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Biochemical characterisation of chymotrypsin from the midgut gland of yellowleg shrimp, *Penaeus californiensis*

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ABSTRACT

Chymotrypsin from shrimp, *Penaeus californiensis*, was compared to *Bos taurus* chymotrypsin, and its structure–function relationship was studied. Catalytic efficiency toward synthetic substrate is lower, but it has a broad specificity and higher activity toward protein substrates, including collagen. It is active at pH 4–10 and fully active up to 50 °C for 2 h and at least nine days at room temperature. The activation peptide is twice as long as bovine chymotrypsinogen, has less disulfide bridges, and is a single polypeptide. Only one activation step is necessary from chymotrypsinogen to the mature enzyme. Postmortem implications in muscle softening and melanisation, resistance to temperature and pH and efficiency with proteinaceous substrates make chymotrypsin useful as a biotechnological tool in food processing. This makes shrimp processing wastes useful as a material for production of fine reagents.

1. Introduction

Shrimp are both a commodity and a delicacy. *Penaeus californiensis* (Holmes, 1900; syn: *Farfantepenaeus californiensis*) is harvested in the Gulf of California and, like *Penaeus vannamei* (syn: *Litopenaeus vannamei*), is susceptible to postmortem melanisation (Díaz-Tenorio, García-Carreño, & Pacheco-Aguilar, 2007; García-Carreño, Cota, & Navarrete del Toro, 2008), mainly in the cephalothorax, by influence of digestive proteases that reduce the value of the product. Another post-harvest concern in crustaceans is muscle softening after extended storage, probably as a result of endogenous proteases (Sriket et al., 2012). Few studies have been done on serine proteases of crustaceans, to relate function with structure (Tsu & Craik, 1996) that could explain postmortem changes. Digestive proteases in penaeids belong to the serine class and trypsins have received more study than have chymotrypsins.

Chymotrypsins (EC 3.4.21.1; MEROPS ID: *S01.152*) cleave peptide bonds formed by the carbonyl groups of hydrophobic amino acid residues and, because of this, are used as biological reagents. Chymotrypsinogens are synthesised in the digestive gland of invertebrates and in the pancreas in vertebrates, as zymogens that need limited-hydrolysis activation to become catalytically functional. Chymotrypsins are excellent models for studying evolutionary divergence to modify function and stability.

Structure-based sequence comparison of well-known bovine chymotrypsin with chymotrypsin in penaeids will help to assess catalytic properties, such as substrate preference, inhibition, and regulation in physiological processes. In this study, we purified and characterised a conspicuous chymotrypsin in *P. californiensis*. The study generated information about purification and biochemical characteristics to contribute to our understanding of physiological, as well as postmortem processes and to provide alternatives for using by-product waste of shrimp processing in various biotechnologies.

2. Materials and methods

2.1. Preparation of enzyme extract

Adult yellowleg shrimp (*P. californiensis*) were harvested at California Gulf and processed. Digestive glands were excised,

Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; pI, isoelectric point; PMSF, phenylmethanesulphonyl fluoride; SAAPFNA, N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine 4-nitroanilide; SBTI, soybean trypsin inhibitor; SDS–PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TPCK, N-p-tosyl-L-phenylalanine chloromethyl ketone.

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pooled, and homogenised in cold distilled water in a ratio of 1:3 w/v, in an ultrasonic liquid processor (XL-2000, Misonix, Farmingdale, NY), using three bursts of 5 s pulses under an ice bath. The homogenate was centrifuged at 10,000×*g* for 30 min at 4 °C. The supernatant was analysed for protein concentration, following the method of Bradford (1976), using bovine serum albumin (BSA, B4287, Sigma–Aldrich, St. Louis, MO) as standard. To keep the enzyme active, the enzyme crude extract was freeze-dried. Powder was kept at −20 °C for further assays. For reconstitution, 100 mg of powder were dissolved in 1 ml of distilled water and analysed for protein content and chymotrypsin activity.

2.2. Activity measurements

Five microlitres of enzyme crude extract were placed in a microplate well and then 300 μl of 0.1 mM succinyl-L-Ala-Ala-Pro-L-Phe-p-nitroanilide (SAAPFNA, S7388, Sigma–Aldrich) in 50 mM Tris–HCl, pH 8.0, containing 20 mM calcium chloride were added. During substrate hydrolysis, the reporter, p-nitroaniline, is liberated and read at 410 nm every 30 s for 3 min at 25 °C in a microplate reader (VersaMax, Molecular Devises, Sunnyvale, CA). Specific activity was calculated as: activity units = (Abs at 410 nm min^{-1} × reaction volume in ml)/8800 × mg protein in the enzyme sample, where 8800 is the extinction coefficient of p-nitroaniline. All assays were done in triplicate.

2.3. Electrophoresis

SDS polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli (1970). Samples were mixed with sample buffer 2× (0.125 M Tris–HCl, pH 6.8, 4% w/v SDS, 20% v/v glycerol, 0.02% w/v bromophenol blue). For reducing conditions, samples were mixed with 2× sample buffer containing 0.2 M DTT and boiled for 10 min, then loaded into a 12% polyacrylamide gel. Low molecular weight standards (17–0446, GE Healthcare Life Sciences, Little Chalfont, UK) were loaded into each gel. Electrophoresis was conducted at 15 mA per gel at 4 °C, using a SE-260 mini-vertical electrophoresis unit (GE Healthcare Life Sciences). After electrophoresis, gels were stained with Coomassie brilliant blue R 250 for 2 h, then de-stained and analysed for protein, using a gel imager (Gel-Doc EZ Imager, Bio-Rad Laboratories, Hercules, CA).

Substrate-SDS–PAGE was assayed to measure proteinase activity in the samples, following the method previously described (García-Carreño, Dimes, & Haard, 1993). Purified chymotrypsin was treated with 2× sample buffer without DTT or boiling and loaded into the gel. After electrophoresis, gels were transferred to a tray and washed with distilled water to eliminate running electrode buffer. Then 60 ml of 3% (w/v) bovine casein (C7078, Sigma–Aldrich) in 50 mM Tris–HCl, pH 8, were gently poured into the tray and placed on an ice bath with orbital shaking to allow the substrate to penetrate the gel, but preventing enzyme activity. After 30 min, the temperature was raised to 25 °C and maintained for 90 min. The gel was then washed with distilled water, stained, de-stained and photo documented. Clear bands on a blue background showed enzyme activity.

