

Concentrates of fish protein from bycatch species produced by various drying processes

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Received 16 July 2005; received in revised form 25 October 2005; accepted 26 October 2005

Abstract

Nine fish species, *Gillichthys seta*, *Oligoplites saurus*, *Eucinostomus entomelas*, *Synodus scituliceps*, *Diplectrum pacificum*, *Pseudopeneus grandisquamis*, *Xenistius californiensis*, *Arius seemanni*, and *Orthopristis redding*, caught as bycatch by shrimp commercial fleet of the Guaymas, Sonora, México, were processed to produce protein concentrates using three drying processes (freeze-drying and heating at 65 and 110 °C). The soluble protein content from each process and species was evaluated. Significant differences were observed among processes. The composition of protein, observed by electrophoresis, was affected by temperature. Raw and freeze-dried protein from *S. scituliceps* showed high proteolytic activity and unwent auto-hydrolysis. The highest proteolytic activity was at 65 °C and its optimum pH was 7.5. When assayed in the presence of several protease inhibitors, the enzymes were completely inactivated by soybean trypsin inhibitor. The in vitro digestibilities of the protein concentrates were higher when obtained by low-temperature methods.

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Keywords: Bycatch; Fish protein; Drying processes; Auto-hydrolysis; Serine proteases

1. Introduction

Protein and its amino acids are essential to the structure of cells, proper functioning of antibodies resisting infections, regulation of enzymes and hormones, and growth and repair of body tissues. Marine organisms possess protein with high proportions of essential amino acids. Protein is also a primary product in the food industry. Keeping or improving nutritive and functional properties depend on appropriate handling of marine organisms during catching, transporting, and processing. Excessive heating for drying, as happens in the production of protein concentrates (commonly called fish meal) yields poor functional properties. Such products can only be used as ingredients in animal feeds and are not fully assimilated. The quality of dried

foods is partly dependent on chemical reactions occurring during processing and storage. If proteins are overheated, the amino acid structure changes. As a result, the protein is not easily digested or absorbed. Savoie, Charbonneau, and Parent (1989) reported that overheating of non-fat dried milk, not only reduced lysine digestibility, but also methionine, phenylalanine, histidine, and cystine. For example, overheating had significant deleterious effects on protein digestibility (–18%) of skim milk powder in rat diets (Sarwar, 1997), leading to failure to grow at normal rates.

Overheating reduces availability of protein either by a denaturing process or, as more commonly occurs, by making the essential amino acids less available. This influences the nutritive quality of food protein. Reduction in availability of cystine, lysine, arginine, threonine, and serine in different protein sources was reported to be a consequence of heat treatment (Papadopulos, 1989). This is particularly true for lysine that forms complexes at high-temperatures

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with carbohydrates, such as reducing sugars, i.e., glucose with lysine (Biemel, Reihl, Conrad, & Lederer, 2001; Gilani & Sepehr, 2003; Papadopulos, 1989). Products of the so called “Maillard reaction”, inhibits the activity of several enzymes, such as trypsin (E.C. 3.4.21.4), pepsin (E.C. 3.4.23.1), carboxypeptidase A (E.C. 3.4.22.17), and aminopeptidase N (E.C. 3.4.11.2) (Schmidt, Nyachoti, & Slominski, 2003).

Because, overheated fish protein concentrates are poorly digestible, some countries, such as Norway, developed a low-temperature fish meal production system for feeds for high-value commercial fish, such as salmon (Hughes & Rumsey, 1991). For some proteins derived from soybeans, thermal treatment is necessary to inactivate anti-physiological compounds (Gilani & Sepehr, 2003). Overheating will cause a reduction in nutritional quality by reducing or even destroying the biological and functional value of proteins (Hwei-Ming, Villaume, & Giannangeli, 2001). Here, we evaluated the effects of drying fish intended as food or as a protein ingredient under different conditions.

