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魚類激肽原純化、特性與分子選殖及其在食品上之應用研究

計畫類別：■ 個別型計畫　　□ 整合型計畫
計畫編號：NSC 94－2313－B－041－009－
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共同主持人：
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成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

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中文摘要

利用酸化處理（pH 4.0）、CM-Sepharose FF 與 Sephacryl S-100 HR 管柱層析的方式，從鯽魚未成熟卵中純化出兩種硫氫蛋白酶抑制劑，分別稱為 cst I 與 cst II。經由 MALDI-TOF 質量光譜儀，鑑定出 cst I 與 cst II 的分子量為 12258 與 12187；此外，更得知它們的 N 端胺基酸序列，分別為 AGIPGGLVDA 與 GIPGGLVDAD。電泳分析（SDS-PAGE）中，經硫氫乙醇（β-Me）處理後的 cst I 分子量增加，而 cst II 分子量卻減少。由此可知，這兩種抑制劑都含有分子內雙硫鍵結，同時它也是維持 cst II 蛋白質中兩個生肽鏈連接在一起的主結構。Cst I 在 60^{o}C、30 分鐘的加熱與 pH 4-11 的環境中，仍保存原有抑制劑活性；cst II 在 70-90^{o}C 的加熱過程後，保有比 cst I 更高的活性，同時可忍受 pH 3-11。這兩種抑制劑對木瓜酵素（papain）的抑制作用，呈現出劑量依存關係與 1：1 結合比例。除木瓜酵素， cst I 與 cst II 尚能微弱地抑制鳳梨酵素（bromelain），而無法對組織蛋白酶（cathepsin）B 與胰蛋白酶（trypsin）產生效用。最後，cst I 與 cst II 的胺基酸序列與鯉魚 cystatin 極為相近，並且類似於鮭魚與鱒魚 cystatin。

關鍵詞：硫氫蛋白酶抑制劑、純化、特性分析、鯽魚、卵細胞

ABSTRACT

Two cystatins (termed as cst I and II) were purified from immature eggs of crucian carp by acidification to pH 4.0, CM-Sepharose FF and Sephacryl S-100 HR chromatographs. The molecular mass (*M*) of cst I and II estimated on MALDI-TOF mass spectrometry were 12258 and 12187. In addition, their N-terminal sequences were determined to be AGIPGGLVDA and GIPGGLVDAD. The *M* raised in cst I and lowered in cst II after treated with β-Me were observed on SDS-PAGE. The data suggested that they had intramolecular disulfide bridges and the cst II could be a nicked protein. The cst I was stable at 60^{o}C for 30 min and pH 4-11, while the stability of cst II within 70-90^{o}C was better than that cst I and could tolerate pH 3-11. Both inhibitors showed a dose-dependent inhibition against papain and had one papain binding domain in each molecule. In addition, they had weak effect on bromelain, but not cathepsin B and trypsin. The comparison of sequence revealed that they were likely identical to common carp cystatin and had highly similarity with chum salmon and rainbow trout.

Keywords: Cysteine proteinase inhibitor, purification, characterization, crucian carp, *Carassius auratus*, 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 ~100 residues without disulfide bridges, whereas the cystatin family is somewhat longer, ~120 residues, and has two disulfide bridges (1-3). Chicken cystatin (5), human cystatin C (6-7) and rat cystatin S (8) were the well characterized members of family II. Moreover, the cysteine proteinase inhibitors occurred in rice (9), corn (10) and potato (11) showed a higher homology to family II cystatin in amino acid sequence; however, the lack of intra-disulfide bridges among them was somewhat similar to stefin. Therefore, the cystatin inhibitors of plant origins should be separately defined as phytocystatin family (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. The autolysis of arrowtooth flounder flesh and gel softening of surimi-based products could be prevented by various cystatins (21-22). Likewise, the pesticidal effect on a virulent insect, western corn rootworm (23) and the antifungal activity against Trichoderma reesei (24) were observed with soybean and sugarcane cystatins respectively. This study aims to purify and characterize the specific cysteine proteinase inhibitor from crucian carp eggs.