2.4. Chymotrypsin purification

Shrimp chymotrypsin was isolated in two steps: affinity chromatography, followed by preparative electrophoresis. Affinity chromatography was used as the first step of purification. In brief, one ml of reconstituted shrimp enzyme crude extract was treated with 100 μl 10 mM N-p-tosyl-L-lysine chloromethyl ketone (T7254, Sigma–Aldrich) to prevent binding of trypsins to the affinity support by inhibiting them in the crude extract. The mixture was loaded into a 1 ml soybean trypsin inhibitor-agarose gel (SBTI, T0637, Sigma–Aldrich) column that was previously equilibrated with 50 mM Tris–HCl pH 7.4 (Buffer A). After incubation for 1 h at 25 °C, the column was washed with 5 ml of Buffer A. Unbounded proteins, including inhibited trypsins were washed out with 5 ml of distilled water. Finally, chymotrypsins were eluted with 5 ml of 10 mM HCl; 1 ml fractions were collected in tubes containing 40 μl of 1 M ammonium bicarbonate to rapidly shift pH and protect chymotrypsin from the acidic medium. All fractions were monitored for chymotrypsin activity; also, possible trypsin contamination from the fractions eluted with HCl was monitored, using benzoyl-Arg-p-nitroanilide (B4875, Sigma–Aldrich), but no trypsin activity was found. Fractions with chymotrypsin activity were pooled and concentrated, using a centrifugal filter 10,000 MW cut-off (Amicon Ultra, EMD Millipore, Billerica, MA) at 4 °C. Then, discontinuous native preparative polyacrylamide gel electrophoresis was used as the second purification step, following instructions in the Mini-Prep Cell manual (Bio-Rad Laboratories). In brief, a 200 μl chymotrypsin sample, obtained from affinity chromatography, was combined with 100 μl of native electrophoresis sample buffer 4× and loaded into a 12% acrylamide rod gel, 7 cm high. Electrophoresis was performed under 1 W at 4 °C. When the tracking dye front reached the bottom of the gel rod, 300 μl fractions were collected and tested for chymotrypsin activity, as follows: 10 μl of each fraction were assayed with 0.1 mM SAAPFNA, as described earlier. All fractions with chymotrypsin activity were pooled, concentrated, and, at the same time, electrode buffer eliminated, by centrifugal filter, as described earlier. Protein composition was assayed by SDS–PAGE. Pure chymotrypsin was sampled in 50 μl aliquots and stored at −20 °C for further analysis.

2.5. Biochemical characterisation

2.5.1. pH and thermal stability

pH stability of shrimp and bovine chymotrypsin were studied by incubating the enzymes in McIlvaine universal buffer from pH 3 to 10 for 2 h; samples of 200 ng were withdrawn every 15 min and assayed for activity, using 0.1 mM SAAPFNA in 50 mM Tris–HCl, pH 8, 20 mM $CaCl_2$ at 25 °C; enzymatic activity was calculated, as described earlier. Relative activity was determined by comparison of the samples with the activity of the enzyme without incubation, designated as 100%. All assays were done in triplicate.

The stability of the enzymes at different temperatures was assayed, as follows: shrimp or bovine chymotrypsin were incubated at 20, 30, 40, 50, and 60 °C; samples containing 200 ng of each enzyme were withdrawn after 15, 30, 60, 90, and 120 min and mixed with 250 μl of 0.1 mM SAAPFNA in Tris–HCl with 20 mM $CaCl_2$ at pH 8 and incubated for 3 min at 25 °C; then, 50 μl of 30% v/v acetic acid were added to stop the reaction. Absorbance of reaction mixture was monitored and enzyme activity was calculated, as described earlier. All assays were performed in triplicate.

2.5.2. Isoelectric point

Once chymotrypsin was purified, it was immediately analysed to find the isoelectric point (pI) by assessing, by electro focussing (PhastGel IEF 3-9, 17-0543-0, GE Healthcare Life Sciences, Uppsala, Sweden) with a pH range of 3–10 and standard calibration kit (17-0471-01, GE Healthcare). Proteins were electrofocussed in a PhastSystem (GE Healthcare Life Sciences) and stained with silver, following the File 210 development technique of the PhastSystem.

2.6. The effect of inhibitors

The effect of specific inhibitors of chymotrypsin activity was assessed as described previously (García-Carreño, 1992). In brief,

8 pmol of shrimp or bovine chymotrypsin were incubated with an excess of 30 pmol of chymostatin (T7274, Sigma–Aldrich) in dimethyl sulfoxide (D5869, Sigma–Aldrich), SBTI (S9003, Sigma–Aldrich), phenylmethanesulphonyl fluoride (PMSF; P7626, Sigma–Aldrich), or N-p-tosyl-phenylalanine chloromethyl ketone (TPCK; T4376, Sigma–Aldrich) in methanol. The enzyme-inhibitor mixtures were incubated for 1 h at room temperature and assayed for activity using 0.1 mM SAAPFNA in 50 mM Tris–HCl pH 8, 20 mM $CaCl_2$ at 25 °C. As the control, the enzyme was incubated with water instead of the inhibitor solution and designated as 100% activity. The effect of the inhibitor solvents was assayed by incubating the enzyme with the respective solvent.

2.7. Hydrolysis of proteinaceous substrate

The ability of shrimp and bovine chymotrypsin to hydrolyse proteinaceous substrates, particularly BSA, used as the protein standard for determination of protein concentration, collagen type III from calf skin (C3511, Sigma–Aldrich), and collagen from rat tail (C8897, Sigma–Aldrich) at 8 mg ml^{-1} final concentration, was measured. In brief, BSA was dissolved in 50 mM Tris–HCl pH 8; pH was readjusted with 0.5 M Tris–HCl. Collagen was dissolved in 500 µl of 0.1 M acetic acid at 40 °C, and then brought to a 1 ml final volume with 0.5 M Tris–HCl, pH 8. For enzyme-substrate reaction mixtures, 2 µg of shrimp or bovine chymotrypsin were mixed with 25 µl of each substrate and incubated for 3 h at 25 °C. Five microlitres subsamples, containing 40 µg of substrate, were withdrawn at 0, 1, 2, and 3 h and mixed with 5 µl of electrophoresis loading buffer containing DTT and boiled for 10 min. All samples were loaded onto a 12% acrylamide gel and electrophoresed to evaluate hydrolysis products. The electrophoresis gels were then stained and destained, as described earlier.