2. Materials and methods

2.1. Protein concentrates

Nine fish species (*Gillichthys seta*, *Oligoplites saurus*, *Eucinostomus entomelas*, *Synodus scituliceps*, *Diplectrum pacificum*, *Pseudopenaeus grandisquamis*, *Xenistius californiensis*, *Arius seemanni*, and *Orthopristis reddinigi*) caught as bycatch by the shrimp commercial fleet based at Guaymas Sonora, México, were obtained. Samples were frozen in the vessel and transported to the laboratory. After thawing, the viscera were removed and each species grounded separately to produce protein concentrates under three drying strategies: freeze-drying and oven-drying at 65 °C for 15 h and at 110 °C for 12 h. Length of oven-drying time was based on bringing the samples to a constant weight. Dried samples are called protein concentrates.

Samples were analyzed for proximal composition, according to standard methods (AOAC, 1990). Amino acid analysis was done by the reverse-phase HPLC method. Soluble protein and electrophoresis of the raw fish and protein concentrates were analyzed from aqueous extracts.

2.2. Extracts preparation

Aqueous extracts were prepared from raw material and their protein concentrate by weighing 250 mg dry material (or its equivalent for raw samples), in 2-ml test tubes, and homogenized in 1 ml of 50 mM Tris–HCl at pH 7.5 at room temperature and stirred 15 min, then centrifuged at 10,000g for 30 min at 4 °C. The supernatant was decanted and placed in 1.5-ml test tubes. To eliminate lipids from the supernatant, 100 µl 1:1 (v/v) methanol/chloroform were added to each extract, homogenized, and centrifuged under the same conditions. The supernatant was recovered and stored at 4 °C until used.

2.3. Solubility of protein and proteolytic activity

Solubility of proteins in protein concentrates obtained from heat drying of raw product and solubility of raw fish muscle were evaluated by quantifying the amount of solubilized protein in the aqueous extracts. The Bradford (1976) method, adapted to micro-assays, uses bovine serum albumin as a standard. For evaluation, diluted samples and the Bradford reactive were placed in a 96-well micro-plate in triplicate. Absorbance was measured at 595 nm in a VersaMax™ micro-plate reader (Molecular Devices, Sunnyvale, CA, USA).

To detect proteolytic enzymes in the protein concentrates and raw muscle, total proteolytic activity in freshly prepared extracts was evaluated using the method described by García-Carreño and Haard (1993), using 0.5% azocasein as substrate in 50 mM Tris–HCl buffer of pH 7.5 at 25 °C for 10 min. After 10 min, the reaction was stopped by adding 20% TCA and centrifuged for 5 min at 10,000g. The amount of peptides released in 300-µl samples of supernatant from the reaction mixture in 96-well micro-plates was evaluated by reading the absorbance at 366 nm in a VersaMax™ micro-plate reader (Molecular Devices, Sunnyvale, CA, USA). Extracts showing proteolytic activity were assayed separately for optimum pH and temperature. One unit of activity (U) is the absorbance at 366 nm min⁻¹ mg protein⁻¹.

The optimum temperature for total proteolytic activity was evaluated, as described above, by incubating the reaction mixture at increasing temperatures of 5 °C in the range from 5 to 70 °C at 5° intervals. The effect of pH on proteases was assessed using universal buffer (composed of citric acid, potassium phosphate, sodium tetraborate, Tris, and potassium chloride) at pH of 5, 6, 7, 8, 9, 10, 11, and 12 at 25 °C (Stauffer, 1989), using azocasein as the substrate (described above).

2.4. Electrophoresis

Electrophoresis was used to determine composition of protein and observe changes to the composition or concentration occurring during the drying process. In brief, sodium dodecyl sulfate, 12% polyacrylamide gel electrophoresis (SDS–PAGE) was used as described by Laemmli (1970). Samples containing 20 µg protein and diluted 1:1 (v/v) with sample buffer were loaded into individual gel wells at 4 °C in a vertical electrophoresis device. Low molecular mass standards of 4 µl loaded on each gel. After electrophoresis, gels were stained with 0.05% Coomassie brilliant blue R-250, and then de-stained. To expose proteases in muscle of fish and their molecular weight, samples were placed in a twin gel after SDS–PAGE, following the technique described by García-Carreño, Dimes, and Haard (1993). Gels were immersed in 3% casein in 50-mM Tris–HCl of pH 7.5 for 30 min at 0 °C (ice bath) to allow the substrate to diffuse into the gel. Temperature was then raised to 25 °C for 90 min. Gels were washed in water and immedi-

ately fixed and stained with Coomassie brilliant blue. To develop activity bands at different temperatures, gels were prepared as above, but after soaking in the ice bath, the temperature of incubation was raised to 37, 50, and 65 °C.

