Purification and Characterization of a Carboxypeptidase from Squid Hepatopancreas (*Illex illecebrosus*)

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The hepatopancreas of squid (*Illex illecebrosus*) extract contains a wide range of carboxypeptidase (CP) activities based on hydrolysis of *N*-CBZ-dipeptide substrates. SDS-PAGE zymograms with *N*-CBZ-Phe-Leu substrate revealed three activity zones (CP-I, 23 kDa; CP-II, 29 kDa; CP-III, 42 kDa). CP-I was purified 225-fold with 86.20% recovery based on *N*-CBZ-Ala-Phe activity by chromatography on DEAE-cellulose, gel filtration, and chromatofocusing. The purified enzyme had broad specificity toward *N*-CBZ-dipeptides; however, it preferred substrates with a hydrophobic amino acid at the C terminus. CP-I had greatest activity with *N*-CBZ-Ala-Phe (specific activity = 7104 units/mg of protein, K_m = 0.40 mM, and physiological efficiency = 22863). CP-I had a p*I* of 3.4 and is a metalloprotease that is activated by Co^{2+} and partially inhibited by Pefabloc, a serine protease inhibitor. With *N*-CBZ-Ala-Phe and Gly-Phe, it had optimum activity at pH 8 and 70 °C. The amino acid composition of squid CP-I is similar to that of CP A from other species.

Keywords: *Carboxypeptidase; squid hepatopancreas; Illex illecebrosus; purification; debitter*

INTRODUCTION

Food products may benefit from treatment with proteolytic enzymes that improve palatability and functional properties of the protein. Proteolytic enzymes are commonly used as processing aids with bread, beer, cheese, fish sauce, and fermented legumes such as soy sauce, miso, and tempeh. A major hindrance to enzymatic hydrolysis of food protein is the formation of bitter-tasting peptides. This appears to be closely related to the content and sequence of hydrophobic amino acids in peptides. Ishibashi et al. (*1*, *2*) established a relationship between peptide structure and bitterness. The bitterness of peptides increases with the hydrophobicity of the C-terminal residue and when a basic amino acid, for example, Arg is located at the N-terminal position. Intense bitterness is also associated with peptides having at least two hydrophobic amino acids at the C terminus (*3*) and increases with the number of Leu (*4*), Phe, and Tyr residues (*5*).

Carboxypeptidases (CP) (EC 3.4.16−19) hydrolyze polypeptide chains from the C terminus. When CP release hydrophobic amino acids, the hydrophobicity of the polypeptide product is reduced, resulting in decreased bitter taste. Several studies support this idea. Hydrolysis of soybean hydrolysate with acid CP from *Aspergillus* lowered bitter taste (*6*). The bitter taste of the hydrolysate from casein (*7*), fish protein concentrate (*8*), and soybean protein (*9*) was eliminated by a wheat CP, which released hydrophobic amino acids from bitter peptides. Squid hepatopancreas (HP) acid CP, named CPase Top, removed the bitter taste of bitter peptides prepared from soy protein and corn gluten (*10*). Moreover, exopeptidase products, free amino acids, and short-chain peptides play an important role in taste and flavor compounds and precursors; for example, the formation of methional and methanethiol from methionine contributes to Cheddar cheese flavor (*11*).

Commercial use of enzymes from fishery byproducts is a growing industry (*12*). The HP, ∼14−20% of the body weight of Atlantic short-finned squid (*Illex illecebrosus*), is a good source of proteolytic enzymes (*13*) and a part of the waste from squid processing plants. Dipeptidyl aminopeptidase I (cathepsin C) was purified from HP (*14*) and used to produce fish sauce lacking bitterness (*15*). A partially purified squid HP aminopeptidase preparation, which had a high ratio of exopeptidase to endoproteinase activity, improved the flavor of Cheddar cheese, but the cheese developed a bitter taste (*16*). In this study, we purified a CP from squid HP that hydrolyzes hydrophobic C-terminal amino acids.

MATERIALS AND METHODS

Materials. The following were purchased from Sigma Chemical Co., St. Louis, MO: 33 *N*-carbobenzoxy (CBZ)-dipeptides, *N*-CBZ-amino acids, sodium pyrophosphate, *O*-phthaldialdehyde (OPA), *Crotalus adamanteus* L-amino acid oxidase, peroxidase type II from horseradish, ethylenediaminetetraacetic acid (EDTA), *O*-phenanthroline, *p*-chloromercuribenzoic acid (*p*CMB), E64 [*N*-(*N*-L-3-*trans*-carboxirane-2-carbonyl-L-leucyl)agmatine], pepstatin, and bestatin [[(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine hydrochloride], cysteine, dithiothreitol (DTT), β-mercaptoethanol (β*M*E), $ZnSO_4$, $CaSO_4$, $MgCl_2$, $CaCl_2$, $MnSO_4$, $MgSO_4$, $CuSO_4$, and Na_2SO_4. Sodium chloride, hydrochloric acid, acetic acid, $ZnCl_2$, $CoCl_2$, $FeSO_4$, $CdSO_4$, KCl, and LiCl were purchased from Fisher Scientific Co. (Pittsburgh, PA). Coomassie Plus 2000 reagent was ordered from Pierce Chemical Co., Rockford, IL. Pefabloc [4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride] was ordered from Boehringer Mannheim Corp. Biochemical Products, Indianapolis, IN. Diethylaminoethyl cellulose (DE 53) was manufactured by Whatman International Ltd. (Maidstone, U.K.,). Toyopearl HW-50F was bought from Tosohaas (Montgomeryville, PA). Protein molecular weight markers were purchased from Pharmacia Biotech, Alameda, CA. Chromatofocusing system, polybuffer exchanger PBE 94 and polybuffer 74, were from Pharmacia Biotech (Uppsala, Sweden). All other chemicals were of analytical grade.

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10.1021/jf010320h CCC: $20.00
Published on Web 09/28/2001

Table 1. Effect of $ZnSO_4$ and NaCl on the Activity of CP Using Different Substrates[a]

substrate	NaCl (0.5 M)[b]	$ZnSO_4$ (10 mM)[b]	relative activity (%)
N-CBZ-Phe-Leu	−	−	100
	−	+	155
	+	−	155
	+	+	364
N-CBZ-Ala-Phe	−	−	100
	−	+	133
	+	−	126
	+	+	170
N-CBZ-Gly-Phe	−	−	100
	−	+	198
	+	−	352
	+	+	421
N-CBZ-Phe-Met	−	−	100
	−	+	138
	+	−	149
	+	+	172
N-CBZ-Val-Leu	−	−	100
	−	+	157
	+	−	148
	+	+	174

[a] Substrate concentrations were 5 mM, and activities were determined at pH 7.5 and 37 °C. The enzyme extract was from step 1 (Table 3) that was dialyzed for 15 h against 50 mM sodium pyrophosphate (pH 7.5). [b] −, not included; +, included.