2.8. Isolation of total RNA and synthesis of complementary DNA

Total RNA was obtained from the *P. californiensis* midgut gland during the intermolt stage, using TRIzol LS reagent (10296-010, Invitrogen, Carlsbad CA), following manufacturer's instructions. Total RNA concentration was quantified at 260 nm, using a spectrophotometer (NanoDrop 2000, Thermo Scientific, Waltham, MA) and processed via 1% agarose-formaldehyde gel electrophoresis for wholeness (Sambrook & Russell, 2001). Genomic DNA was eliminated by treating the sample with DNAse I (AMDP1, Sigma–Aldrich), following manufacturer's instructions. One microgram of total RNA was used to synthesise single-strained complementary DNA (cDNA), using a reverse transcription system kit (A-3500, Promega, Madison, WI) and oligo-dT primers, following manufacturer's instructions. To amplify the full length of the cDNA of chymotrypsin by PCR, Gotaq Green Master Mix was used (M7122, Promega); oligonucleotides primers were based on *P. vannamei* mRNA for chymotrypsin I (GenBank ID: X66415.1).

2.9. Sequence analysis

2.9.1. Amino acid sequence analysis

Once chymotrypsin was purified, it was immediately analysed by PAGE under reduced conditions and the protein was blotted onto a PVDF membrane for amino terminal sequence. This was determined by the Edman degradation technique at Eötvös Loránd University, Hungary. Mass spectrometry analysis was performed, using 2 µg of shrimp chymotrypsin on a 12% acrylamide gel. After, the gels were stained with Coomassie Blue to identify protein bands that were then cut from the gel. The sequence analysis was done at The Scripps Centre for Metabolomics and Mass Spectrometry (http://masspec.scripps.edu/). After digestion by trypsin, peptides were analysed by nano-flow LC-tandem mass spectrometry in data-dependent scanning mode, using an ion trap mass spectrometer.

2.9.2. Nucleotide sequence analysis

Nucleotide sequences of the gene were translated to protein with ChromasPro 1.5 software (Technelysium, Brisbane, Australia). Database nucleotide homology searches used the BLAST programme (Altschul et al., 1997). Theoretical isoelectric point and relative molecular masses of the deduced protein were predicted, using the ExPASy Compute pI/Mw tool (http://web.expasy.org/compute_pi/). Sequences were analysed using structural superposition alignment, using the VAST algorithm (Gibrat, Madej, & Bryant, 1996), that compared protein structures of *P. vannamei* chymotrypsinogen BI and *Fenneropenaues chinensis* chymotrypsin-like serine protease with crab collagenase of *Uca pugilator* (*syn*: *Celuca pugilator*; PDB ID: 1AZZ), fire ant *Solenopsis invicta* chymotrypsin (PDB ID: 1EQ9), and bovine chymotrypsinogen and chymotrypsin (PDB ID: 2CGA and 5CHA). The nucleotide sequences of the shrimp chymotrypsins were deposited in GenBank (ID: JX889715, JX889716, and, JX889717).

3. Results and discussion

3.1. Purified and deduced cDNA sequence of shrimp chymotrypsin

A chymotrypsin from the *P. californiensis* digestive gland was purified to homogeneity as observed after electrophoresis, this enzyme has a molecular mass of 35.7 kDa (Fig. 1A, lane 1); when the shrimp chymotrypsin was reduced with DTT and boiled, the protein migrated with a mass band of 27 kDa (Fig. 1A, lane 2). Three different clones were found; they were composed of 770 bp that coded for 256 amino acids. Clone Ch_20 (GenBank ID: JX889717) was used in further comparisons because the insert was confirmed from the analysis of many plasmids. Variations of the other two isoforms were semi-conserved; the deduced chymotrypsinogen was identified as Chymo_P.cal. The deduced amino acid sequence was confirmed by the N-terminal (seven residues) and mass spectrometry of the purified shrimp chymotrypsin (79 residues in six peptides covering 40% of the deduced sequence; Fig. 2, dotted boxes). Results from mass spectrometry could not differentiate between the nucleotide sequences that were obtained. When the molecular mass was derived from the deduced primary structure, the result was 23.72 kDa. Differences between theoretical weight (23.7 kDa) and mobility in electrophoresis (27 kDa) could be related to post-traductional modification, such as glycosylation predicted by the public NetOGlyc software (Shirai, 2008) at residues Thr135 and Thr137 (Fig. 2, residues double underlined). The molecular weight difference with unreduced pure shrimp chymotrypsin (35.7 kDa) could be explained to conformation changes between folded and unfolded proteins. Bovine chymotrypsin has a basic pI of 8.7, whereas shrimp chymotrypsin, like most crustacean serine proteases, has an exceptionally acidic pI of 3.6 (Table 1). Only one protein was obtained by the isoelectric point, which supports the idea that a unique isoform was purified. Although pure shrimp chymotrypsin was stored at −20 °C in 50 µl samples to avoid thawing and refreezing, two proteins appeared after five months (Fig. 1C, lane 1), probably as a result of autohydrolysis or conformations changes. However, both proteins had proteolytic activity (Fig. 4, insert).

The deduced primary structure of *P. californiensis* chymotrypsinogen share identity of sequence with other penaeid chymotrypsins (Fig. 2); its closest homologues are chymotrypsin BI from *P. vannamei* (UniProt ID: Q00871), and chymotrypsin-like serine protease from *F. chinensis* (UniProt ID: Q00871). A putative activation peptide of 30 residues, including one Arg at the cleavage site at