To characterize the class, type, and composition of proteinases when proteolytic activity was found in extracts from raw samples, extracts from lyophilized samples were incubated in the presence of proteinase inhibitors. Solutions of soybean trypsin inhibitor (SBTI), TLCK (1-chloro-3-tosylamido-7-amino-2-heptanone), E64 (HCl, *N*-(*N*-L-3-trans-carboxirane-2-carbonyl-lleucyl-*agmatine*)), phenylmethyl sulfonyl fluoride (PMSF), chymostatin, EDTA, and 4-(2-aminoethyl)-benzenesulfonyl fluoride, hydrochloride (PEFABLOC) were separately added to enzyme extracts containing 3 mU in a ratio of 1:5 inhibitor:extract, and incubated for 60 min at 25 °C. Distilled water replaced inhibitors in controls. Samples were mixed with sample buffer, as described above, and loaded into the gels. After electrophoresis, gels were stained for activity, as described previously. Bands of enzymes mixed with proteinase inhibitors were compared with those of the controls without inhibitors.

2.5. Degree of hydrolysis

The degree of protein hydrolysis (DH) of the protein concentrates and casein (reference protein) was evaluated by the pH-stat method, using a mixture of commercial enzymes (1.6 mg ml⁻¹ trypsin (E.C. 3.4.21.4), 3.1 mg ml⁻¹ chymotrypsin (E.C. 3.4.21.1), 1.3 mg ml⁻¹ peptidase from porcine intestinal mucosa (E.C. 3.4.24.x, SIGMA P7500), and 7.95 mg ml⁻¹ pronase Type XIV (E.C. 3.4.24.x, SIGMA P5147)), as described by Ezquerra, Garcia-Carreño, Civera, and Haard (1997). Measurements were made with a 718 Stat Titrino (Metrohm Ion Analysis, Herisau, Switzerland) with a computer interface (Metrodata Menu Computer Program 718 STAT TitrinoPC). The appropriate amount of each protein concentrate was weighed to yield 0.08 g protein. The feed was stirred with distilled water in the hydrolysis vessel, adding sufficient water to give a 10-g substrate mixture (substrate + water + enzyme = 10 g). The pH of this mixture was adjusted to 7.9 with a 1 mol l⁻¹ NaOH solution and stirred for 1 h

to facilitate complete solubilization of protein and stabilization of the pH. Prior to starting the reaction, the pH was raised to 8.0 automatically by the pH-stat by adding 0.1 mol l⁻¹ NaOH. To start the reaction, volume equivalent to 4.0 units (measured with azocasein as substrate) of enzyme activity (pH adjusted to 8.0) were added. All assays were done in triplicate. The reaction mixture was maintained at 28 °C using a jacketed reaction vessel and a circulating water bath. The DH was calculated as described in Córdova-Murueta, Garcia-Carreño, and Navarrete-del-Toro (2003). The DH of casein was used as a reference protein, using the same procedure as for protein concentrates. An auto-hydrolysis assay, with no addition of enzymes, was performed with protein concentrates from lyophilized dried samples at 65 °C. Auto-hydrolysis was followed by SDS-PAGE. Samples were taken at 0, 15, 30, and 60 min.

2.6. Statistical analysis

All results are reported as means of three replicates. ANOVA was carried out to compare mean values. Differences were determined at $P < 0.05$.

3. Results and discussion

3.1. Proximate chemical composition and amino acid content

Protein content (dry basis) among species varied from 57% to 77% (Table 1). *G. seta* had the highest water content of all the species studied. The species with the highest lipid contents were *X. californiensis* and *Orthopristis reddinigi* with more than 16%. *S. scituliceps* had the lowest quantity of lipid (<1%). Highest ash content was for *D. pacificum* and the lowest for *G. seta*. In general, ash content was high in all species because samples included the entire fish, except for viscera. There were no differences of protein content among samples of different drying methods.