Carboxypeptidase Assay. CP activity was assessed by using 5 mM *N*-CBZ-dipeptides or *N*-CBZ-amino acids in 50 mM sodium pyrophosphate buffer (pH 7.5) containing 500 mM NaCl and 2 mM $ZnSO_4$. In some experiments, the buffer system was varied as stated below. The substrate solution (0.45 mL) was equilibrated at 37 °C before the enzyme (50 μL) was added. Sample aliquots (100 μL) were taken at $t = 0$ and 10 min and boiled in a water bath for 2 min to stop the reaction. α-Amino group formation was determined by using the OPA method (*17*), and Leu was selected as a standard amino acid. One unit of CP activity was defined as the amount of enzyme that releases the equivalent of 1 μmol of Leu in 1 min under the specified conditions of the assay. Specific activity was expressed as CP units per milligram of protein. Linearity of reaction was established between 0 and 10 min of assay. Reported data of CP activity are based on the average of two determinations during purification and three determinations for characterization. The variation for replicates was <5%.

Preliminary Study. The squid (*I. illecebrosus*) HP extract was examined for CP activity as described above. Squid HP extract (step1, Table 3) was dialyzed against 50 mM sodium pyrophosphate buffer (pH 7.5) for 15 h and assayed as described above. Thirty-three *N*-CBZ-dipeptides were used as substrates to detect the activity of CP. The reaction was carried out at both pH 7.5 and 3.1. Sodium citrate (50 mM) was used as a buffer for assay at pH 3.1.

An extract of squid HP (dialyzed solution of step1, Table 3) was assayed with substrate solution containing either 10 mM $ZnSO_4$ or 0.5 M NaCl or both in 50 mM sodium pyrophosphate buffer (pH 7.5). Five *N*-CBZ-dipeptide substrates (5 mM) were tested. The product formed was determined by OPA method as described under Carboxypeptidase Assay.

Cleavage Position of Squid CP on CBZ-Dipeptides. *N*-CBZ-dipeptides (5 mM; Leu-Tyr, Ala-Phe, Phe-Met, and Phe-Leu) in 50 mM sodium pyrophosphate buffer containing 0.5 M NaCl and 2 mM $ZnSO_4$ were incubated with partially purified squid CP (step 5, Table 3, without gel filtration) for 10 min. The reaction was stopped by heating for 2 min in boiling water. Squid CP incubated in sodium pyrophosphate buffer without an *N*-CBZ-dipeptide substrate was used as a control. An aliquot of the hydrolysis product was analyzed for free amino acids at the Molecular Structure Laboratory at the University of California, Davis, using Beckman 6300 sodium citrate based amino acid analyzer (*18*).

Table 2. Specific Activities (Units per Milligram of Protein) of Squid HP Crude Extract on *N*-CBZ-Dipeptides at Assay pH 7.5 and 3.1[a]

N-CBZ-dipeptide	pH 7.5	pH 3.1	*N*-CBZ-dipeptide	pH 7.5	pH 3.1
Phe-Leu	57.97	4.65	Gly-Ala	3.05	5.21
Ala-Leu	57.87	64.71	Ile-Ile	2.72	2.27
Ala-Phe	41.48	2.58	Ala-Ala	2.00	2.95
Leu-Tyr	35.70	26.73	Ala-Glu	1.96	3.56
Phe-Met	28.76	11.28	Ile-Met	1.64	0.62
Val-Leu	26.84	2.40	Gly-Ile	1.56	1.45
Val-Phe	26.40	0.38	Leu-Gly	1.55	2.97
Gly-Phe	8.99	3.13	Gly-Pro	1.26	2.29
Ala-Ile	8.59	0.86	Ile-Phe	1.11	0.00
Ile-Leu	6.51	4.35	Ile-Ala	0.97	0.00
Phe-Ala	6.09	2.91	Phe-Gly	0.61	2.37
Pro-Phe	5.85	4.15	Glu-Phe	0.00	3.87
Leu-Ala	5.56	3.94	Ile-Pro	0.00	2.98
Ala-Met	4.35	2.56	Ala-Pro	0.00	2.59
Glu-Tyr	4.15	3.21	Pro-Ala	0.00	1.95
Gly-Leu	3.41	1.06	Gly-Val	0.00	1.56
Ala-Val	3.19	0.00			

[a] Squid HP extract (step 1, Table 3) was dialyzed against 50 mM sodium pyrophosphate buffer (pH 7.5) for 15 h at 4 °C prior to assay. Assay was at 37 °C under standard conditions described under Materials and Methods.

Molecular Weight by Gel Filtration. Squid CP MW was determined by using the gel filtration column described under CP purification. Ribonuclease A (13700), chymotrypsinogen A (25000), ovalbumin (43000), bovine serum albumin (67000), and blue dextran 2000 (2000000) were used as standard proteins. Each protein (2 mg) was loaded independently through the column and eluted with 50 mM sodium phosphate buffer containing 0.1 M NaCl (pH 7.5) at a flow rate of 0.2 mL/min. Void volume was 63 mL.

Electrophoresis. Sodium dodecyl sulfate−12.5% polyacrylamide gel electrophoresis (SDS-PAGE) of the purified CP fraction was performed according to the method of Laemmli (*19*). The protein staining solution contained 0.1% Coomassie blue R-250 in water, methanol, and acetic acid (50:40:10 v/v/v). Standard proteins used as molecular weight markers were phosphorylase *b* (94000), albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), trypsin inhibitor (20100), and α-lactalbumin (14400).

Native gel electrophoresis was also run to provide evidence for homogeneity of the squid CP. Native gel electrophoresis was done by using the Laemmli system without the addition of SDS, which was replaced by deionized-distilled water. The pH values of this system were as follows: running gel, pH 8.8; stacking gel, pH 6.8; and tank buffer, pH 8.3. A second native gel system was prepared by adjusting the pH of the running gel to pH 7.5, that of the stacking gel to pH 5.5, and that of the tank buffer to pH 7.0 using HCl.

Activity Gel Electrophoresis of Carboxypeptidases. A zymogram method to detect CP activity after electrophoresis was modified from that of Lewis and Harris (*20*). Substrate agar contained 15 mL of 2% agar solution and 15 mL of 0.2 M Tris-HCl buffer (pH 7.5) containing 3 mM *N*-CBZ-dipeptides, 5 mg of *Crotalus adamanteus* L-amino acid oxidase, 3 mg of peroxidase, 5 mg of *O*-dianisidine, and 0.5 mL of 0.1 M $ZnSO_4$. A slab of substrate agar was prepared fresh before being layered over the SDS-PAGE gel. The enzyme gel slab was prewashed two times, 15 min each, with a solution containing 0.2 M Tris-HCl buffer (pH 7.5), 5 mM $ZnSO_4$, and 1% Triton X-100 (ratio 50:50 v/v) at 4 °C before it was layered with the substrate gel. The appearance of brown bands within 90 min after incubation at 37 °C indicated CP zones of activity.

