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ABSTRACT

Two cystatins (cst-I and cst-II) were purified from crucian carp eggs by acidification, and chromatography on CM-Sepharose and Sephacryl S-100 HR. The molecular masses of cst-I and cst-II analyzed on SDS-PAGE were 11.9 and 14.4 kDa under reducing condition, while those were 13.5 and 12.7 kDa under non-reducing condition. The cst-I and cst-II were stable after 30 min of incubation respectively within 60°C and 50°C. No significant loss in the inhibitory activity of both cst at pH range of 4-11. They could affect the proteolysis of papain, cathepsin L and bromelain, but could not inhibit cathepsin B and trypsin. The partial N-terminal amino acid sequences of both cst inhibitors were homologous and that of cst-I was recognized as NH_2-AGIPGGLVDADINDADVQ. This fragment shared 88.9% identity to common carp cystatin and 44.4-55.6% to cystatins of other aquatic animals. Therefore, the two cst inhibitors were members of family 2 cystatin.

Keywords: *Carassius auratus*; crucian carp; cystatin; cysteine proteinase inhibitor; eggs.

INTRODUCTION

Cystatin superfamily comprises a number of cysteine proteinase inhibitors that are widely distributed in vertebrate and plant tissues. These proteinaceous inhibitors have been subdivided into three individual families on the basis of their size and structure. Members of families I and II are also respectively recognized as stefin and cystatin families. Family III is larger glycoproteins (60-120 kDa), previously known as kininogen existing in blood plasma [1-4]. Inhibitors of families I and II are structurally related but differ in certain aspects. The stefin family has a polypeptide of about 100 residues without disulfide bridges, whereas the cystatin family is somewhat longer, approximate 120 residues, and has two disulfide bridges [1-3]. Chicken cystatin, human cystatin C and rat cystatin S were the well-characterized members of family II [5-8]. Moreover, the cysteine proteinase inhibitors occurred in rice, corn and potato revealed a higher homology to family II cystatin in amino acid sequence [9-11]; however, the lack of intra-disulfide bridges among them was somewhat similar to stefin. Therefore, the cystatin inhibitors originated from plants should be separately defined as a new family, phytocystatin [12].

These cystatins can inactivate lysosomal cysteine proteinases such as cathepsins B, H, and L, as well as several structurally related plant proteinases such as papain and actinidin, by forming a tightly equimolar complex. The enzyme-inhibitor complex has a very low dissociation constant of 20 nM-10 fM, which effectively blocked the reactions of target proteinases [5-7, 13-15].

They are thus considered to be the physiological regulators for cysteine proteinases [16-17]. Bode *et al*. [18] and Stubbs *et al*. [19] proposed the docking model interaction between papain and chicken cystatin or recombinant human stefin. They further identified the possible binding regions between papain and these inhibitors. Cystatin has a central well-conserved motif of 53-QVVAG-57 (number in chicken cystatin), an N-terminus of 9-GA-10, and a C-terminus of 103-PW-104, which can provide the substrate-like binding edges to papain. Accordingly, these proteins can hinder the active cleft of proteolytic area, and consequently inactivates the papain proteolysis with a competition manner [18]. Arai *et al*. [20] further confirm the necessity of the central motif of the cystatin superfamily for inhibiting cysteine proteinases.

The cystatins have become more attractive for utilization on food processing and protection of agricultural crops. For instance, the autolysis of arrowtooth flounder flesh and gel softening of surimi-based products could be prevented by various cystatins [21-22]. On the other hand, the pesticidal effect of soybean cystatin on a virulent insect, western corn rootworm; and the antifungal activity against *Trichoderma reesei* by sugarcane cystatin were apparently observed [23-24]. These results suggested that the cystatins had potential for improving the cysteine proteinase-related issues. Therefore, the study aims to purify and characterize the cysteine proteinase inhibitor from crucian carp eggs.

MATERIALS AND METHODS

Materials. Papain (2-fold crystallized), trypsin, cathepsin B (from bovine spleen), cathepsin L (from human liver), E-64 (1-trans-epoxysuccinyl-leucylamino-4-guanidinobutane), Nα-benzoyl-DL-arginine-2-naphthylamide (BANA), and *p*-dimethylaminocinnamaldehyde were purchased from Sigma Chemical Co. (MO, USA). Bromelain was commercially available from Merck (Dermstadt, Germany). Benzyloxycarbonyl-phenylalanylarginine-7-(4-methyl)coumarylamide (Z-Phe-Arg-MCA) was obtained from Peptide Institute (Osaka, Japan). CM-Sepharose FF, Sephacryl S-100 HR, and low molecular mass calibration kit were products of Amersham Biosciences (Uppsala, Sweden). Live crucian carp (*Carassius auratus*) was purchased from a local fish market in Tainan.