Based on the analyses performed, quality and quantity of fish protein are appropriate for use in food products. Amino acids in all species were higher than international recommendations (FAO/WHO, 1985) for children over 2

Table 1
Proximate composition of nine fish species

Sample ^a	Moisture	Protein ^b	Lipids ^b	Ash ^b	Fiber ^b	N.F.E. ^c	Cal g ⁻¹
A	82.57	77.82	3.73	8.15	0.42	9.88	5015.92
B	69.97	66.37	10.4	16.49	0.91	5.83	4917.72
C	70.2	68.88	11.23	14.99	0.68	4.22	4891.72
D	74.87	68.37	0.62	18.68	0.45	11.88	4291.27
E	75.2	63.86	4.57	20.27	0.57	10.73	3888.43
F	74.22	59.93	11.44	15.88	0.79	11.96	4930.88
G	74.70	57.46	16.54	16.4	0.72	8.88	4244.93
H	75.62	59.2	8.49	16.96	0.58	14.77	4949.03
I	71.10	58.85	16.15	13.09	0.79	11.12	4290.87

^a A = *Gillichthys seta*, B = *Oligoplites saurus*, C = *Eucinostomus entomelas*, D = *Synodus scituliceps*, E = *Diplectrum pacificum*, F = *Pseudopeneus grandisquamis*, G = *Xenistius californiensis*, H = *Arius seemanni*, and I = *Orthopristis reddinigi*.

^b Values expressed as dry weight.

^c Difference of 100%.

years and adults, with the exception of *G. seta* that was deficient in methionine and threonine (Table 2, column A). These species synthesize protein that was suitable either for human or animal consumption.

3.2. Soluble protein and proteolytic activity

Solubility of protein is a paramount functional property of dehydrated products. Protein solubility of protein concentrates was species-specific, showing characteristic behaviour after drying under varying conditions (Fig. 1). Drying affected the solubility of proteins. In general, raw samples possess higher protein solubility. Diminishing solubility was found with increase in drying temperature, but this was not universal. We expected some changes since dehydration contributes to denaturation of proteins (heat or freeze-drying). These changes may alter the secondary, tertiary, or quaternary structures of the molecules. Signs of denaturation of protein are reflected in changes in solu-

bility. The method of processing affects the solubility of protein especially if they are exposed to heat (Kilara & Harwalkar, 1996). When proteins are exposed to freezing or heating processes, dehydration stresses occur. Solubility of proteins from *O. saurus* was severely affected by freeze-drying and the 110 °C heat-drying processes (Fig. 1). Drying at 65 °C is an intermediate and safe, but a slow dehydration process, thus reducing excessive damage to the structure of proteins. We found many samples that were dried at 65 °C having similar or even higher quantities of soluble protein than the freeze-dried samples (Fig. 1, letters: A, B, C, D, G, H, and I). Damage to protein structure, reflected in solubility, was greater for proteins of most species when exposed to 110 °C. Electrophoresis also showed differences in the protein composition among the drying processes and raw samples (Fig. 2). The samples dried at 110 °C did not show defined bands of proteins, probably from inter- and intra-molecular cross-linking during the long exposure to high-temperature.

Table 2

Amino acid composition of whole fish (viscera removed), expressed as mg/g of protein compared with FAO/WHO (1985) recommendations for children over 2 years and adults

AA	Children ^a	Adults ^a	A ^b	B ^b	C ^b	D ^b	E ^b	F ^b	G ^b	H ^b	I ^b
Ala			43	45	63	58	64	12	7	11	71
Arg			41	38	64	57	27	47	53	62	59
Asp			73	76	104	93	89	106	108	96	111
Glu			118	109	163	150	143	152	153	149	154
Gly			46	40	65	58	60	66	76	60	81
His			12	17	20	16	26	27	27	21	27
Ser			14	17.0	13	10	19	14	18	18	15
Ile	28	38	38	42	55	49	54	55	55	54	45
Leu	66	65	61	62	85	78	84	88	86	80	78
Lys	58	50	63	63	95	85	79	90	84	71	84
Met	25*	25*	6	20	30	28	30	29	30	29	26
Phe	63**	65**	28	33	41	37	45	47	43	46	40
Thr	34	25	16.0	21	24	22	27	23	26	32	32
Tyr			8	8	6	4	2	113	122	122	6
Val	35	35	38	42	57	51	56	55	58	60	51

^a Values are reported in Young and EL-Khoury (1995).