Carboxypeptidase Purification. Temperature was controlled at 4 °C during the purification procedure. All purification steps were monitored with activity determination (*N*-CBZ-Phe-Leu) and total activity after each step was also examined by using five other *N*-CBZ-dipeptides (Table 4). Protein

Table 3. Purification Scheme of 25 kDa Squid CP Using *N*-CBZ-Ala-Phe as Substrate[a]

step	purification step	vol (mL)	activity (units/mL)	total activity	protein (mg/mL)	specific activity	yield (%)	fold
1	squid hepatopancreas extract	27	734.5	19830.2	23.2	31.6	100.0	1
2	25−50% ammonium sulfate fractionation	20	842.9	16858.3	10.5	80.6	85.0	2.5
3	DEAE	5	1271.2	6356.1	2.8	458.9	32.1	14.5
4	gel filtration	7	815.9	5711.1	1.1	748.5	28.8	23.7
5	chromatofocusing and dialysis (5 mM $ZnSO_4$)	13.2	1295.0	17094.0	0.2	7103.7	86.2	224.5

[a] Substrate concentrations were 5 mM, and activities were determined at pH 7.5 and 37 °C.

Table 4. Purification Yield of Squid CP-I with Different Substrates[a]

N-CBZ-dipeptide	specific activity (units/mg of protein)	yield (%)	fold
Ala-Phe	7104	86.2	225
Gly-Phe	2583	78.6	205
Val-Leu	2349	31.9	83.2
Leu-Tyr	1710	27.5	71.6
Phe-Met	1808	15.8	41.2
Phe-Leu	2361	13.6	35.5

[a] Substrate concentrations were 5 mM, and activities were determined at pH 7.5 and 37 °C.

quantification of column fractions was done by A_{280nm}. Protein content of fractions recovered after each of the purification steps was done according to the method of Lowry et al. (*21*) except for the fraction obtained after chromatofocusing, which was done by Coomassie Plus 2000 reagent. Bovine serum albumin was used as the protein standard.

Squid HP was homogenized (Polytron Kinematica GmbH, Brinkmann Instruments) with 4 volumes of 50 mM sodium pyrophosphate buffer (pH 7.5) and centrifuged at 10000*g* (Sorvall, RC-5B refrigerated superspeed centrifuge, DuPont Instruments) for 20 min. The fat layer on the top was discarded, and the supernatant was fractionated with solid ammonium sulfate (25−50% saturation). The ammonium sulfate fraction recovered after centrifugation at 10000*g* for 15 min was redissolved in a minimum quantity of cold 50 mM sodium phosphate buffer (pH 7.5) and dialyzed against 50 mM sodium phosphate buffer (pH 7.5) for 12 h (Spectrapor membrane, MW cutoff 6000−8000). The dialyzed solution was centrifuged at 10000*g* for clarification before being applied to an anion exchange DEAE column (5 × 10 cm). Flow rate was controlled at 1 mL/min. The enzyme was eluted from the column in a stepwise (0.1, 0.2, and 0.3 M NaCl in 50 mM sodium phosphate buffer, pH 7.5) manner. Active fractions were pooled, concentrated by ultrafiltration (Amicon microconcentrator, Diaflo PM10 membrane), and dialyzed against 50 mM pyrophosphate containing 0.1 M NaCl (pH 7.5). The dialyzed enzyme solution was further fractionated by gel filtration chromatography with a Toyopearl HW-50F column (1.5 × 115 cm), having an exclusion limit 80 kDa for globular protein. The enzyme was eluted with buffer (50 mM pyrophosphate, pH 7.5, and 0.1 M NaCl) using a peristatic pump with a flow rate of 0.2 mL/min. Active fractions were pooled, concentrated, and dialyzed. The dialysate was then loaded on a chromatofocusing column, polybuffer exchanger PBE 94 (1 × 30 cm). The active fraction (Figure 4) was dialyzed against 50 mM sodium pyrophosphate buffer (pH 7.5) containing 5 mM $ZnSO_4$ for 12 h. The purification steps are summarized in Table 3. The purified squid HP CP was measured for substrate specificity and characterized as follows.

Kinetic Parameters. The K_m and V_{max} for *N*-CBZ-Ala-Phe, *N*-CBZ-Phe-Met, and *N*-CBZ-Gly-Phe were graphically calculated from Lineweaver−Burk plots. The range of substrate concentrations was 0.25−5 mM. Otherwise, conditions were as described under Carboxypeptidase Assay.

Effect of pH. The pH optima of CP activities on *N*-CBZ-Ala-Phe, *N*-CBZ-Phe-Met, and *N*-CBZ-Gly-Phe were obtained by assay at pH 3−12 with 50 mM universal buffer [composed of boric acid, citric acid, monobasic sodium phosphate, and NaOH (*22*)]. The effect of pH on CP stability was determined by incubation of the enzyme in a series of universal buffers at 37 °C for 6 h. After incubation, activity was determined at pH 7.5. All other conditions were kept constant.

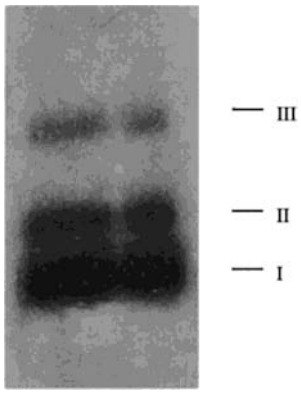

Figure 1. Activity gel of crude squid HP extract using *N*-CBZ-Phe-Leu as a substrate.

Effect of Temperature. Assays for determining the temperature optimum of squid CP were performed from 25 to 80 °C at pH 7.5 in 50 mM sodium pyrophosphate buffer. Thermal stability was studied by incubation of squid CP in 50 mM sodium pyrophosphate buffer (pH 7.5) at various temperatures for 30 min (25−80 °C), and residual activity was measured with three *N*-CBZ-dipeptides at 37 °C. All other conditions were kept constant.

Effect of Inhibitors, Thiol Compounds, and Metal Cations. An enzyme aliquot (10 μg of protein) was incubated with inhibitors, thiol compounds, or metal salts at different concentrations for 10 min at 37 °C. Assays proceeded by adding the substrate (*N*-CBZ-Ala-Phe, *N*-CBZ-Phe-Met, or *N*-CBZ-Gly-Phe) to evaluate the activity of squid CP. For the study of the effect of metal cations, neither NaCl nor $ZnSO_4$ was included in the substrate solution. The activity was reported as a percentage of the original activity.

Amino Acid Composition Analysis. Purified squid CP-I was sent to the Molecular Structure Laboratory of the University of California, Davis, to analyze the amino acid composition (*18*). Sample was hydrolyzed in 6 N HCl for 24 h at 110 °C before loading on a Beckman 6300 amino acid analyzer. Cys and Met were determined by oxidation with performic acid, yielding the acid stable forms prior to the HCl hydrolysis. Mercaptoethanesulfonic acid hydrolysis was used to determine Try content. Norleu was used as an internal standard for amino acid quantification.