Materials and Methods

Materials. Papain (2-fold crystallized), trypsine, cathepsin B, E-64 (1-trans-epoxysuccinyl-leucylamino-4-guanidinobutane), *N*α-benzoyl-DL-Arginine-2-naphthylamide (BANA), *p*-dimethylaminocinnamaldehyde and mannose were purchased from Sigma Chemical Co. (MO, USA). Bromelain was commercially available from Merck (Dermstadt, Germany). 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 obtained from a local fish market in Tainan.

Purification. The immature eggs from comatose crucian carp was homogenized with 5 volumes of 20 mM Tris-HCl buffer, pH 7.5 containing 10 mM EDTA. After 20 min of centrifugation at 15000 g, the floating lipid was removed. The homogenate was acidified to pH 4.0 with 1 N HCl and the precipitated contaminant proteins were eliminated by 20 min of centrifugation at 40000 g. The treated sample was dialyzed against 30 mM Tris-HCl buffer, pH 8.8 containing 2 mM EDTA and 0.01% NaN_3 overnight and then centrifuged again. For chromatography, crude cystatin was loaded onto a CM-Sepharose FF (2.6 x 18 cm) which pre-equilibrated with dialysis buffer. The flow rate and collection were 1.5 mL/min and 10 mL/fraction. Until the absorbance at 280 nm of column elute 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 minimum volume using ultrafiltration (cutoff: 10 kDa; Millipore). 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 1.7 mL/fraction. Fractions containing the papain inhibitory activity were pooled, dialyzed against 10 mM Tris-HCl buffer, pH 7.5 and concentrated.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Cystatin in dissociating buffer (62.5 mM Tris-HCl buffer, pH 6.8, containing 3% SDS, 0.002% bromophenol blue and 5% β-mercaptoethanol) 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 of gels were fixed in 12% TCA and then stained with Coomassie Brilliant Blue G-250 (26). Low molecular mass (M) 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 and Saccharide content. Protein concentration was determined by dye-binding method (27). Bovine serum albumin was used as a standard protein. The total saccharide content of cystatin was measured with the phenol-sulfuric acid reaction (28). The mannose was employed as a standard saccharide.

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. (29). The concentration of papain was determined by active-site titration with E-64 as described by Barrett and Kirschke (30). The inhibitory activity of cystatin was assayed indirectly by measuring the residual papain activity using BANA (31). Papain in 0.2 M sodium phosphate buffer (pH 6.5), containing 8 mM β-mercaptoethanol 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 μg of papain, 0.1 mL 200 mM sodium phosphate buffer (pH 6.5) containing 8 mM β-mercaptoethanol and 2 mM EDTA and 0.05 mL of cystatin. Reaction was initiated by adding 0.05 mL of 10 mM BANA and stopped by adding 0.5 mL mixed reagent (2% HCl and 0.1% p-dimethylaminocinnam- aldehyde in methanol). After 30 min of color development at room temperature, the reaction solution was measured at 540 nm using a spectrophotometer (Hitachi U-2800A, Japan). In addition, the inhibitions of cystatin toward various proteinases were performed in 0.1 M sodium acetate buffer, pH 4.5 for bromelain and pH 5.0 for cathepsin B, and 0.1 M Tris-HCl buffer, pH 8.0 for trypsin. 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 1 unit of absorbance at 540 nm within 1 min of reaction at 40°C.

Assessment of stability. The cystatin in 20 mM Tris-HCl buffer (pH 8.0) was 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 the 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) were incubated at 40°C for 1 hr and the residual activity of treated cystatins were then assayed.

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry and Protein sequencing. The M was determined by MALDI-TOF mass spectrometry. Purified cystatin was syringe injected into a QSTAR XL Q-Tof mass spectrometer (Applied Biosystems, Foster City, CA, USA) and detected at 600-2000 *m/z*. The obtained data was analyzed with AnalystTMQS software (Applied Biosystems). For the N-terminus sequencing of protein, 10 pmole of cystatin was loaded and analyzed on an Applied Biosystems Procise Sequencer (Applied Biosystems).