Purification. The eggs obtained from crucian carp was homogenized with 5 volume of 20 mM Tris-HCl buffer, pH 7.5 containing 10 mM EDTA. After 20 min of centrifugation at 15000 x*g*, the floating lipid was removed. The homogenate was acidified to pH 4.0 with 1 N HCl and the acid-precipitated proteins were eliminated by 20 min of centrifugation at 40000 x*g*. The treated sample was adjusted to pH 8.8 by using 1 N NaOH and dialyzed against 30 mM Tris-HCl buffer, pH 8.8 containing 2 mM EDTA and 0.01% NaN_3 overnight. Proteins precipitated during dialysis were discarded by centrifugation at 40000 x*g*. For chromatography, crude cystatin solution was loaded onto a CM-Sepharose FF (2.6 x 18 cm), which pre-equilibrated with the dialysis buffer. The flow rate and collection were 1.5 ml/min and 10 ml/fraction. Until the absorbance of column elute determined at 280 nm

reached the baseline, a linear gradient of 0-0.5 M NaCl in the same buffer was applied. Fractions with papain inhibitory activity were pooled and concentrated to a minimal volume using ultrafiltration (cutoff: 10 kDa, Millipore, MA, USA). The concentrated sample was chromatographied on a Sephacryl S-100 HR (1.6 x 75 cm) which pre-equilibrated with 20 mM Tris-HCl buffer, pH 8.0 containing 2 mM EDTA and 0.01% NaN_3. Flow rate and collection were 0.5 ml/min and 1.6 ml/fraction. Two fractions containing the papain inhibitory activity were pooled, dialyzed against 10 mM Tris-HCl buffer, pH 7.5 and concentrated. Furthermore, the second inhibitor fraction eluted from Sephacryl S-100 was chromatographied again on the same column as described above. Gel filtration low molecular mass calibration kit (conalbumin, 75 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa; ribonuclease A, 13.7 kDa; and aprotinin, 6.5 kDa) was used as standard.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Inhibitors in sample buffer (62.5 mM Tris-HCl buffer, pH 6.8, containing 3% SDS and 0.002% bromophenol blue) with or without 5% β-mercaptoethanol (β-Me) was heated at 95°C for 5 min. SDS-PAGE was performed on a 15% of polyacrylamide slab gel according to the method of Laemmli [25]. After electrophoresis, the proteins were fixed in gel by using 12% trichloroacetic acid and then stained with Coomassie Brilliant Blue G-250 [26]. Low molecular mass calibration kit (phosphorylase b, 94 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; α-lactalbumin, 14.4 kDa) was used as protein marker.

Protein concentration. Protein concentration was determined by dye-binding method [27]. Bovine serum albumin was used as a standard protein.

Assay of inhibitory activity. Papain was used as proteinase for the assay of inhibitory ability of cystatin during purification, which was further purified according to the method of Machleidt *et al*. [28]. The concentration of papain was determined by active-site titration with E-64 as described by Barrett and Kirschke [29]. The inhibitory activity of cystatin was assayed indirectly by measuring the residual papain activity using BANA as substrate [30]. Papain (0.43 nmol) in 0.2 M sodium phosphate buffer, pH 6.5, containing 8 mM β-Me and 2 mM EDTA, with or without cystatin was pre-incubated at 40°C for 10 min. The enzyme mixture (0.2 mL) comprised 10 g of papain, 0.1 ml 200 mM sodium phosphate buffer (pH 6.5, containing 8 mM β-Me and 2 mM EDTA) and 0.05 ml of cystatin. Reaction was initiated by adding 50 μl of 10 mM BANA and stopped by adding 0.5 ml mixed reagent (0.1% *p*-dimethylaminocinnamaldehyde/2% HCl/methanol). After 30 min of color development at room temperature, the absorbance at 540 nm was measured by using a spectrophotometer (Hitachi U-2800A, Japan). One unit of inhibitory activity was defined as the amount of cystatin that could inhibit 1 unit of proteolytic activity of papain, whereas 1 unit of proteolytic activity was defined as the amount of papain that could hydrolyze BANA and increase one absorbance unit at 540 nm within 10 min of reaction at 40°C. Furthermore, the actions of purified cst toward bromelain (1.8 nmol), cathepsins B (3.2 nmol) and L (2.2 nmol) as well as trypsin (4.2 nmol) were

evaluated. In addition to the cathepsin L was determined by using Z-Phe-Arg-MCA as substrate [4]. The rest of target proteinases were assayed in the appropriate buffers by using synthetic substrate of BANA as described above.

Assessment of stability. Cystatins in 20 mM Tris-HCl buffer (pH 8.0) were incubated at 25, 30, 40, 50, 60, 70, 80 and 90°C for 30 min. After being cooled in ice water for 20 min, the remaining inhibitory activity was measured. For pH stability, cystatins in various buffers (50 mM citric acid-Na_2HPO_4, pH 2.6-7.5; 50 mM Tris-HCl, pH 7.5-8.5; 50 mM glycine-NaOH, pH 8.5-10.5; 50 mM Na_2HPO_4-NaOH, pH 11.0-11.5) were incubated at 40°C. After 1 h of incubation, an equal volume of neutralization buffer (0.2 M sodium phosphate, pH 7.0) was added and cooled in ice bath for 20 min. Then, the residual activity of the pH-treated cystatins was determined.

Protein sequencing. Purified cst-I and cst-II bands were excised from polyvinylidene fluoride (PVDF) membrane that had been electroblotted from a 15% SDS-PAGE under reducing condition. Their N-terminal amino acid sequences were resolved by using an automated Edman degradation sequencer (Applied Biosystems Procise 492, Foster City, CA, USA).