The Metzger difference index (MDI) was used to compare the amino acid composition of squid CP-I with that of CP A from different sources (*23*). Two proteins with no amino acid in common have an MDI of 100, and two proteins with the same composition have an MDI of 0.

RESULTS AND DISCUSSION

Effect of $ZnSO_4$ and NaCl on the Activity of CP. The CP assay was developed from the method of Lacko and Neurath (*24*), which included 0.5 M NaCl in the substrate solution. Also, the majority of CP are zinc enzymes. Therefore, the effect of NaCl and $ZnSO_4$ on the CP activity of squid HP extract with five of the effective CBZ-dipeptide substrates (see Table 2) was

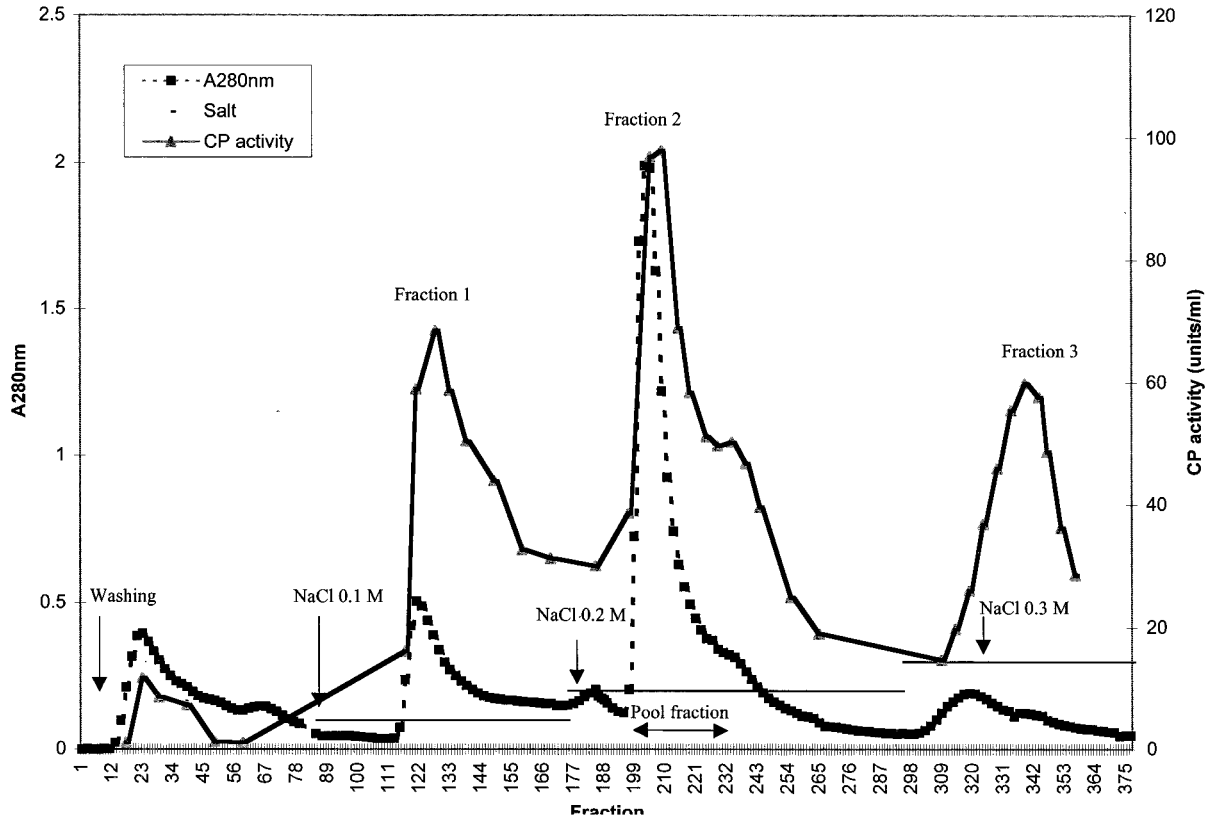

Figure 2. Anion exchange chromatography of squid HP CP on a DEAE column. The column was eluted stepwise with buffer containing increasing concentrations of NaCl. Activity was assayed with 5 mM *N*-CBZ-Phe-Leu as described under Materials and Methods.

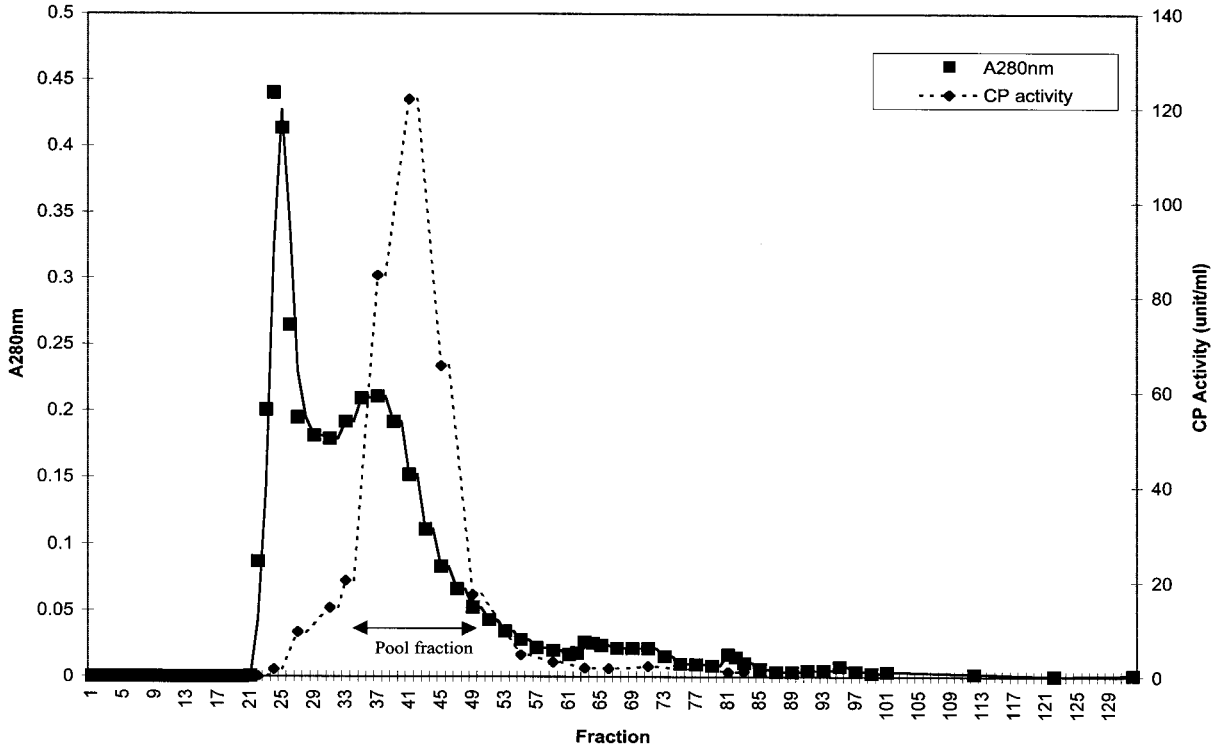

Figure 3. Gel filtration of squid HP CP (peak 3 from Figure 2) on a Toyopearl HW50F column. The column was eluted with 50 mM sodium phosphate buffer (pH 7.5), containing 0.1 M NaCl, at a flow rate of 0.2 mL/min.