Results and Discussion

Purification. Massive proteins were extracted from the immature eggs of crucian carp. The pH of crude extract was therefore adjusted to pH 4.0 by adding 1 N HCl. This acidification step could eliminate about 81% of unstable proteins and most of inhibitory activity was still left in solution (**Table 1**). Furthermore, the treated sample was chromatographied on a CM-Sepharose FF. There was about half of inhibitory activity loss in wash portion and only few proteins with highly activity were absorbed on this column at pH 8.8, which could be eluted out with ~0.2 M sodium chloride buffer (**Figure 1**). According to **Table 1**, this pooled fraction contained 35.8% of inhibitory activity and had merely several protein bands on SDS-PAGE (**Figure 3**, lane 3). The distribution of proteins would make the gel filtration chromatography more effective for isolation of low molecular mass cystatin. Two contiguous inhibitory activity peaks, I (fraction number 60-66) and II (fraction number 67-70), following the contaminant protein were occurred on Sephacryl S-100 HR chromatography (**Figure 2**). Analysis on SDS-PAGE showed that these two inhibitors were purified to electrophoretic homogeneity, which briefly termed as cst I and II (**Figure 3**, lanes 4 & 5). As summarized in **Table 1**, approximate 7.3 mg of cst I and 4.6 mg of cst II were obtained from eggs after acidification and two chromatographs.

Molecular mass (*M*). The *M* of cst I and II determined in MALDI-TOF mass spectrometry were 12258 and 12187 (data not shown). As analysis on 15% of SDS-PAGE in the absence of β-Me, they were showed to be about 13.5 and 12.7 kDa (**Figure 3**, lanes 6 & 7). Furthermore, the *M* slightly raised in cst I and lowered in cst II were also observed after treated with β-Me (**Figure 3**, lanes 4 & 5). The data implied that cst I had intramolecular disulfide bridge and cst II might be a nicked protein, the smaller peptide chain was thus separated through the disruption of disulfide bond. According to the data, crucian carp cst I and II had *M* similar to family 2 cystatin, such as chicken (*5*), common carp (*32-33*), rainbow trout (*34*) and chum salmon (*35*).

Stability. The inhibitory activities of both cst were almost remained during 25-60^{o}C incubation for 30 min. As represented on **Figure 4**, the residual activity of cst I within 70-90^{o}C was dramatically, even declined as <10%. However, it was gradually decreased in cst II and still had about 27% of the

original activity after 30 min of incubation at 90°C. For pH stability, these two inhibitors were stable at pH 4.0-11.0 and 3.0-11.0, respectively. Although they had the same resistance at alkaline pH, the cst I was showed to be more acid-labile compared with cst II (**Figure 5**). A previous study had reported that the common carp cystatin was stable at pH 6.0-11.0 and 70°C incubation for 5 min (*33*). Therefore, the stabilities of cystatins obtained in this study were much better than that of common carp.

Inhibition of proteinases. The proteolytic activity of papain was inhibited by both cst I and II with a dose-dependent pattern. The concentration of both inhibitors for inactivating 50% papain (0.43 nmole) was about 0.22 nmole (**Figure 6**). According to the data, it suggesting that there was one binding domain for papain-like cysteine proteinase occurred in both cst. In addition, they also revealed weak inhibitory action against bromelain. Although the excess amounts of both cst were added, the proteolytic activities of cathepsin B and trypsin were remained constant (**Figure 7**).

Protein sequence. The N-terminal sequence of crucian carp cst I was determined to be AGIPGGLVDA, while the first amino acid was truncated in cst II, which had sequence alignment of GIPGGLVDAD. The almost identical sequences implied that these two inhibitors could be derived from the same precursor. It is necessary for investigating whether the cst II was an autolytic product during purification or was the isoform of cst I. The cst I and II were homologous with common carp cystatin, this result further supported that these two species were classified as the same family, *Cyprinidae* (**Figure 8**).