RESULTS

Purification. There was an abundance of soluble proteins occurred in the homogenate of crucian carp eggs. Because the cystatin had been examined to be a stable inhibitor family at a wide pH range, the acidification was thus utilized for denaturing the acid-labile proteins. After acidified to pH 4.0,

approximate 81% contaminant proteins were removed from the homogenate. Otherwise, there was 92% inhibitory activity left after this treatment (Table 1). The crude sample was then alkalized to pH 8.8 by using 1 N NaOH and followed by dialysis. The chromatography of crude cystatins in CM-Sepharose FF was shown in Figure 1, first inhibitor peak was eluted in the wash portion and the other one was bound to the column, which could be desorbed with 0.16 M NaCl in the equilibrium buffer. Because the first inhibitor peak displayed obviously brown color and also had more protein constituents than that of the bound fractions when analyzed on SDS-PAGE (Table 1; Figure 3, lanes 2 and 3). The inhibitor peak occurred in the bound fractions (tube number: 90-105) was therefore used for further isolation. Chromatography of the resulting sample in Sephacryl S-100 HR provided an efficient separation of contaminant and inhibitor proteins. According to Figure 2, one major (tube number: 60-66) and one descending shoulder (tube number: 67-70) peaks were observed among the inhibitory fractions, which were designated cst-I and cst-II respectively in this study. The cst-I was analyzed to be electrophoretic homogeneity by SDS-PAGE under reducing and non-reducing conditions (Figure 3, lanes 4 and 6). However, there was still a smear of cst-I band occurred among the cst-II fraction (data not shown). Hence, the re-chromatography of cst-II was carried out by using the same column and gave a single protein band on SDS-PAGE under both conditions (Figure 3, lanes 4 and 7). As shown in Table 1, the cst-I and cst-II were purified 312-fold and 273-fold and the yields of them were 28% and 3.6%

separately.

Molecular mass. The molecular masses of cst-I and cst-II were 11.9 and 14.4 kDa, as determined by SDS-PAGE under reducing (with β-Me) condition (Figure 3, lanes 4-5). However, under non-reducing (without β-Me) condition they were shown to be 13.5 and 12.7 kDa (Figure 3, lanes 6-7). When chromatographied on Sephacryl S-100 gel filtration column, the elution of these two cst protein peaks were observed near that of ribonuclease A, a calibration protein with molecular mass of 13.7 kDa (Figure 2). The data suggested that the native form of both cst inhibitors was monomer.

Stability. As shown in Figure 4, the inhibitory activity was almost left within 30 min of incubation at 60°C for cst-I or at 50°C for cst-II. Although the cst-II was somewhat heat-labile than cst-I, it could retain about 65% original activity at 60°C. When the incubation at higher than 70°C, both cst inhibitors were mostly inactivated. For pH stability, no significant loss in activities of both cst inhibitors were observed within pH range of 4.0-11.0, at 40°C (Figure 5).

Inhibition of proteinases. Proteolytic activity of papain was decreased by both cst inhibitors with a dose-dependent manner. The concentrations of cst-I and cst-II for inactivating 0.43 nmol of papain were estimated as 0.426 nmol and 0.443 nmol separately (Figure 6). The reaction ratios suggested that both cst inhibitors might have one papain-like binding domain within each molecule, which was in agreement with most cystatins and stefins [2-3, 18-19]. Moreover, they could affect the proteolysis of cathepsin L and bromelain, but could not inhibit cathepsin B and trypsin (Figure 7). These two cst proteins

therefore behaved as natural inhibitors for papain-like cysteine proteinases.

Protein sequence. To analyze the partial N-terminal amino acid sequences, the purified cst bands on SDS-PAGE (Figure 3, lanes 4-5) were blotted onto PVDF membrane, and then subjected to protein sequencing. A sequence of 18 amino acid residues in cst-I was identified as AGIPGGLVDADINDADVQ. The decapeptide sequence determined from cst-II was found to agree with that of cst-I. A search in Genbank databases revealed that cst-I fragment shared highest homology to common carp ovarian cystatin [32].

DISCUSSION

Two cysteine proteinase inhibitors, cst-I and cst-II, were purified from crucian carp eggs by the procedure described in this study. The molecular masses of cst-I and cst-II were shown to be 11.9 and 14.4 kDa on SDS-PAGE under reducing condition, whereas they were 13.5 and 12.7 kDa under non-reducing condition. Both cst had the molecular mass similar to those of family 2 cystatin originated from other species, such as chicken [5, 31], common carp [32-33], chum salmon [34], and rainbow trout [35].

The little difference of molecular mass occurred in both cst inhibitors between under reducing and non-reducing conditions could be due to the conformational change arose from the dissociation of native disulfide bonds. The β-Me treatment led to the molecular mass was decreased in cst-I, but it was increased in cst-II (Figure 3). This phenomenon suggested that part of peptide could be liberated from native cst-I after reduced with sulfide

reductant. However, the peptide piece was too small to be appeared on SDS-PAGE pattern, the molecular mass of cst-I was therefore lowered under reducing condition. The data speculated that cst-I was a nicked protein which had two peptides linking together by intramolecular disulfide bond. On the other hand, since the protein structure stabilized by disulfide bonds was unfolded while treated with sulfite reductant, the migration of cst-II on gel was slightly retarded. Thus, the cst-II could be a single peptide inhibitor comprising disulfide bonds. Although the opposite electrophoretic behavior of the two cst between under reducing and non-reducing conditions was observed, their partial N-terminal amino acid sequences were analyzed to be identical. It was possible that both cst inhibitors were derived from a common precursor, or the cst-I was proteolytically modified from the cst-II during oocyte maturation and/or purification. As reported by Tsai *et al*. [32], the cystatin obtained from ovarian fluid of common carp was shown to a nicked form, which was consisted of two peptides holding together by disulfide bond. In the previous study, however, its intact form was isolated directly from ovary tissue [33]. Comparison of the data implied that proteinase specific for cleaving cystatin could be accumulated within the ovulated fluid through the spawning stage of common carp. In spite of the peptide backbone was restrictedly cleaved, its inhibitory feature was still maintained by native intramolecular disulfide bonds of cystatin [32-33].