tested (Table 1). The squid HP extract was dialyzed prior to assay to removal of small peptides and free amino acids that cause high background reading in the OPA method. Sodium pyrophosphate buffer was used in the assay because sodium phosphate precipitates with Zn^{2+} and Tris-HCl interferes with OPA measurement. $ZnSO_4$ (10 mM) increased the activity 133−198%, whereas 0.5 M NaCl increased the activity 126−352%

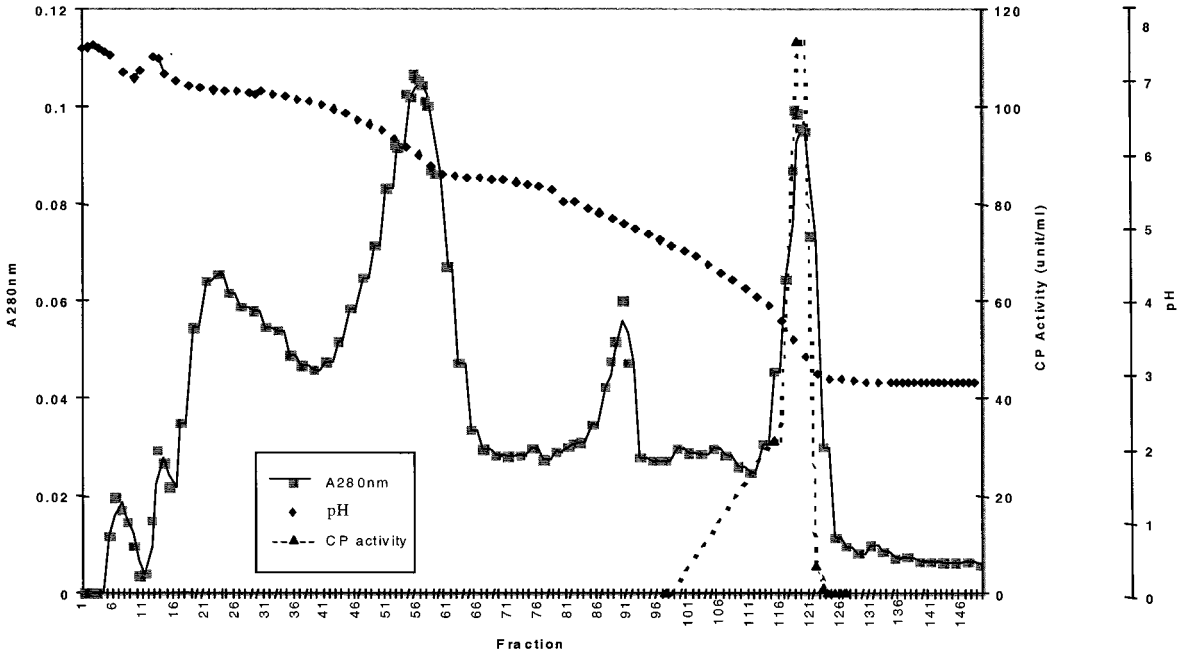

Figure 4. Chromatofocusing of squid HP CP. The polybuffer pH gradient was from 7 to 3.

(Table 1). When both salts were included in the substrate solution, the CP activity was increased to 170−421%. With *N*-CBZ-Gly-Phe, a substrate for CP A, the strongest effect was observed for both salts. Accordingly, subsequent assays included 0.5 M NaCl and 2 mM $ZnSO_4$ with the substrate−buffer system. However, NaCl and $ZnSO_4$ were not part of the basic assay system when the effect of metal ions on purified CP-I was studied (Table 9).

Carboxypeptidase Activities in Squid Hepatopancreas Extract. Some of the 33 *N*-CBZ-dipeptide substrates tested were more effectively hydrolyzed by the dialyzed extract of squid HP (Table 2). These were Phe-Leu, Ala-Leu, Val-Leu, Ala-Phe, Val-Phe, Leu-Tyr, and Phe-Met. In general, squid CP extract was more active at a reaction pH of 7.5 than at pH 3.1 (Table 2). The literature reports other CP that have such broad specificity, being active on several dipeptide substrates (*25*−*28*). Six *N*-CBZ-dipeptide substrates were used to monitor activity after each purification step (Table 4).

Carboxypeptidase Purification. Three bands of CP activity with *N*-CBZ-Phe-Leu activity were detected in squid HP extract by using SDS-PAGE activity gel electrophoresis (Figure 1). CP-I (band I, 23 kDa) with highest intensity was selected for further purification. CP-II and CP-III molecular weights were estimated at 29 and 42 kDa, respectively. Activity of squid HP CP was determined after each step by using *N*-CBZ-Ala-Phe (Table 3). The purification yield of activities with all six *N*-CBZ-dipeptides is shown in Table 4. The greatest recovery of activity was for hydrolysis of Ala-Phe and Gly-Phe. The latter is typically used as a CP A substrate.

DEAE-cellulose separated the ammonium sulfate fraction components into three activity peaks. Fraction 2 (Figure 2), which was the major activity peak with *N*-CBZ-Phe-Leu, was pooled and loaded on a gel filtration column (Toyopearl HW50F; Figure 3). Gel filtration significantly increased specific activity without a major decrease in yield (Table 3). The fraction recovered after gel filtration was further purified by chromatofocusing. Polybuffer interferes with protein determination according to the Lowry method because copper ions may form a complex with polybuffer. Therefore, the Bradford (Coomassie Plus) protein assay was selected to determine the amount of protein after chromatofocusing because it does not interfere with polybuffer (*29*). The purified enzyme was also dialyzed against 50 mM sodium pyrophosphate to remove polybuffer. The activity peak p*I* was ∼3.4 (Figure 4). The p*I* determination may not be precise because pH 3.4 is lower than the buffer range (4−7) of polybuffer. This enzyme is quite acidic. Crab HP CP has a p*I* value of 4.5 (*30*), and ostrich CP A has a p*I* of 6.6 (*31*). Squid HP CP-I activity with *N*-CBZ-Ala-Phe was purified 224-fold with 86.20% recovery. The enzyme was also active in hydrolyzing *N*-CBZ-Phe-Leu and *N*-CBZ-Gly-Phe, a CP A substrate. The recovery of activities with *N*-CBZ-Ala-Phe (Table 3) as well as *N*-CBZ-Gly-Phe (data not shown) increased between steps 4 and 5 possibly because of the dialysis against $ZnSO_4$ or the removal of an inhibitor by chromatofocusing.