^b As explained in Table 2 footnote.

* Values correspond to the sum of methionine and cysteine.

** Values correspond to the sum of phenylalanine and tyrosine.

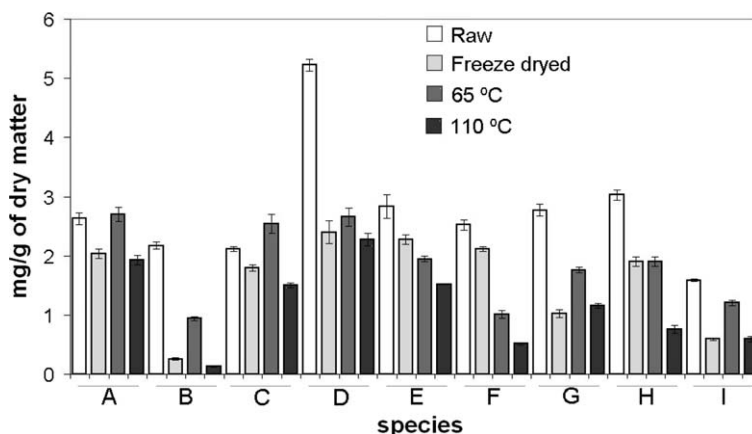


Fig. 1. Soluble protein measured in samples from nine fish species and drying processes. Species are listed in Table 1 footnote.

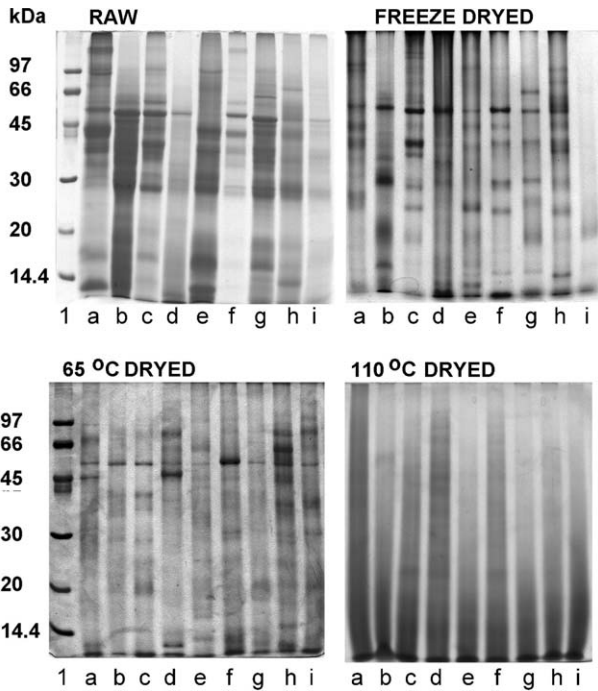


Fig. 2. Electrophoresis (SDS–PAGE) of fish muscle showing the protein profile of nine species using different drying methods. Lanes labeled 1 are the molecular weight markers. Each letter corresponds to a fish species (listed in Table 1 footnote).

The proteolytic activity measured in test tube for the extracts from freeze dried and raw samples of fish showed that only the specie *S. scituliceps* had proteolytic activity ($0.122 \text{ U ml}^{-1} (\pm 0.02)$). Also it was evidenced in S-SDS PAGE.

The optimum temperature of the enzyme(s) responsible for the proteolytic activity was 65 °C, showing activity in the interval of 5–70 °C, and the highest temperature assayed (Fig. 3). The highest activity from the *S. scituliceps* muscle enzymes was achieved at pH 7 (Fig. 4), but there was some activity from pH 5 to 12. The lowest activity was registered at pH 12.

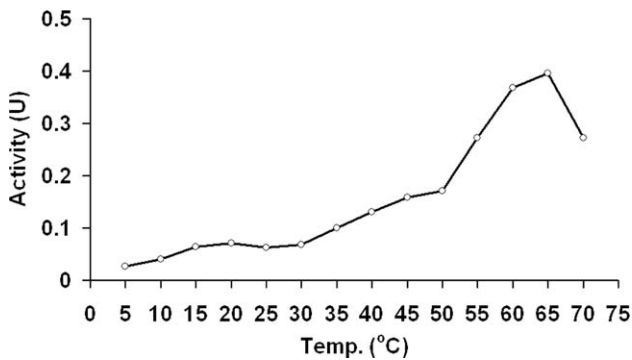


Fig. 3. Activity of enzymes in *Synodus scituliceps* muscle measured at different temperatures.