The characterization indicated that both cst had thermal stability similar to common carp cystatin [33] and Chinese sturgeon cystatin [36], nevertheless, it

was inferior to cystatins obtained from terrestrial organisms, such as chicken [22, 31] and tomato leaf [37]. The broad pH stability occurred among pig plasma kininogen [4], chicken recombinant cystatin [22, 31], tomato leaf cystatin [37], and rabbit muscle cystatin [38], was also observed in the two inhibitors. Comparison of the N-terminal sequences of the two cst with those of aquatic cystatins and the well-characterized chicken egg white cystatin was showed in Figure 8. The conserved glycine residue, which had been proposed to interact with the active cleft of papain-like proteinases [18-19], was observed at position 5 of both cst inhibitors. The cst-I fragment shared 88.9% amino acid sequence identity to common carp cystatin [32], 55.6% to horseshoe crab cystatin [39], 52.9% to Chinese sturgeon cystatin [36], 50.0% to rainbow trout [35] and zebra fish cystatins [40], 44.4% to chum salmon cystatin [34], and 33.3% to chicken cystatin [41]. This data elucidated the close relationship between crucian carp and common carp as well as confirmed that both cst inhibitors could be the members of family 2 cystatin.

According to the investigation of the underused fishes including mackerel [42-43], Pacific whiting [44], and chum salmon [45], it was indicated that catheptic cysteine proteinases resident in the lysosome of flesh could play the critical role not only in disintegration of myofibrillar proteins during postmortem also in softening of surimi gels. In order to make such fish proteins a valuable ingredient or basal component for a wide range of reconstituted food products, the action of endogenous proteinases that impaired the functionalities of muscle proteins should be prevented. Hence,

the cystatin was a preferable alternative for improving the protein degradation and the textural properties owing to its inhibition against autolytic proteinases [21-22]. Otherwise, the defensibility against digestive proteinases of coleopteran larvae [23, 46] and growth of fungi [24, 46] speculated that cystatin family inhibitors could protect crops from injuries caused by pest insects and pathogens during the cultivation or storage.

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Table 1. Purification of cst inhibitors from crucian carp eggs.

Procedure	Total protein (mg)	Inhibitory activity (units)*	Specific activity (unit/mg)	Yield (%)	Purification fold
Homogenate	8156.1	1320	0.16	100	1
Acidification (pH 4.0)	1517.7	1214	0.80	92	5
CM-Sepharose FF	81.3	473	5.82	36	36
Sephacryl S-100 HR					
cst-I	7.3	364	49.86	28	312
cst-II	1.3	56	43.08	4.2	269
2nd Sephacryl S-100 HR of cst-II	1.1	48	43.64	3.6	273

* One unit of inhibitory activity was defined as the amount of cystatin that could inhibit 1 unit of proteolytic activity of papain, whereas 1 unit of proteolytic activity was defined as the amount of papain that could hydrolyze BANA and increase one unit of absorbance at 540 nm within 10 min of reaction at 40°C.

Figure Captions

Fig. 1 Chromatography of cst inhibitors on CM-Sepharose FF. The column (2.6 x 18 cm) was equilibrated with dialysis buffer. The flow rate and collection were 1.5 ml/min and 10 ml/fraction. A linear gradient of 0-0.5 M NaCl in same buffer was developed. The fractions with papain inhibitory activity (indicated by bar) were pooled and concentrated

Fig. 2 Chromatography of cst inhibitors on Sephacryl S-100 HR (1.6 x 75 cm). The elution buffer was 20 mM Tris-HCl buffer, pH 8.0, containing 2 mM EDTA and 0.01% NaN_3. Flow rate and collection were 0.5 ml/min and 1.6 ml/fraction. Gel filtration low molecular mass calibration kit (conalbumin, 75 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa; ribonuclease A, 13.7 kDa; and aprotinin, 6.5 kDa) was used as standard

Fig. 3 SDS-PAGE analysis of cst inhibitors from crucian carp eggs. The acrylamide concentrations of stacking and resolving gels were 3.75 and 15% separately. Lane M, low molecular mass protein marker; lane 1, homogenate of eggs; lane 2, homogenate after acidified to pH 4.0; lane 3, cst inhibitors eluted in the bound portion of CM-Sepharose FF; lane 4, cst-I eluted from Sephacryl S-100 HR; lane 5, cst-II eluted from the second chromatography of Sephacryl S-100 HR; lane 6, cst-I without β-Me; lane 7, cst-II without β-Me