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Tables and Figures

Table 1. Purification of cystatin 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					
peak I (cst I)	7.3	364	49.86	28	312
peak II (cst II)	4.6	72	15.65	5.5	98

* 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 1 unit of absorbance at 540 nm within 1 min of reaction at 40^{o}C.

Figure legends

Figure 1. Chromatography of cystatin on CM-Sepharose FF. The column (2.6 x 18 cm) was equilibrated with 30 mM Tris-HCl buffer, pH 8.8 containing 2 mM EDTA and 0.01% NaN_3. 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 were pooled and concentrated.

Figure 2. Chromatography of cystatin 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 at a flow rate of 0.5 mL/min. Collection was 1.7 mL/fraction. The peaks I (fraction number: 60-66, cst I) and II (fraction number: 67-70, cst II) containing the papain-inhibitory activity were pooled, concentrated and characterized, respectively.

Figure 3. SDS-PAGE analysis of cystatins from crucian carp eggs. The acrylamide concentration of stacking and resolving gels were 3.75 and 15%. Lane M, protein marker; lane 1, homogenate; lane 2, acidification to pH 4.0; lane 3, chromatography on CM-Sepharose FF, lane 4, peak I on Sephacryl S-100 HR (cst I); lane 5, peak II on Sephacryl S-100 HR (cst II); lane 6, cst I without β-Me; lane 7, cst II without β-Me.

Figure 4. Thermal stability of cst I and II. Purified cst I and 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.

Figure 5. pH stability of cystatin. Purified cystatin 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) were incubated at 40°C for 1 hr and the residual inhibitory activity cystatin was assayed.

Figure 6. Inhibition of papain by cst I and II. Papain (0.43 nmole) was incubated with cst I and II at room temperature for 10 min, and the remaining proteolytic activity was assayed respectively.

Figure 7. Effect of cst I and II on proteinases. Bromelain, cathepsin B and trypsin were incubated with cystatins at room temperature for 30 min. The remaining proteolytic activities of

these enzymes were determined by using BANA as substrate.

Figure 8. Comparison of the N-terminus amino acid sequences of cst I and II with other origins. The identical amino acids were indicated as shadows.