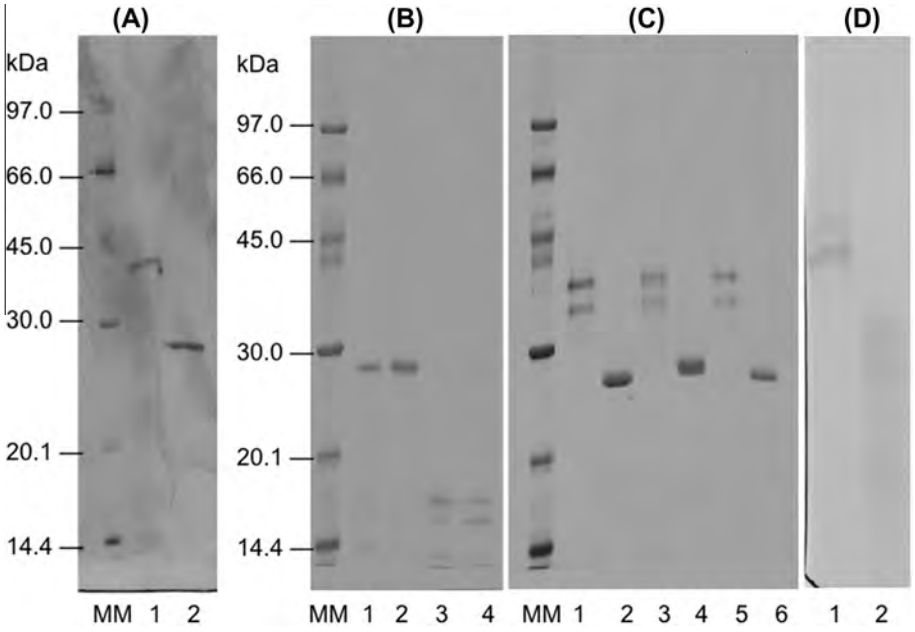

Fig. 1. The effect of temperature denaturation, reduction, or alkylation on molecular behaviour of chymotrypsin, by SDS– and Native-PAGE. Footnote: (A) Intact shrimp chymotrypsin lane 1, chymotrypsin treated with SDS; lane 2, chymotrypsin treated with SDS, DTT and boiled for 10 min (B) bovine chymotrypsin and (C) stored pure shrimp chymotrypsin. Lane 1 pure sample mixed with loading buffer 2×; lane 2, sample with loading buffer and boiled for 10 min; lane 3, sample reduced with DTT; lane 4, sample reduced and boiled for 10 min; lane 5, sample reduced with DTT and treated with iodoacetamide to block the disrupted disulfide bridges; lane 6, sample with loading buffer, DTT, iodoacetamide, and boiled for 10 min. (D) Shrimp chymotrypsin in native conditions. Lane 1, sample reduced with DTT; lane 2 sample reduced with DTT and boiled for 10 min.

nucleotide 93, was present in the cDNA (Fig. 2, solid triangle). Though there is no experimental evidence to prove where the activation peptide of crustacean zymogens begins, sequence comparison with *P. vannamei* chymotrypsins (Sellos & Van Wormhoudt, 1992), and *U. pugilator* collagenase (Tsu & Craik, 1996) suggests that the activation peptide of *P. californiensis* chymotrypsin is twice as long as that of bovine chymotrypsinogen (Fig. 2). Signal sequence and the putative proteolytic cleavage site for *P. vannamei* were obtained by a hydrophobic score (Kyte & Doolittle, 1982; Sellos & Van Wormhoudt, 1992). We found a similar peptide in the deduced amino acid sequence of *P. californiensis*, using the SignalP 4.1 server software (Petersen, Brunak, von Heijne, & Nielsen, 2011). Large activation peptide (29–51 residues) is a landmark of crustacean serine proteinases (Hernández-Cortés, Cerenius, García-Carreño, & Söderhäll, 1999; Kristjánsdóttir & Gudmundsdóttir, 2000; Sellos & Van Wormhoudt, 1992; Tsu & Craik, 1996). This long activation peptide in crustaceans is important for expression of recombinant proteins and may be essential for correct folding or processing of the enzyme (Kristjánsdóttir & Gudmundsdóttir, 2000). Because the pro-chymotrypsin activation site is similar to those of other chymotrypsinogens (Fig. 2, Arg15 marked as a solid triangle), trypsin seems to be responsible for activation of shrimp chymotrypsinogen; however, because of the broad specificity of this chymotrypsin, an *in trans* self-activation process is not excluded (Tsu & Craik, 2004). If shrimp chymotrypsin could be self-activated or processed by trypsin, it may have deleterious implications in post-mortem changes because unwanted characteristics, such melanisation, may appear. It was demonstrated in other shrimp species that haemocyanin plays a main role in post-mortem melanosis (García-Carreño et al., 2008) through the conversion of haemocyanin to phenoloxidase activity by chymotrypsin proteolysis. This modified haemocyanin uses mono- and diphenols as substrates that finally oxidises melanin that is responsible for black spots. Both chymotrypsin and haemocyanin are synthesised in the digestive gland and are abundant. Even during storage at 4 °C, black spots were observed after 24 h (Díaz-Tenorio et al., 2007).

Five disulfide links are conserved in mammalian chymotrypsins (Lesk & Fordham, 1996); only three of these are conserved in crustacean chymotrypsins (Fig. 2), including the one adjacent to the catalytic His57 (Cys 42:58), the bond that holds domain 2 (Cys 168:182), and the link in a hairpin containing Ser195 (Cys 191:220). Hence, the disulphide links that hold the propeptide (A with B chain, Cys 1:122) and B with C chain (Cys 136:201) in mammalian chymotrypsins are missing in Arthropoda chymotrypsinogens, such as in Crustacea (Sellos & Van Wormhoudt, 1992; Shi, Zhao, & Wang, 2008; Tsu & Craik, 1996) and Insecta (Broehan et al., 2010; Herrero et al., 2005). Chymotrypsin from *Daphnia* (GenBank ID: EFX79602.1), a fresh-water crustacean, retains Cys122, but lacks their counterpart Cys1 to form a disulphide bridge. A similar case occurs in fire ant, *Solenopsis invicta*, chymotrypsin (GenBank ID: Q7SIG2.1) that possesses Cys136 but lacks Cys201. The absence of these disulphide bridges implies that they are not essential, but may still affect enzymatic activity. Whether this character influences post-mortem food properties remains unknown.

P. californiensis chymotrypsin lacks the activation peptide and the mature protein begins at residue Ile16, as in other shrimp chymotrypsins (Hernández-Cortés, Whitaker, & García-Carreño, 1997), because shrimp chymotrypsinogen lacks Cys1 (Fig. 2, first black dot). The liberation of the propeptide does not affect function. The loss of the activation peptide is confirmed by the N-terminal amino acid sequence of the purified shrimp chymotrypsin (Fig. 2, residues underlined). The second step of bovine chymotrypsin maturation does not happen in invertebrate chymotrypsins; we found a continuous sequence that corresponds to four peptides in shrimp chymotrypsin from residue Leu108–Lys188 by mass spectrometry, where residues Thr147-Asn148 are cleavage sites in bovine chymotrypsin (Fig. 2, residues dotted underlined). Denaturation by high temperature changes conformation of