Fig. 4 Thermal stability of cst-I (○) and cst-II (●). Purified cst-I and cst-II in 20 mM Tris-HCl buffer, pH 7.5 containing 4 mM EDTA, were incubated at 25-90°C for 30 min. After being cooled in ice bath for 20 min, the residual

inhibitory activity was assayed

Fig. 5 pH stability of cst-I (hollow symbols) and cst-II (solid symbols). Purified cst-I and cst-II in pH buffers (50 mM citric acid-Na_2HPO_4, pH 2.6-7.5; 50 mM Tris-HCl, pH 7.5-8.5; 50 mM glycine-NaOH, pH 8.5-10.5; 50 mM Na_2HPO_4-NaOH, pH 11.0-11.5) were incubated at 40°C. After 1 h of incubation, an equal volume of 0.2 M sodium phosphate (pH 7.0) was added and cooled in ice bath for 20 min. The residual activity of the pH-treated cst inhibitors was determined

Fig. 6 Inhibition of papain by cst-I (○) and cst-II (●). Papain (0.43 nmol) was pre-incubated with cst inhibitors at room temperature for 10 min and the remaining proteolytic activities of papain were assayed by using BANA as substrate

Fig. 7 Effect of cst-I (hollow symbols) and cst-II (solid symbols) on bromelain, cathepsin B, cathepsin L and trypsin. Proteinases were pre-incubated with cst inhibitors at room temperature for 30 min respectively and the remaining proteolytic activities were assayed

Fig. 8 Comparison of the partial N-terminal amino acid sequences of cst inhibitors with those of common carp [32], horseshoe crab [39], Chinese sturgeon [36], rainbow trout [35], zebra fish [40], chum salmon [34], and chicken egg white [41] cystatins. Amino acids identical to those of cst-I were shadowed, and the boldface indicated the conserved residue among cystatin family. The sequences were numbered according to the original references, and the alignment gaps were represented by dashes

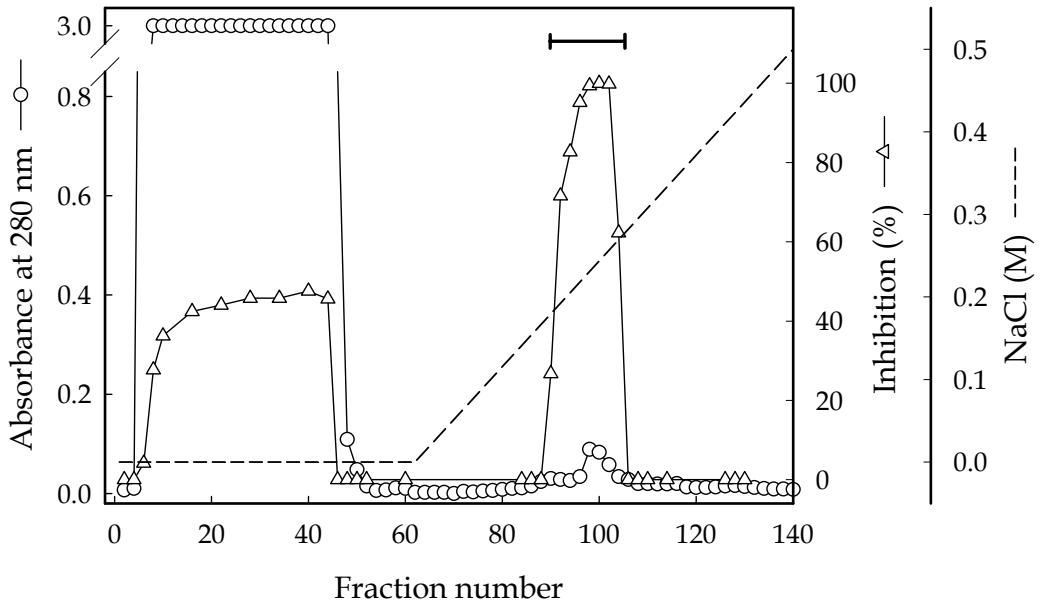
Absorbance at 280 nm
Inhibition (%)
NaCl (M)
Fraction number
3.0
0.8
0.6
0.4
0.2
0.0
100
80
60
40
20
0
0.5
0.4
0.3
0.2
0.1
0.0
0
20
40
60
80
100
120
140

Fig. 1

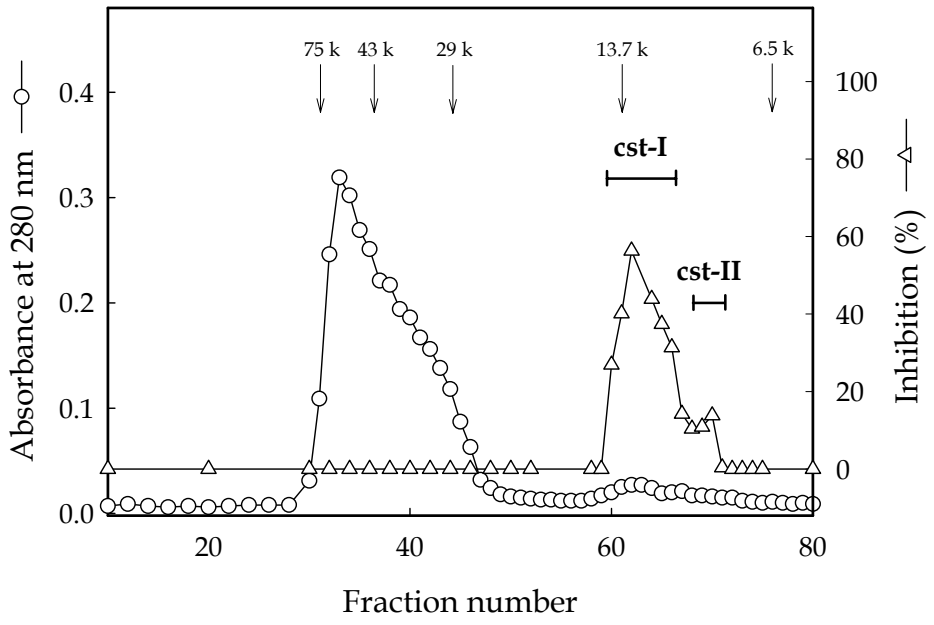
75 k
43 k
29 k
13.7 k
6.5 k
cst-I
cst-II
Absorbance at 280 nm
Inhibition (%)
Fraction number
0.0
0.1
0.2
0.3
0.4
0
20
40
60
80
100
20
40
60
80

