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Purification and biochemical characterization of chymotrypsin from the viscera of Monterey sardine (*Sardinops sagax caeruleus*)

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Abstract

Chymotrypsin was isolated from the viscera of Monterey sardine by ammonium sulphate fractionation, gel filtration, and ionic exchange chromatography. The approximate molecular weight was 26,000 and its isoelectric point was about 5. Identity as chymotrypsin was established by its catalytic specificity for amide or ester bonds on the synthetic substrates succinyl-L-ala-ala-pro-L-pheil-alanine-*p*-nitroanilide and benzoyl-L-tyrosine-ethyl-ester, showing esterase activity 3.2-fold higher than amidase. It was inhibited by phenylmethylsulfonyl-fluoride and soybean trypsin inhibitor, partly inhibited by the specific chymotrypsin inhibitor *N*-toluenesulfonyl-L-phenylalanine chloromethyl-ketone, but not inhibited by EDTA or Benzamidine. Chymotrypsin showed its maximum activity at pH 8.0 and 50 °C for the hydrolysis of SAAPNA. The Michaelis–Menten constant was 0.074 mM with a catalysis constant of 18.6 seg^{-1}, and catalytic efficiency of 252 seg^{-1} mM^{-1}. Results indicated that Monterey sardine chymotrypsin is a good catalyst and could be used as a biotechnological tool in food processing and using sardine industry wastes as a material for production of fine reagents.

Keywords: Monterey sardine; Chymotrypsin; Biochemical characterization; Viscera; Purification; Chromatography; Enzyme activity

1. Introduction

The Gulf of California, Mexico is the habitat of a great variety of marine organisms, including Monterey sardine (*Sardinops sagax caeruleus*), the main commercial fish (SAGARPA, 2003). During processing, large quantities of waste are generated and discarded directly into the sea, causing pollution (Doode, 1996). Viscera are one of the most important by-products of the sardine industry and are recognized as a potential source of digestive enzymes, especially proteases with high activity over a wide range of pH and temperature conditions (Cancre et al., 1999; Gildberg, 1992; Martinez & Serra, 1989; Simpson & Haard, 1987). Proteases from fish viscera could be used in industrial applications, so its recovery from viscera might be a partial solution to the pollution problem generated by the sardine industry.

Monterey sardine, like other small pelagic fish, is very susceptible to fast abdominal deterioration caused mainly by digestive enzymes, suggesting that viscera are a source of digestive enzymes (Martínez & Gildberg, 1988). Fish digestive proteases belong to the aspartic- and serine-proteases families, specifically trypsin and chymotrypsin the main alkaline proteolytic enzymes in fish viscera (Heu, Kim, & Pyeun, 1995; Pyeun, Kim, & Godber, 1990). Most

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studies of fish digestive enzymes have focused on cold-water fish; studies of enzymes of tropical fish are scarce. Among the digestive enzymes, trypsin-like proteases have received more interest while information about chymotrypsins is less available (Kristjansson & Nielsen, 1992). Studies of fish chymotrypsins purified from dogfish (*Squalus acanthias*) (Prahl & Neurath, 1966; Racicot & Hultin, 1987), carp (*Cyprinus carpio*) (Cohen, Gertler, & Birk, 1981a, 1981b), Atlantic cod (*Gadus morhua*) (Asgeirsson & Bjarnason, 1991; Raae & Walther, 1989), rainbow trout (*Oncorhynchus mykiss*) (Kristjansson & Nielsen, 1992) and anchovy (*Engraulis japonica*) (Heu et al., 1995), have revealed that fish chymotrypsins are similar to bovine and porcine chymotrypsins; however, some important differences have been detected in fish chymotrypsin, including higher catalytic activity, lower thermostability, and differences in polypeptide amino acid composition (Cohen et al., 1981a; Racicot & Hultin, 1987; Ramakrishna, Hultin, & Racicot, 1987).