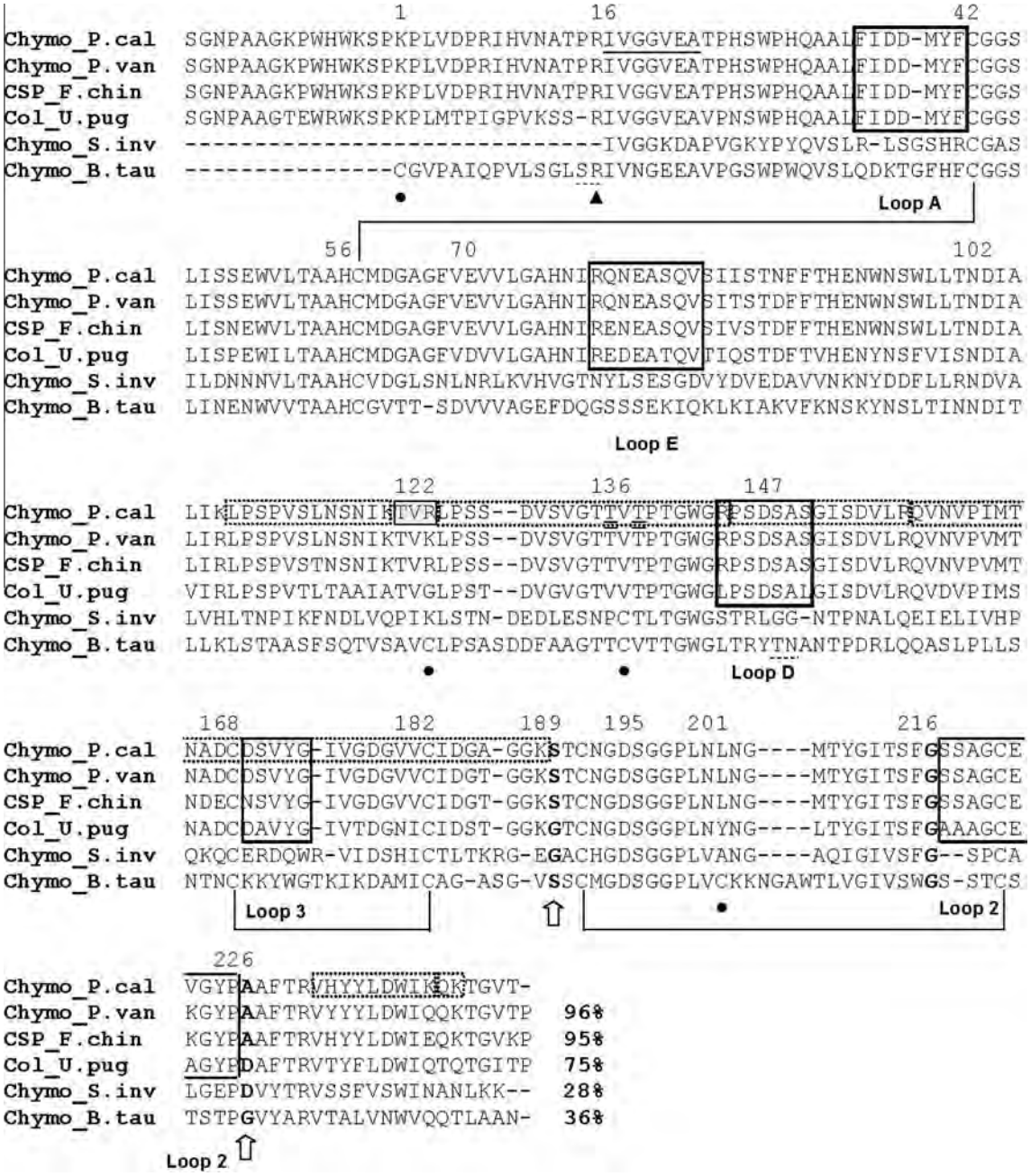

Fig. 2. Amino acid sequence alignment of serine proteases with chymotrypsin and/or collagenolytic activity. Footnote: *P. californiensis* chymotrypsinogen isoform 20 (Chymo_P.cal), *P. vannamei* chymotrypsinogen BI (Chymo_P.van), *F. chinensis* chymotrypsin-like serine protease (CSP_P.chin), *U. pugilator* serine collagenase 1 (Col_U.pug), *S. invicta* chymotrypsin (Chymo_S.inv) and *B. taurus* chymotrypsinogen (Chymo_B.tau). Chymotrypsinogen numbering system is used (Hartley & Neurath, 1970). Solid circles denote non-conserved cysteine residues and lines between cysteines indicates conserved disulfide bonds. The amino acid residues of the specific pocket are in bold; non-conserved residues are marked with an arrow. Solid triangle indicates conserved trypsin cleavage site and residues that are released during bovine chymotrypsinogen activation are dotted underlined. Loops involved in collagen recognition by crustaceans are indicated with boxes. N-terminal sequence of *P. californiensis* chymotrypsin obtained by Edman reaction is underlined. Six peptides from the intact shrimp chymotrypsin in dotted boxes were confirmed by mass spectrometry; residues not found in the stored shrimp chymotrypsin are in a grey box. Potential glycosylated residues are double underlined. Percentages of identical amino acids (compared to the sequence of Chymo_P.cal) are indicated at the end of the sequences.

Table 1
Biochemical and catalytic properties of chymotrypsins.

Species	pI	K_M (mM)	k_{cat} (s^{-1})	$10^{-5} \times k_{cat}/K_M$ ($M^{-1}s^{-1}$)	The effect of Ca^{2+} relative activity (%)	The effect of inhibitors Inhibitory activity (%)			
						Chymostatin	SBTI	PMSF	TPCK
P. californiensis	3.6	0.12	21.5	1790	160	66	96	100	1
Bos taurus	8.7	0.08	60.0	7500	120	96	1	95	56

shrimp chymotrypsin (see below) and only one polypeptide chain was found (Fig. 1A, lane 2 and Fig. 1C, lanes 2, 4, and, 6).

3.2. Primary structure of shrimp chymotrypsin

To further study some structural characteristics of shrimp chymotrypsin, we assayed it by gel electrophoresis and compared it to bovine chymotrypsin. Bovine and shrimp control samples (Fig. 1B and C, lanes 1) were diluted in the loading buffer containing SDS. Treatments included denaturation by boiling (lanes 2), reduction (lanes 3), denaturation by boiling and reduction (lanes 4), alkylation without boiling (lane 5) and, alkylation with boiling (lane 6). Iodoacetamide was used to avoid disulphide bonding after reduction.

For bovine chymotrypsin, a single band of 28 kDa was observed, either unboiled (Fig. 1B, lane 1) or boiled (Fig. 1B, lane 2); however, three bands, about 13, 16, and 18 kDa, were observed when bovine chymotrypsin was exposed to DTT (Fig. 1B, lanes 3 and 4). This indicates that active bovine chymotrypsin is composed of three polypeptides joined by disulfide bonds, which agrees with the mechanisms of maturation of chymotrypsinogen in mammals. Bovine chymotrypsinogen is activated by limited hydrolysis at Arg15-Ile16 to release the activation peptide, the so-called A chain; the activation peptide remains covalently anchored by disulfide bond Cys 1:122. Then, a second limited hydrolysis, this time autohydrolysis, releases Thr147-Asn148 (Fig. 2, dotted underline), generating the so-called chains B and C. These two peptides are held together by a disulphide bond; Cys136 in chain B reacts with Cys201 in chain C; the whole mechanism yields three interwoven peptides that form the active chymotrypsin.