Fig. 2

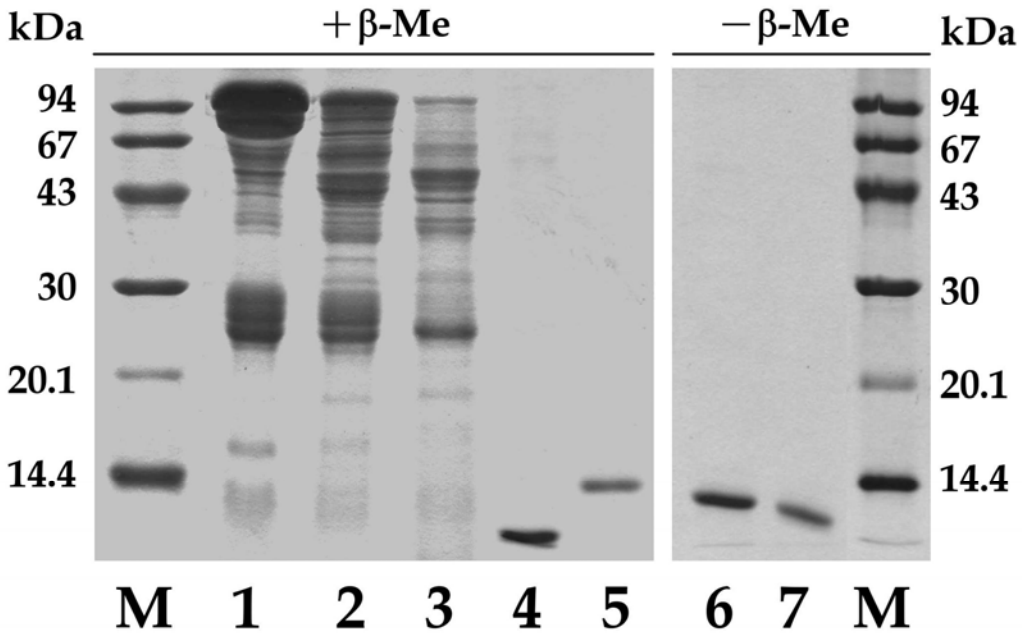

Fig. 3

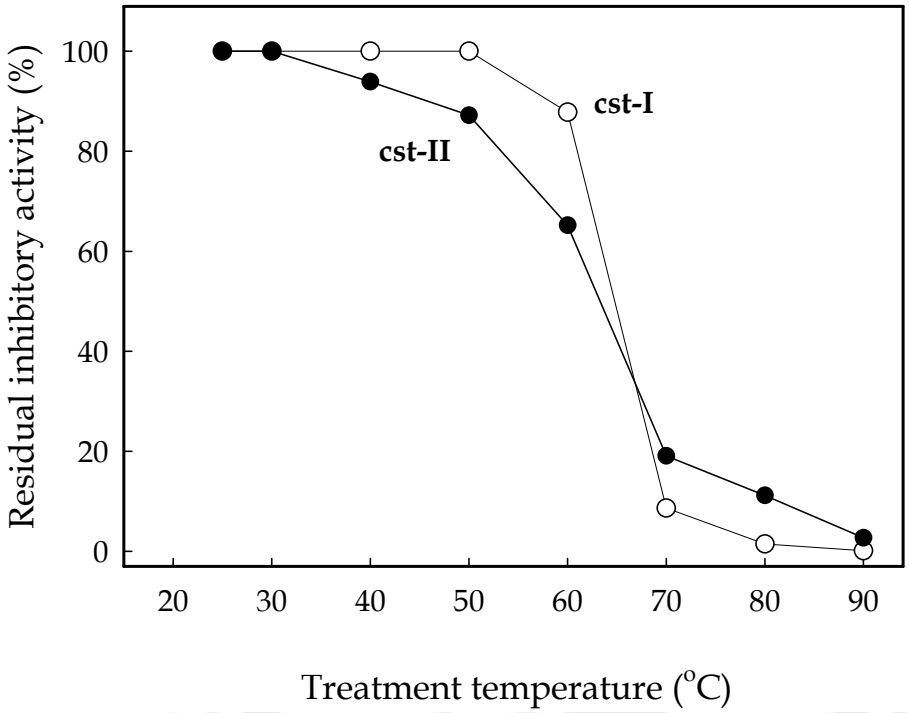
Residual inhibitory activity (%)
100
80
60
40
20
0
cst-I
cst-II
20
30
40
50
60
70
80
90
Treatment temperature (°C)