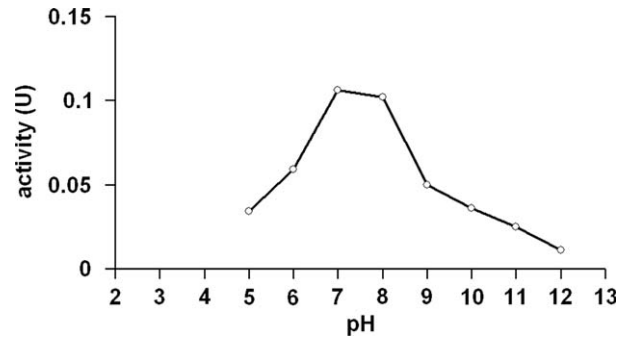


Fig. 4. Proteolytic activity of enzymes in *Synodus Scituliceps* muscle at different pH values.

3.3. Degree of hydrolysis

Proteins in the concentrates from most samples were better hydrolyzed during the determination of DH when they were dried at low-temperatures, except for *S. scituliceps*, which showed lower DH when dried at 65 °C than at 110 °C (Table 3). In general, freeze-drying yielded better DH. The freeze-dried protein from *E. entomelas*, *S. scituliceps*, *D. pacificum*, and *A. seemanni* almost doubled the DH as compared to the other species. Proteins dried at 110 °C had the lowest DH.

The low DH obtained for *S. scituliceps* protein dried at 65 °C was unexpected; the DH of protein dried at 110 °C was higher (Table 3). It was attributed to a pre-hydrolysis

Table 3
Degree of hydrolysis of the fish species under different drying methods

Species	Process	DH (%)
A	1	9.4 ^a
A	2	7.6 ^b
A	3	6.3 ^c
B	1	9.6 ^a
B	2	6.8 ^b
B	3	5.2 ^c
C	1	18.7 ^a
C	2	8.3 ^b
C	3	5.8 ^c
D	1	18.6 ^a
D	2	5.25 ^c
D	3	7.24 ^b
E	1	18.9 ^a
E	2	nd*
E	3	6.7 ^b
F	1	11.8 ^a
F	2	7.6 ^b
F	3	6.5 ^c
G	1	11.3 ^a
G	2	7.8 ^b
G	3	5.5 ^c
H	1	18.3 ^a
H	2	7.13 ^b
H	3	5.5 ^c
I	1	12.6 ^a
I	2	7.2 ^b
I	3	5.5 ^c

nd = not determined.

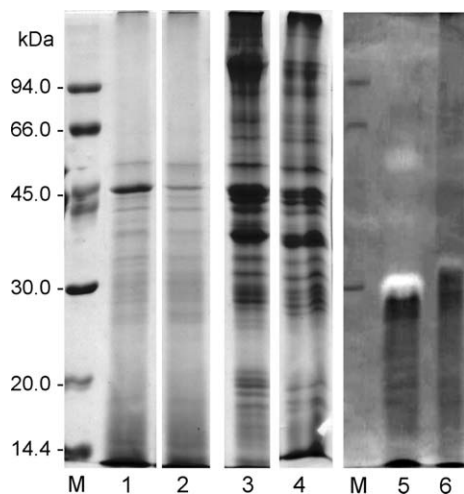


Fig. 5. Auto-hydrolysis in SDS-PAGE of extracts from protein concentrates from *Synodus scituliceps* dried at 65 °C (Lanes 1 and 2 are at 0 and 60 min, respectively) and lyophilized (Lanes 3 and 4 are at 0 and 60 min, respectively). Substrate-SDS-PAGE of the raw sample shown in Lane 5, and the remaining activity after drying at 65 °C is shown in Lane 6.

process occurring when drying at 65 °C, but not occurring at 110 °C. Endogenous enzymes remained active (Fig. 5, Lane 5) and a reduction in the number and intensities of protein bands was found in electrophoresis (Fig. 5, Lanes 1–4). We confirmed that some proteins were partially hydrolyzed during the drying process (lasting 12–15 h for 65–110 °C, respectively), and that enzymes were completely denatured at 110 °C. Others reported that the pH-stat method is not suitable for previously hydrolyzed proteins (Córdova-Murueta & García-Carreño, 2002; Dimes et al., 1994), which is the reason for these DH results.