Electrophoresis of Purified CP-I. Purity of the purified CP was evaluated by using native gel electrophoresis at two different pH values. There was one protein band on both gels (Figure 5a,b). On SDS-PAGE, the CP migrated as a single band of M_r 23 kDa (Figure 6a). This enzyme showed Ala-Phe CP activity on the gel zymogram (Figure 6b). The brown color intensity varied on three different *N*-CBZ-dipeptide substrates, *N*-CBZ-Ala-Phe > *N*-CBZ-Gly-Phe > *N*-CBZ-Phe-Met (data not shown). However, CP zymogram activity may not be quantitative because (1) the coupled L-amino acid oxidase reacts at different rates against different amino acids and (2) band broadening often occurs because of diffusion of reaction product in the agar-based medium. This makes the precise location of the bands difficult (*20*).

Squid CP-I has a monomeric structure with molecular weight of 23 kDa by SDS-PAGE (Figure 6a) and 25 kDa by gel filtration chromatography. The peak of squid HP CP−I eluted at the same volume (101 mL) as chymotrypsinogen A (25 kDa). Chemical and physical proper-

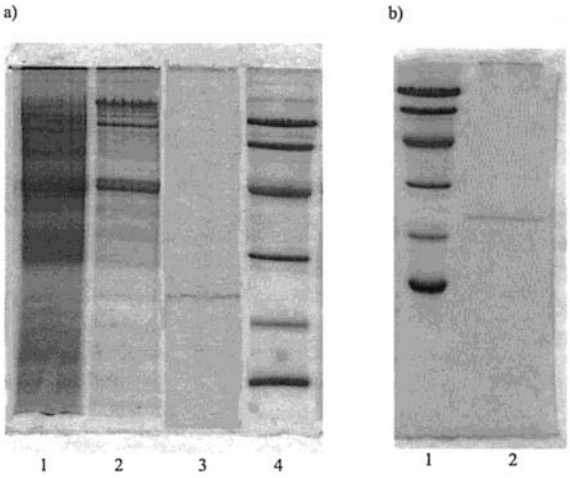

Figure 5. Native gel electrophoresis at (a) pH 8.8 (lane 1, ammonium sulfate fraction; lane 2, DEAE fraction; lane 3, purified CP; lane 4, standard molecular weight) and (b) pH 7.5 (lane 1, standard molecular weight; lane 2, purified CP).

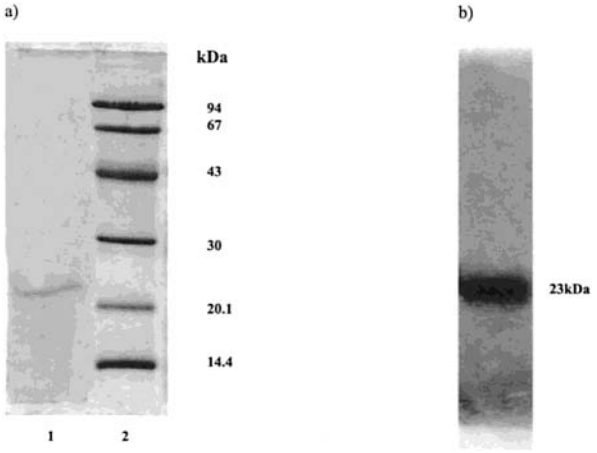

Figure 6. SDS-PAGE of purified squid HP CP: (a) protein gel; (b) activity gel (using *N*-CBZ-Ala-Phe as substrate).

ties of purified CP−I were evaluated and compared with other available CP information in the literature as follows.

Cleavage Position of Squid CP on CBZ-Dipeptides. The CP assay used carbobenzoxy-blocked dipeptides as substrate. The enzyme could possibly cleave either the peptide bond or the bond between the blocking group and the dipeptide. The gel zymograms provide evidence that CP-I cleaves the peptide bond between two amino acids and releases a free amino acid. Free amino acids (*20*), but not dipeptides (*32*), are oxidized by L-amino acid oxidase, giving H_2O_2. Hydrogen peroxide reacts with *O*-dianisidine and peroxidase, resulting in the brown color of oxidized dianisidine.

To further verify the reaction mechanisms of squid CP-I, we analyzed the reaction products. Analysis of the reaction products from each of four *N*-CBZ-dipeptide substrates by amino acid analysis revealed only C-terminal amino acids were formed, that is, Tyr from *N*-CBZ-Leu-Tyr, Phe from Ala-Phe, Met from Phe-Met, and Leu from Phe-Leu. This result indicates that squid CP-I cleaves the peptide bond between the penultimate and the terminal amino acid of the *N*-CBZ-dipeptide substrate and is not a dipeptidyl hydrolase.

Substrate Specificity. Thirty-three *N*-CBZ-dipeptides were tested against CP-I to gain information about substrate specificity. Relative activities of squid HP CP are shown in Table 5. A broad substrate specificity of CP-I was demonstrated by its ability to cleave amino acid from several *N*-CBZ-dipeptides. However, CP-I prefers substrate with Phe or Leu at the C terminus adjacent to Ala. Activities toward most substrates were <10% that with *N*-CBZ-Ala-Phe. Some dipeptides were not hydrolyzed at all, that is, *N*-CBZ-Gly-Pro, *N*-CBZ-Ala-Glu, *N*-CBZ-Pro-Phe, and *N*-CBZ-Ile-Ala. The penultimate amino acid residue of the peptide also affected the rate of hydrolysis by CP-I, that is, Ala-Phe ≫ Val-Phe, Glu-Phe, Ile-Phe, and Pro-Phe. The activity with CP A substrate (Gly-Phe) was 36% of that with Ala-Phe (Table 5). CBZ-Ile-X and CBZ-Pro-X had lower activity than the others did (X = amino acid residue). CP from the fungus *Mucor racemosus* (*25*) and the yeast *Candida albicans* (*33*) also had a strong activity against Ala-X when compared to other CBZ-dipeptides including Gly-X. CP from *Paecilomyces carneus* preferred substrates with bulky amino acids such as Phe, Tyr, and Ile at the penultimate position (*28*). Kawabata et al. (*10*) found that a CP (Top) from squid (*Toardes pacificus*) HP had a higher relative activity on CBZ-Phe-Leu than on CBZ-Phe-Ala and CBZ-Tyr-Phe compared to other dipeptide substrates.