Fig. 4

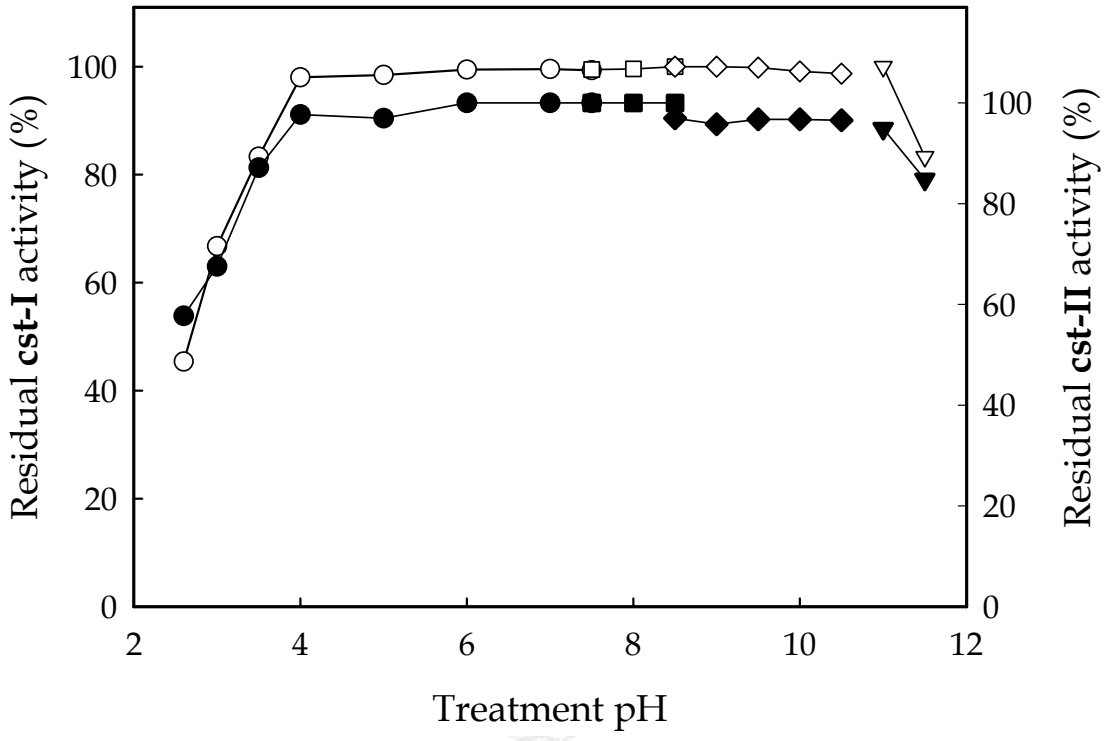
Residual cst-I activity (%)
Residual cst-II activity (%)
Treatment pH
0
20
40
60
80
100
2
4
6
8
10
12

Fig. 5

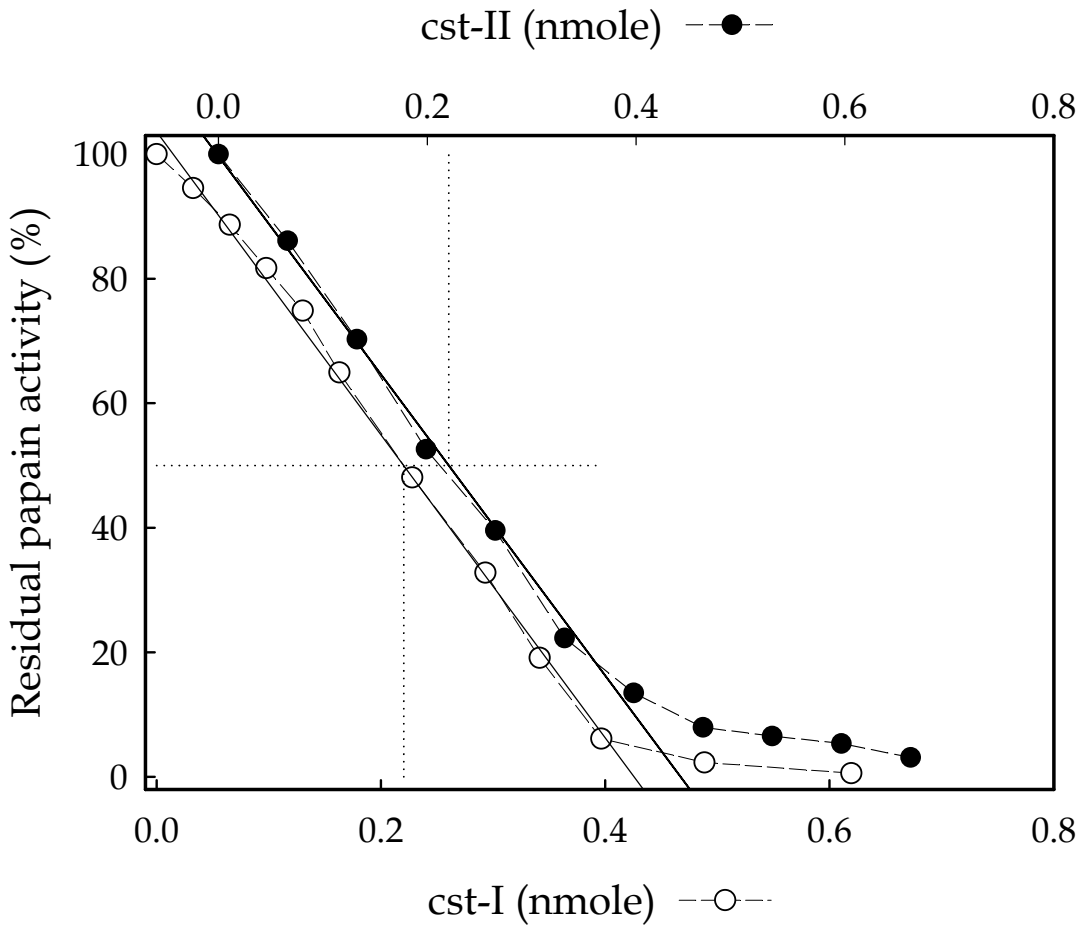
cst-II (nmole)
0.0
0.2
0.4
0.6
0.8
100
80
60
40
20
0
Residual papain activity (%)
0.0
0.2
0.4
0.6
0.8
cst-I (nmole)