Figure 1

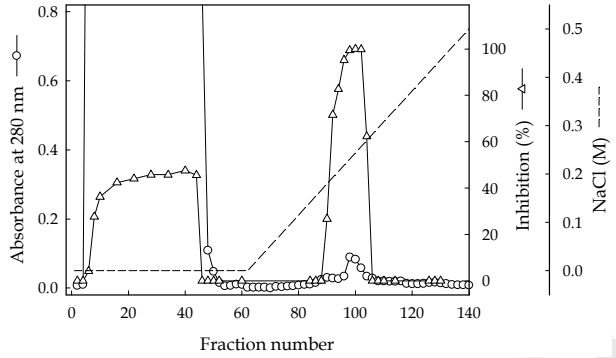

Figure 2

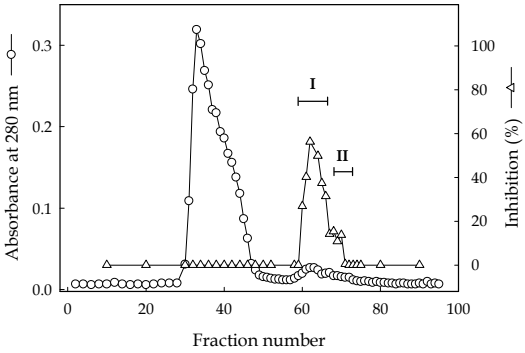

Figure 3

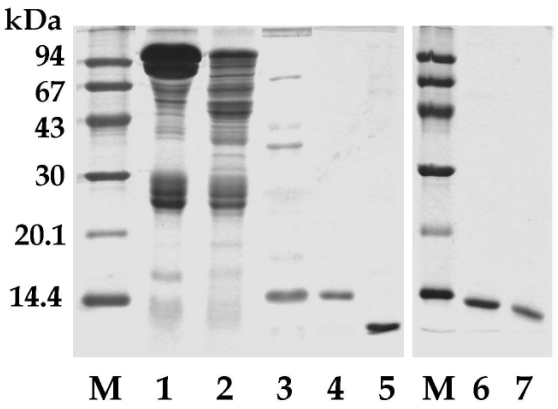

Figure 4

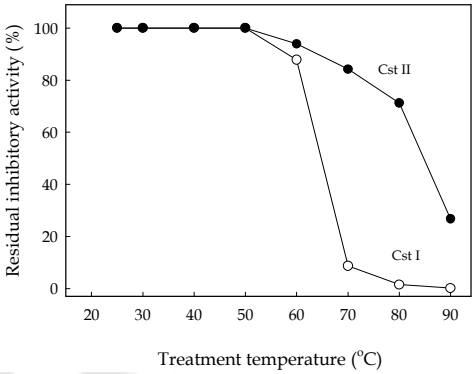

Figure 5

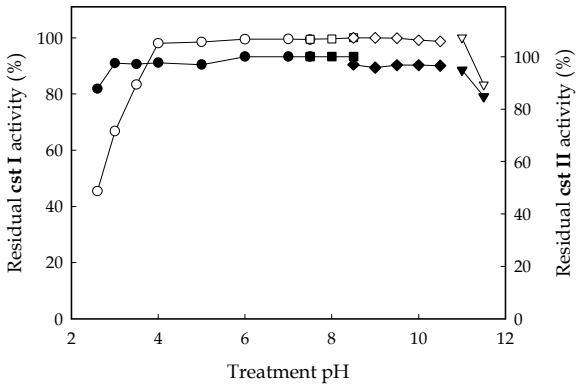

Figure 6

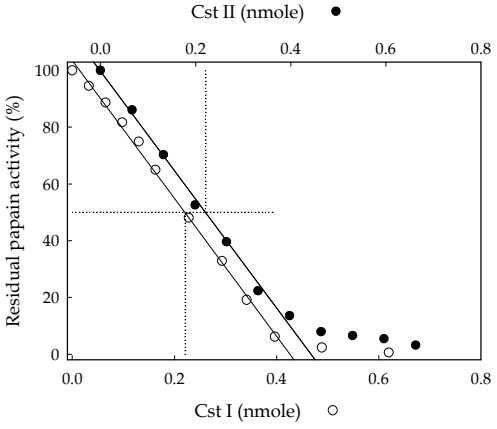

Figure 7

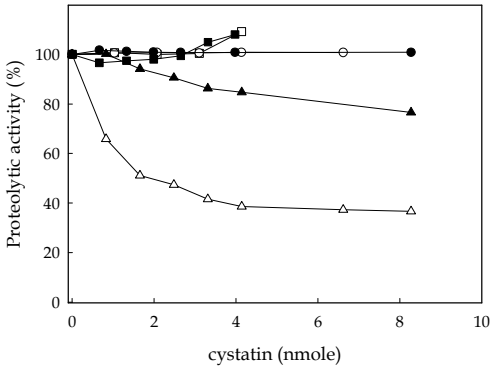

Figure 8

Sources			**Ref.**
Crucian carp cst I	1	AGIPGGLVDA 10	**This study**
Crucian carp cst II	1	GIPGGLVDAD 10	**This study**
Common carp	1	TGIPGGLVDAD 11	**Tsai et al., 1996 (L23572)**
Chum salmon	-1	AGLVGGPMDAN 10	**Yamashita and Konagaya, 1996 (D86628)**
Rainbow trout	-1	AGLIGGPMDAN 10	**Li et al., 1998 (U33555)**
Chicken	5	SRLLGAPVPVD 15	**Colella et al., 1989 (J05077)**
Human C	7	PRLVGGPMDAS 17	**Grubb and Lofberg, 1982**
Wheat 1	19	GPLVGGISDSP 29	**Kuroda et al., 2001 (AB038392)**