Table 5. Activity of Purified Squid CP-I on Various *N*-CBZ-Dipeptides[a]

CBZ-dipeptide	specific activity (units/mg of protein)	relative activity (%)	CBZ-dipeptide	specific activity (units/mg of protein)	relative activity (%)
Ala-Phe	7104	100.0	Ile-Pro	208	2.9
Ala-Leu	6297	88.6	Phe-Gly	195	2.7
Glu-Tyr	3225	45.4	Leu-Ala	167	2.3
Gly-Phe	2583	36.4	Leu-Gly	145	2.0
Val-Phe	2569	36.2	Gly-Ala	75	1.1
Glu-Phe	2544	35.8	Ile-Ile	74	1.0
Phe-Leu	2361	33.2	Gly-Ile	70	1.0
Val-Leu	2349	33.1	Ile-Met	67	0.9
Phe-Met	1808	25.5	Ala-Pro	61	0.9
Leu-Tyr	1710	24.1	Ala-Ala	25	0.4
Ala-Ile	928	13.1	Pro-Ala	23	0.3
Gly-Leu	732	10.3	Gly-Val	7	0.1
Ile-Leu	533	7.5	Gly-Pro	0	0.0
Ala-Met	477	6.7	Pro-Phe	0	0.0
Phe-Ala	416	5.9	Ala-Glu	0	0.0
Ile-Phe	326	4.6	Ile-Ala	0	0.0
Ala-Val	258	3.6			

[a] Substrate concentrations were 5 mM, and activities were determined at pH 7.5 and 37 °C. Reaction mixture contained 0.5 M NaCl and 2 mM $ZnSO_4$.

Activities against *N*-CBZ-amino acids (Table 6) were generally slower than with *N*-CBZ-dipeptide substrates (Table 5). *N*-CBZ-Lys (100%) was the most active substrate, followed by *N*-CBZ-Gly (17%) and *N*-CBZ-Glu (13%). Whereas the specificity of squid CP for *N*-CBZ-Ala-Phe was 7103 units/mg of protein, the specificity of *N*-CBZ-Lys was only 2213 units/mg of protein (31% of the *N*-CBZ-Ala-Phe). No activity was observed with *N*-CBZ-Phe, -Pro, -Ala, and -Val. Because there was no penultimate amino acid, the reaction did not occur even with an aromatic amino acid such as Phe at the C terminus. This result also indicates the importance of the penultimate amino acid position. CP from *Sulfolobus solfataricus* cleaved several CBZ-Gly-X and CBZ-X substrates (*34*). It preferred CBZ-Arg > CBZ-His > CBZ-Lys. It did not hydrolyze CBZ-Trp or CBZ-Pro. However, the rate in hydrolyzing CBZ-dipeptides was double the rate in hydrolyzing CBZ-amino acids. Whereas the hydrolysis rate of CBZ-Gly-Lys (preferred substrate)

Table 6. Activity of Squid CP-I on *N*-CBZ-Amino Acids[a]

N-CBZ-AA	specific activity (units/mg of protein)	relative activity (%)
L-Lys	2212	100.0
L-Gly	375	16.9
L-Glu	289	13.1
L-Leu	203	9.2
L-Met	150	6.8
L-Arg	71	3.2
L-Tyr	57	2.6
L-Val	0	0.0
L-Pro	0	0.0
L-Phe	0	0.0
L-Ala	0	0.0

[a] Substrate concentrations were 5 mM, and activities were determined at pH 7.5 and 37 °C. Reaction mixture contained 0.5 M NaCl and 2 mM $ZnSO_4$.

Table 7. Kinetic Parameters of Squid CP-I with Three Substrates[a]

kinetic parameter	*N*-CBZ-Ala-Phe	*N*-CBZ-Phe-Met	*N*-CBZ-Gly-Phe
K_m (mM)	0.40	0.74	3.77
V_{max} (units/mg of protein)	9145	3428	5485
V_{max}/K_m	22863	4632	1455

[a] Substrate concentration was 5 mM, and activities were determined at pH 7.5 and 37 °C. Universal buffer was used as described under Materials and Methods.

was 27.2 μmol/min·mg of protein, the hydrolysis rate of CBZ-Lys was only 13.0 μmol/min·mg of protein.

In activity measurement on *N*-CBZ-dipeptides, values on some dipeptides may be overestimated because there may be simultaneous cleavage of the bond between the blocking group (CBZ) and the amino acid. On the other hand, some *N*-CBZ-X are not hydrolyzed by squid CP, such as *N*-CBZ-Val, -Pro, -Phe, and -Ala (Table 6). Because CP-I did not hydrolyze *N*-CBZ-Ala, activity on *N*-CBZ-X does not complicate the analysis of the preferred substrates (*N*-CBZ-Ala-Phe and *N*-CBZ-Ala-Leu). Also, amino acid analysis of the hydrolysis products of Ala-Phe, Phe-Met, Leu-Tyr, and Phe-Leu revealed only the C-terminal amino acid was released (see above).

Kinetic Parameters. The K_m value of CP-I toward *N*-CBZ-Ala-Phe was 1 order of magnitude lower than with the CP A substrate (*N*-CBZ-Gly-Phe) (Table 7). CP-I from crab HP has a $K_m = 1.94$ mM with CBZ-Gly-Phe (*30*). CP-I from *Aspergillus oryzae* has a K_m value of 0.24 mM with CBZ-Ala-Phe (*35*), which is the most similar to that of squid CP-I ($K_m = 0.4$ mM on *N*-CBZ-Ala-Phe). K_m values of squid HP CP-I are in agreement with those (0.16−3.57 mM on nine *N*-CBZ-dipeptides) of a serine CP from *Paecilomyces carneus* (*28*). From V_{max}/K_m ratios, an estimate of catalytic efficiency of the enzyme, *N*-CBZ-Ala-Phe was ∼5 times more efficient than *N*-CBZ-Phe-Met and 15 times more than *N*-CBZ-Gly-Phe.

Effect of pH. CP-I showed optimum activity at pH 8 with *N*-CBZ-Ala-Phe and Gly-Phe, and a pH optimum of 10 with CBZ-Phe-Met (Figure 7). At pH 10.0, the activities against Ala-Phe and Gly-Phe were still 80−85% that at pH 8, whereas they dropped sharply at acidic pH and were 0 at pH 4.0. This enzyme is not suitable for use in acid food fermentation because only 10% of activity remains at pH 5.0 with *N*-CBZ-Ala-Phe. Squid CP-I optimum pH is in the same range as ostrich pancreas CP A and CP B (pH 8) (*36*). Study of the influence of pH on the stability of CP-I showed best stability under mildly alkaline and neutral conditions but rapid inactivation under acidic conditions (Figure 8). A similar effect has been observed for crab CP (*30*).