As mentioned earlier, after five months, two bands appeared in pure shrimp chymotrypsin during gel electrophoresis (Fig. 1C, lane 1), as well, when this sample was treated with DTT (Fig. 1C, lane 3) and, treated with DTT and iodoacetamide (Fig. 1C, lane 5). These proteins have molecular masses of 35.7 and 33.4 kDa. The 35.7 kDa protein seems to be the intact shrimp chymotrypsin (Fig. 1A, lane 1). The other protein (33.4 kDa) was also sent for mass spectrometry analysis and five peptides were obtained. Peptide sequences matched with the intact pure shrimp chymotrypsin, except that residues 120–122 were missing (Fig. 2, grey dotted box), probably related to technical problems. Otherwise, another two peptides should have appeared in gel electrophoresis because disulfide bonds are not interconnected. The smaller protein could be a consequence of autolysis of the pure shrimp chymotrypsin.

When stored pure shrimp chymotrypsin was boiled (Fig. 1C, lane 2), treated with DTT and boiled (Fig. 1C, lane 4) or, treated with DTT, iodoacetamide and, boiled (Fig. 1C, lane 6), a single band, about 27 kDa, was observed. There were no differences in the mobilities between the reduced and non-reduced samples or intact and stored pure shrimp chymotrypsin if they were boiled (Fig. 1A, lane 2; 1C, lanes 2, 4 and 6). Instead of auto-activation by bovine chymotrypsinogen (Tyr146 and Asn148), shrimp chymotrypsin has residues not suitable for recognition at the autolytic site (Asp146 and Ala148); besides, Ser147 and Ala148 were detected by mass sequencing. Shrimp chymotrypsin also lacks the activation peptide and the mature protein begins at residue Ile16 (Fig. 2). To discard the SDS influence, stored pure shrimp chymotrypsin was analysed in a native gel. Again, even with DTT, two proteins were found (Fig. 1D, lane 1). When this sample was boiled, their mobility changed and remained separated (Fig. 1D, lane 2). When SDS was used, both proteins had the same net negative charge and migrated as a single band; however, in a native gel, a small difference in charge can be noticed.

3.3. Structural stability: effects of pH, temperature, and calcium ions on catalytic activity

Shrimp chymotrypsin (Fig. 3B) was differently affected by time and pH compared to bovine chymotrypsin (Fig. 3A). Shrimp chymotrypsin remained fully active for 120 min between pH 5 and

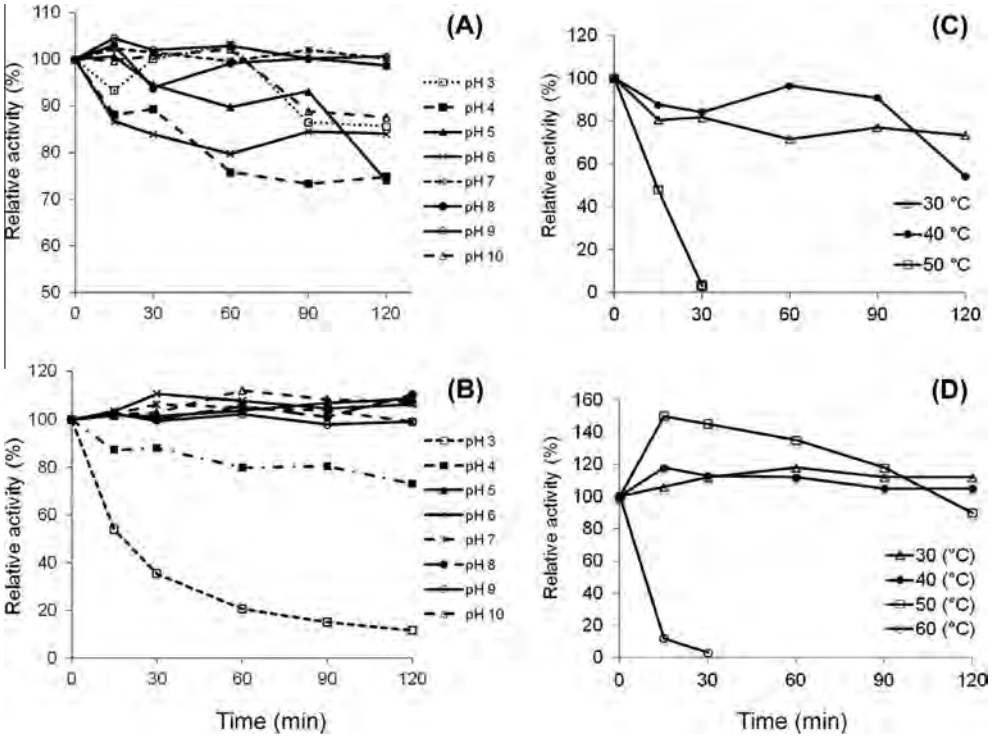

Fig. 3. Effect of pH and temperature on activity. Footnote: For pH: bovine chymotrypsin (A) and shrimp chymotrypsin (B). For temperature: bovine chymotrypsin (C) and shrimp chymotrypsin (D). Statistics with standard values are provided as supplementary material (*Table* 2).

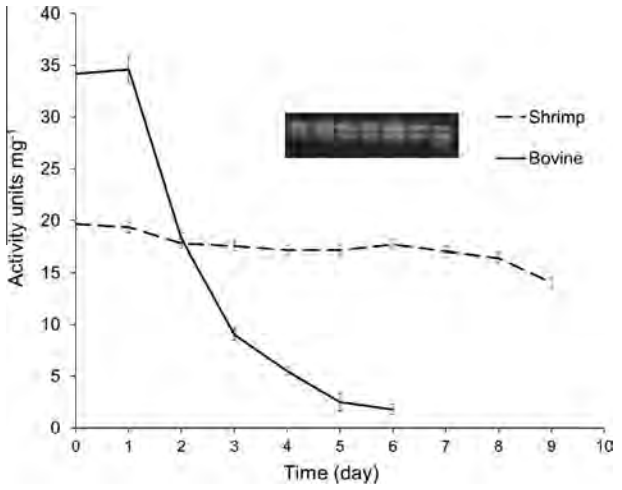

Fig. 4. Activity of bovine and shrimp chymotrypsin during storage at 25 °C. Footnote: Insert shows that both forms of shrimp chymotrypsin remain active at room temperature for up to eight days. Samples were not heated before electrophoresis.