S. scituliceps is among the four most abundant species in the incidental catch (Martínez-Tovar et al., 2004). The large volume of bycatch of fish species during shrimp fishing encourages alternative uses of these species. Traditionally, it has been underutilized because the muscle texture of these fish is not as firm as what the human consumption market considers palatable after cooking. Enzymes in fish muscles are responsible for hydrolyzing muscle protein, leading to softness of the meat. If whole-fish consumption is not expected, the presence of active enzymes in the fish muscle can be advantageous for processing. Because enzymes remain active at 65 °C, partially hydrolyzed protein are more easily assimilated by the digestive system of marine organisms and other animals (Cahu, Zambonino infante, Quazuguel, & Le Gall, 1999; Clemente, 2000; Córdova-Murueta & García-Carreño, 2002; Hardy, 1991). Enzymes have also been used in feed supplements to improve the biological value of proteins (Haard, 2001).

3.4. Characterization of muscle enzymes

The proteolytic activity of *S. scituliceps* sample incubated with soybean trypsin inhibitor (SBTI), was completely inhibited, (Fig. 6, Lane 4). SBTI inhibits trypsin,

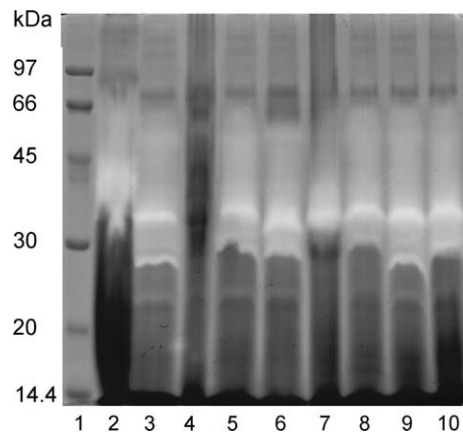


Fig. 6. Substrate-SDS-PAGE of enzymes from *Synodus scituliceps* raw muscle in the presence of inhibitors. Lanes 1–10: molecular weight markers, control without inhibitors (not de-lipidized), control de-lipidized, SBTI, TLCK, E64, PMSF, chymostatin, EDTA, and PEFABLOC, respectively.

and to a lesser extent chymotrypsin and plasmin. SBTI also inhibits proteases with mechanisms of catalysis similar to trypsin (serine proteases). Some activity bands were also diminished with phenylmethyl sulfonyl fluoride (PMSF) and 4-(2-aminoethyl)-benzenesulfonyl fluoride, hydrochloride (PEFABLOC) (Fig. 6, Lanes 7 and 10), PMSF inhibits serine proteases like chymotrypsin, trypsin, and thrombin. PEFABLOC is specific also for serine proteases, including trypsin and chymotrypsin. These results indicate that the main protease in the muscle of *S. scituliceps* is a serine protease. It has maximum activity at 50–60 °C, and inhibition by SBTI and PEFABLOC. Occurrence of serine proteases in fish muscle is not unusual (Cao et al., 1999; Ohkubo et al., 2004; Toyohara, Kinoshita, Shimizu, & Sakaguchi, 1991; Yatsunami & Takenaka, 2000), the physiologic role of serine proteases in fish muscle has not been fully studied and more research is needed in this field.

4. Conclusions

The results during drying of *S. scituliceps* meat demonstrated that the effect of heat on food proteins could be used to induce desirable properties or functionality. It is important to learn what characteristics of species used in food processing can be enhanced by appropriate methods to obtain maximum benefits. Here, we demonstrated that thermal treatments modify protein structure and make some proteins inaccessible to digestive enzymes, as demonstrated by the pH-stat hydrolysis method using commercial enzymes.

Acknowledgments

This project was supported by Grants SAGARPA-CONACyT 106 and CIBNOR EP4.0. Editing staff at CIBNOR reviewed the English text.

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