Fig. 6

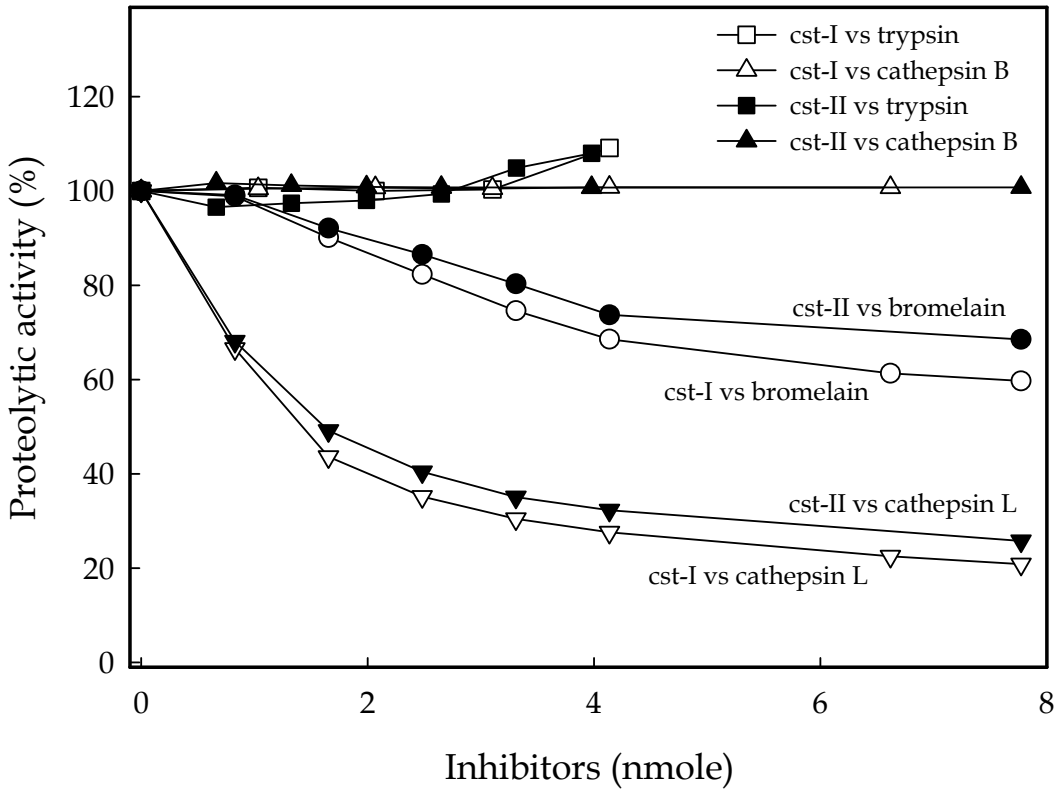
cst-I vs trypsin
cst-I vs cathepsin B
cst-II vs trypsin
cst-II vs cathepsin B
cst-II vs bromelain
cst-I vs bromelain
cst-II vs cathepsin L
cst-I vs cathepsin L
Proteolytic activity (%)
Inhibitors (nmole)
0
20
40
60
80
100
120
0
2
4
6
8

Fig. 7

Cystatins	Start site	Amino Acid Sequences	Identity (%)
Crucian carp cst-I	1	AG-IP**G**GLVDADINDADVQ	
Crucian carp cst-II	1	AG-IP**G**GLVDA	
Common carp	1	TG-IP**G**GLVDADINDKDVQ	88.9 (16/18)
Horseshoe crab	-1	GQIP**G**GWIDANVGDTDVK	55.6 (10/18)
Chinese sturgeon	1	GL-V**G**GPMDADIGEEGVQ	52.9 (9/17)
Rainbow trout	-1	AGLI-**G**GPMDANMNDQGTR	50.0 (9/18)
Zebra fish	19	AGL-V**G**GPTDADM-DKDSES	50.0 (9/18)
Chum salmon	-1	AGL-V**G**GPMDANMNDQGTR	44.4 (8/18)
Chicken egg white	5	SRL-L**G**APVPVDENDEGLQ	33.3 (6/18)

Fig. 8