10 (Fig. 3B). At pH 4, it retained 75% activity for 120 min. At pH 3, 50% of its activity was lost after 15 min. Invertebrate chymotrypsins loses activity at pH below 5 because of their relatively low structural stability to this variable (Tsu & Craik, 2004). Another serine protease, from the crayfish, *Astacus leptodactylus*, shows similar behaviour (Molnár et al., 2013). Sensitivity to low pH could be used to control this shrimp chymotrypsin in biotechnological processes or to stop deleterious reactions.

When comparing the effect of time and temperature on bovine and shrimp chymotrypsins, striking differences were found. For the bovine enzyme, higher temperatures immediately reduced activity, compared to room temperature (Fig. 3C). At 120 min, between 20% and 40% of its activity was lost at 30 °C and 40 °C and it was reduced to zero at 50 °C in 30 min. Shrimp enzyme activity increased by 50% at 50 °C in the first 30 min (Fig. 3D). After 120 min at 50 °C, the enzyme retained 90% of its starting activity. After 120 min at 30 and 40 °C, it remained almost unchanged. At 60 °C, the shrimp enzyme was inactivated in 30 min. Thermostability may be a useful characteristic of *P. californiensis* chymotrypsin in processes at room temperature or at moderately high temperatures.

Serine proteinases bind calcium for stability, to protect against autolysis and hydrolysis by co-synthesised enzymes, as in the case of human cationic trypsin (Szmola & Sahin-Tóth, 2007). This was assayed by the effect of 20 mM $CaCl_2$ on the enzymes. Bovine chymotrypsin activity increased by 20%, but shrimp chymotrypsin increased by 60% (Table 1). Ca^{2+} binds to acidic residues in bovine chymotrypsin (Adebodun & Jordan, 1989) and shrimp chymotrypsin shares (with bovine chymotrypsin) Asp153 for calcium-binding. However, shrimp chymotrypsin possesses more unique acidic sites (Asp37, 38, 60, 146, 167, 169 and, 184; Glu50, 65, 77, 92 and, 221), than does bovine chymotrypsin, that can bind calcium and be more active than bovine chymotrypsin. The higher content of Asp and Glu makes shrimp chymotrypsin exceptionally acidic, as demonstrated by the pI of 3.6 (Table 1).

Stability is a crucial factor when using proteinases in biotechnology. To further compare the bovine and shrimp chymotrypsins, the effect of storage at 25 °C of enzyme preparations for several days was assessed. Bovine chymotrypsin was fully active for 1.5 days and then decreased sharply until day 6. Shrimp chymotrypsin gradually declined until day 9 but still retained >50% activity (Fig. 4). As with the giant river prawn collagenase (Sriket, Benjakul, Visessanguan, & Kishimura, 2011) and its trypsin (Sriket et al., 2012), shrimp chymotrypsin could cause softening of the flesh if proper cold storage is not applied. From a biotechnological viewpoint, this shrimp chymotrypsin could be a candidate for processes where anionic detergents are involved because SDS do not affect their function.

3.4. Catalytic parameters and sensitivity to different chymotrypsin inhibitors

Bovine and shrimp chymotrypsins were compared for catalytic variables on the synthetic substrate, SAAPFNA (Table 1). Shrimp chymotrypsin had three times less catalytic turnover (k_{cat}) and four times less catalytic efficiency (k_{cat}/K_M) than had bovine chymotrypsin. Although TPCK is a well-known synthetic and irreversible inhibitor of mammalian chymotrypsins, it only slightly inhibited shrimp chymotrypsin, as in other crustacean chymotrypsins (Hernández-Cortés et al., 1997; Tsai, Liu, & Chuang, 1986; Tsai, Lu, & Chuang, 1991). TPCK is an inhibitor of chymotrypsins because it possesses a Phe residue at P1 that is recognised by the enzyme. It reacts covalently and irreversibly with His at the catalytic site. Crustacean chymotrypsins have low catalytic efficiency (<0.1) toward single residue ester substrates (Tsai et al., 1986, 1991; Tsu & Craik, 1996), but compensate by binding extended polypeptide substrates. This could explain why TPCK has no effect on crustacean chymotrypsin (Hernández-Cortés et al., 1997; Tsai et al., 1986, 1991), whilst oligopeptide or proteinaceous inhibitors do.

To further compare shrimp and bovine chymotrypsins, the rate of inhibition by assorted proteinase inhibitors was assayed; one is a protein and the others are synthetic proteins that mimic substrates. Table 1 shows that the four inhibitors have different effects on proteinases. Chymostatin is specific for chymotrypsins and inhibits the bovine enzyme but is less effective on shrimp chymotrypsin activity. PMSF inhibits all serine proteinases, including trypsins and chymotrypsins, which was the case in our study. It almost completely inhibited bovine and shrimp chymotrypsins. PMSF and SBTI inhibited shrimp chymotrypsin because this enzyme belongs to the S1 family. What was unexpected is that SBTI inhibited shrimp chymotrypsin, but failed to inhibit the bovine chymotrypsin. TPCK only partly inhibited the bovine chymotrypsin and did not inhibit shrimp chymotrypsin.

3.5. Hydrolysis of proteinaceous substrates

Bovine chymotrypsin partly hydrolysed BSA (Fig. 5 BSA, lanes B1, B1, B3), which occurred after hour 1 and remained the same until hour 3. The main band of BSA, ~66 kDa, was hydrolysed immediately, yielding a polypeptide, ~50 kDa. Shrimp chymotrypsin produced smaller polypeptides than did BSA (Fig. 5 BSA, lanes S1, S2, S3). After an hour 1 reaction time, lower molecular mass bands were present, mainly one at ~25 kDa. At hour 3 of reaction time, a polypeptide, ~50 kDa, was present, but not at hour 1 and 2 reaction time. We think this band was further hydrolysed to yield the lower molecular weight bands. The broader specificity of shrimp chymotrypsin may be related to structural elements that are distant from the substrate binding sites that are crucial determinants of specificity.

