsen, H. H. (2001). Purification and characterization of cathepsin D from herring muscle (*Clupea harengus*). *Comparative Biochemistry and Physiology, 128B*, 351–363.

Olivas-Burrola, H., Ezquera-Brauer, M. J., Rouzaud-Sández, O., & Pacheco-Aguilar, R. (2001). Protease activity and partial characterization of the trypsin-like enzyme in the digestive tract of the tropical sierra *scomberomorus cocncolor*. *Journal of Aquatic Food Product Technology, 10*(4), 51–63.

Popova, V., & Pishtiyski, I. (2001). Isolation of cyclodextrine glucanotransferase preparations of different purities. *European Food Research Technology, 213*, 67–71.

Regenstein, J. M., & Regenstein, E. C. (1991). *Specialty products and by-products: Introduction to fish technology* (pp. 169–174). New York: Van Nostrand Reinhold.

Shahidi, F., & Kamil, J. Y. V. A. (2002). Enzymes from fish and aquatic invertebrates and their application in the food industry. *Trends in Food Science and Technology, 13*, 1–30.

Wang, S., Hsiao, W., & Ghang, W. (2002). Purification and characterization of an antimicrobial chitinase extracellularly produced by *monascus purpureus* CCRC31499 in a shrimp and cramb shell powder medium. *Journal of Agricultural Food Chemistry, 50*, 2249–2255.

Wilhite, S. E., Elden, T. C., Brzin, J., & Smigocki, A. C. (2000). Inhibition of cysteine and aspartyl proteinases in the alfalfa weevil midgut with biochemical and plant-derived proteinase inhibitors. *Insect Biochemistry and Molecular Biology, 30*, 1181–1188.

Zotos, A., & Taylor, K. D. A. (1996). Partial purification and characterization of proteases from Norway lobster (*Nephrops norvegicus*) and their role in the phenolase activation. *Food Chemistry, 56*(1), 61–68.