Most studies of fish chymotrypsins have shown that it is common to find two isoforms with the same specific activity. Like mammalian chymotrypsins, they are endopeptidases that cleavage the peptide bond of proteins on the carboxyl side of phenylalanine, tyrosine, and tryptophane and also synthetic substrates, such as SAAPNA and BTEE. Likewise, they are susceptible to specific inhibitors such as *N*-toluenesulfonyl-L-phenylalanine chloromethyl-ketone (TPCK) and *N*-carbobenzoyi-L-phenylalanine chloromethyl ketone (ZPCK) (De Vecchi & Coppes, 1996; Simpson, 2000). They have molecular weight in the range of 22,000 and 30,000 Da, with an optimum activity range of pH 7.5–9 and 45–55 °C; are unstable at temperatures >55 °C and acidic conditions (Cohen et al., 1981a; Heu et al., 1995). This study generated data on purification and the main biochemical characteristics of chymotrypsin from Monterey sardine viscera to contribute alternatives for the commercial use of this by-product.

2. Materials and methods

2.1. Reagents

Phenyl methyl sulfonyl fluoride (PMSF), *N*-toluenesulfonyl-L-phenylalanine chloromethyl ketone (TPCK), benzamidine, soybean trypsin inhibitor (SBTI), ethylene-diamine-tetracetic acid (EDTA), succinyl-L-ala-ala-pro-L-phenylalanine-*p*-nitroanilide (SAAPNA), benzoyl-L-tyrosine ethyl-ester (BTEE), *N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), glycine, citric acid, TRIS buffer, trichloroacetic acid (TCA), ammonium sulfate, sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE), and molecular weight markers (14,000–66,000) were purchased from Sigma (Mexico). Sodium dodecyl sulphate (SDS), acrylamide, ammonium persulfate (APS), tetramethyl ethylene diamine (TEMED), and coomassie blue G and R were obtained from Bio-Rad Laboratories (Mexico). Diethylene amino ethyl sepharose (DEAE-sepharose) fast flow, Sephadex G-75, and dialysis tubing were purchased from Amersham Pharmacia Biotech (Sweden). All reagents were of analytical grade.

2.2. Samples

Monterey sardine specimens were obtained from Productos Pesqueros de Guaymas, S.A. (Guaymas, Mexico). Sardines were collected within 6 h of death from the fishing vessel's storage vault, where they were stored at 8 °C. Samples were placed in a portable cooler between layers of crushed ice and transported to the CIAD Seafood Products Laboratory in Hermosillo, Mexico. The viscera were extracted at low temperature, placed in hermetically sealed polyethylene bags, immediately frozen, and kept at −80 °C until analysis.

2.3. Purification procedure

Portions of pyloric caeca (50 g) were separated from the viscera and homogenized with 250 ml extraction buffer (50 mM Tris–HCl pH 7.5, 10 mM $CaCl_2$, 0.5 M NaCl) for 1 min. The homogenate was incubated for 8 h at 25 °C, defatted with 50 ml of CCl_4, and centrifuged at 26,000*g* for 30 min at 2–4 °C. The supernatant was considered the crude enzyme extract (Heu et al., 1995; Whitaker, 1994).

The crude enzyme extract was mixed with ammonium sulfate and the fraction between 30% and 70% saturation was collected. After 2 h in an ice-bath, this fraction was centrifuged at 20,000*g* for 20 min. The pellet was dissolved in 30 ml buffer A (50 mM Tris–HCl pH 7.5, NaCl 0.5 M) (Janson & Rydén, 1998), and loaded into a 1 × 80 cm Sephadex G-75 gel filtration chromatography column (Amersham Pharmacia Biotech, Uppsala, Sweden). Buffer A was used as a mobile phase at 0.5 ml/min flow rate and 5-ml fractions were collected. Fractions with chymotrypsin activity were combined and dialyzed against 6 l 20 mM Tris–HCl, pH 7.5 buffer (Cohen et al., 1981a; García-Carreño & Haard, 1993; Simpson & Haard, 1984).

Dialyzed fractions were loaded into a DEAE-Sepharose column (1.6 × 20 cm) and equilibrated with 20 mM Tris–HCl pH 7.5 buffer. Unabsorbed protein was washed with equilibration buffer, and the column was eluted with a 400-ml linear gradient ranging from 0.0 to 0.4 M NaCl (Amersham Pharmacia Biotech, 1999; Cohen et al., 1981a; García-Carreño & Haard, 1993).

Protein concentration was evaluated using $Abs_{280\,nm}$ and the method of Bradford (1976). Trypsin- and chymotrypsin-specific activities in the eluted fractions were also evaluated, using specific substrates according to