Another distinctive difference between shrimp and bovine chymotrypsin is the capacity of shrimp chymotrypsin to cleave native collagen. Some crustacean serine proteinases developed the capacity to hydrolyse the main connective tissue protein, the highly fast collagen. Collagen fastness comes from the structural organisation of cross-linking proteins, which makes them also highly resistant to proteolytic enzymes. Most collagenases are metallo-proteinases from bacteria and vertebrates. Collagenases, mostly in decapods, are synthesised in the digestive gland and digest connective tissue

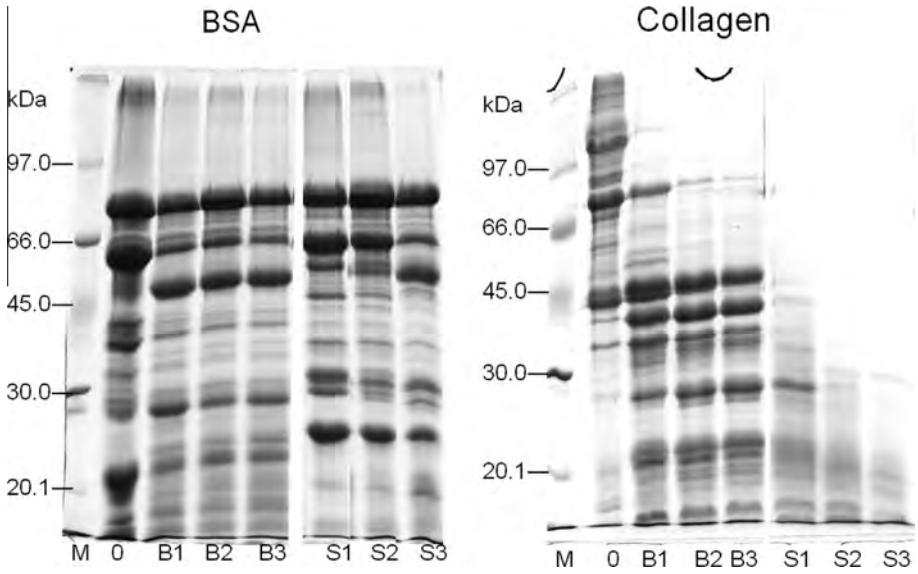

Fig. 5. Hydrolysis of proteinaceous substrates. Footnote: The progress of hydrolysis of BSA and rat tail collagen is followed for 3 h; M is MW markers; 0 is the substrate before adding the enzyme; B1, B2, and B3 are bovine chymotrypsin and S1, S2, and S3 are shrimp chymotrypsin.

in food. Penaeid shrimp prey on animals, hence digestion of connective tissue is paramount for obtaining amino acids. Crustacean collagenases were first described in the Brachyura, the subgroup of true crabs; serine proteinases with collagenolytic activity are called "brachyurins" (Rudenskaya, 2003; Tsu & Craik, 2004). However, the term must be analysed carefully because the protease purified in this work has all the characteristics of a chymotrypsin that can hydrolyse collagen.

Bovine chymotrypsin partially hydrolysed type VII collagen (Fig. 5 Collagen, lanes B1–B3), but shrimp chymotrypsin fully hydrolysed the connective protein. Only a few low molecular weight polypeptides remained after three hours of hydrolysis (Fig. 5, Collagen, lanes S1–S3). No doubt, *P. californiensis* chymotrypsin possesses a collagenolytic capacity, which is compatible with a biological function of digesting animal food. Previously, we mentioned that bovine chymotrypsin showed higher activity when the effect of pH and temperature on enzyme activity was analysed. For that analysis, the synthetic substrate SAAPFNA was used. With the use of natural proteinaceous substrate, it is obvious that the shrimp chymotrypsin is better suited, than is the bovine chymotrypsin, to deal with proteinaceous substrates. This is tied to physiological function and derives from the three-dimension structure.

Some brachyurins have Gly189 and Asp226, compared to Asp189 and Gly226 in the binding pocket of trypsin (Tsu & Craik, 1996). These mutations significantly contribute to determining the broad substrate specificity of such serine proteases (Tsu, Perona, Fletterick, & Craik, 1997). Because of specificity to basic, polar, and hydrophobic amino acids, brachyurins are the only serine proteases capable of hydrolysing collagen (Tsu, Perona, Schellenberger, Turck, & Craik, 1994). We demonstrated that shrimp chymotrypsin hydrolysed collagen. Another protein substrate, BSA, was extensively hydrolysed by shrimp chymotrypsin, compared to the activity of bovine chymotrypsin. This indicates broader substrate specificity. For brachyurins, loop interactions seem to be important for collagen recognition (Perona, Tsu, Craik, & Fletterick, 1997). The shrimp chymotrypsin structure that is deduced from the amino acid sequence is similar to that of crab brachyurin in those regions (Fig. 2, boxes), which include loops A (100%), E (75%), D (71%), 2 (70%), and 3 (80%) (Tsu et al., 1997) where percentage represents similarity between shrimp chymotrypsin and crab brachyurin at the residues that formed those loops. Most substitutions are conserved or semi-conserved, keeping their interaction with collagen. Even though shrimp chymotrypsin has an amino acid composition (at the specific pocket) similar to bovine chymotrypsin (Ser-189, Gly-216 and Gly/Ala-226); it can hydrolyse collagen and has broader substrate specificity, but it does not recognise Arg as P1 as does crab brachyurin. Ant chymotrypsin has the same amino acid composition in the specificity pocket as crab brachyurin (Gly189, Gly 216 and, Asp226), but the collagen recognition loops have A (0%), E (20%), D (14%), 2 (37%) and, 3 (2%) identity between bovine and shrimp chymotrypsins. Similar cases occur with a lepidoptera chymotrypsin (Herrero et al., 2005) and a beetle chymotrypsin (Broehan et al., 2010). Both enzymes have Gly189, Gly216 and, Asp226 in the specificity pocket, but low identity at the collagen recognition loops. It seems that those loops are responsible for collagen recognition, whatever residues are at the specific pocket. However, it is unknown how they are involved in the proteolysis of other substrates. Because shrimp chymotrypsin can hydrolyse simple proteins as extensively as BSA or complex ones like collagen, this kind of shrimp protease easily digests myofibrillar proteins. Even during ice storage, shrimp muscle quality can deteriorate in a few days (Okpala, Choo, & Dykes, 2014). Although shrimp muscle collagen was not tested as a substrate, it is expected that it can be hydrolysed by shrimp chymotrypsin, as other crustacean collagenases hydrolyse their own muscle-soluble collagen and other collagen sources (Sriket et al., 2012).

4. Conclusions

Based on comparisons of amino acid sequences and the ability to hydrolyse chymotrypsin substrates, we identified a serine protease isolated from *P. californiensis* as a chymotrypsin. Compared to bovine chymotrypsin, shrimp chymotrypsin is more stable at higher temperatures (50 °C); sensitive to low pH, possesses an acidic pI and, is activated by calcium. The mature enzyme is a single polypeptide chain, activated in a single step. These characteristics match the primary structure and have implications for post-harvest properties and biotechnological uses, including food processing. Structural features and broad substrate specificity,

including chymotryptic and collagenolytic activity, of the *P. californiensis* protease, have been demonstrated and linked to its primary structure.

Conflict of interest

Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.09.160.

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