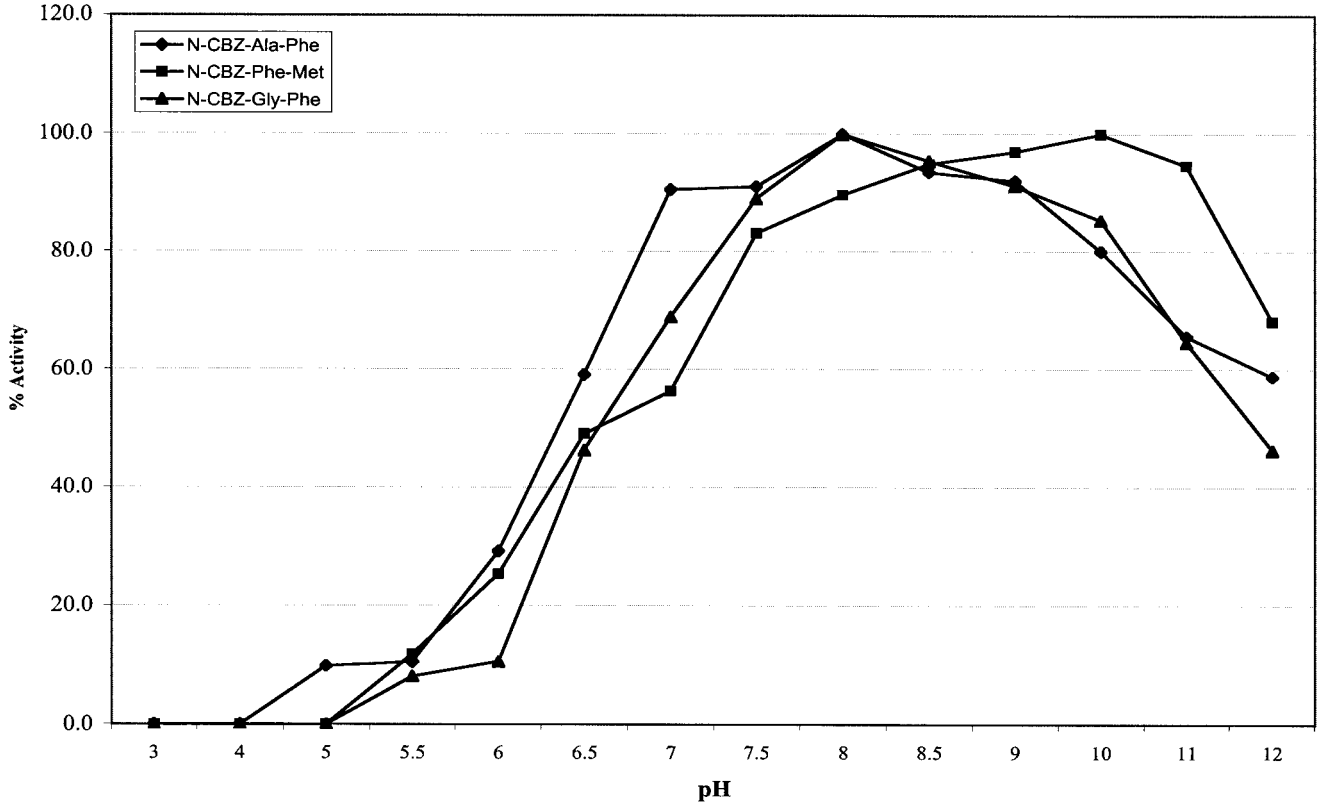

Figure 7. pH optimum of squid CP with three *N*-CBZ-dipeptide substrates. CP activities were determined at pH 3−12 using 50 mM universal buffer at 37 °C.

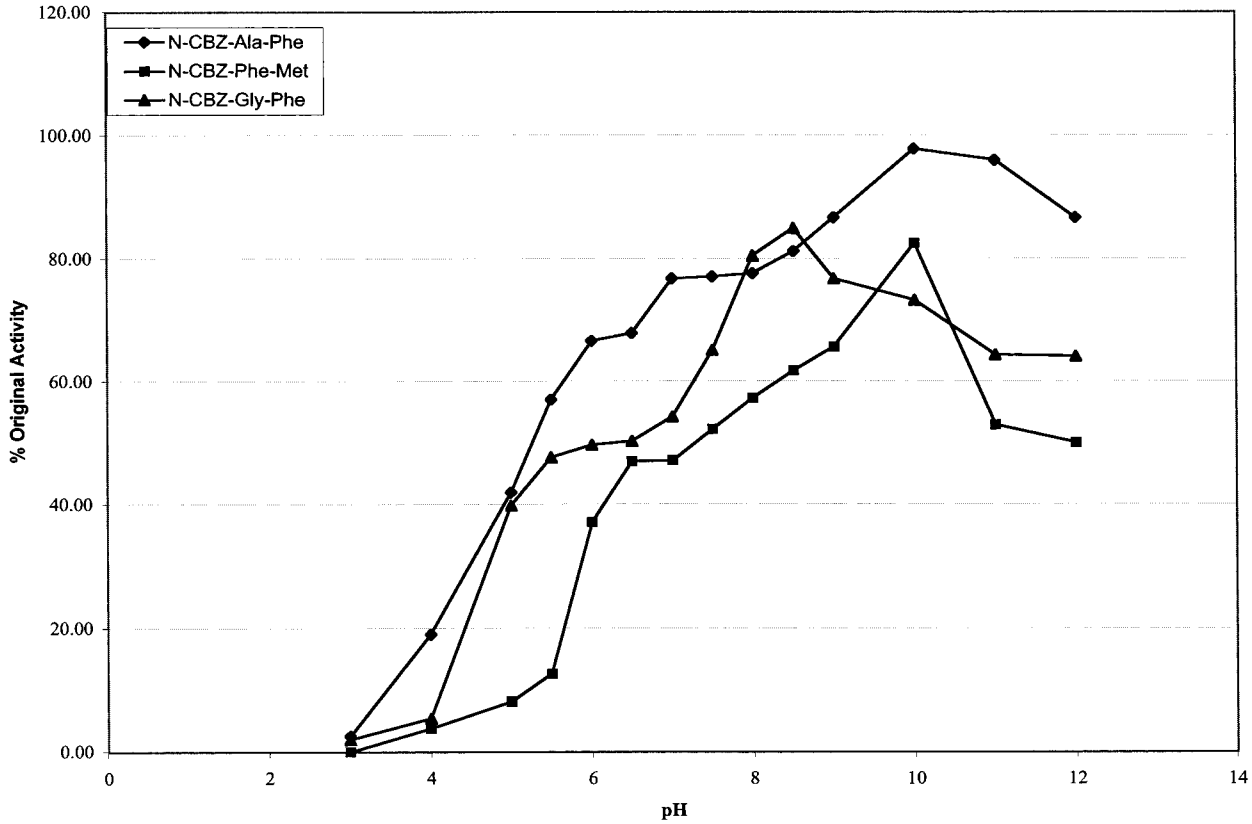

Figure 8. pH stability of squid CP with three *N*-CBZ-dipeptide substrates. CP was incubated in a series of universal buffers (pH 3−12) at 37 °C for 6 h. After incubation, remaining activity was determined at pH 7.5.

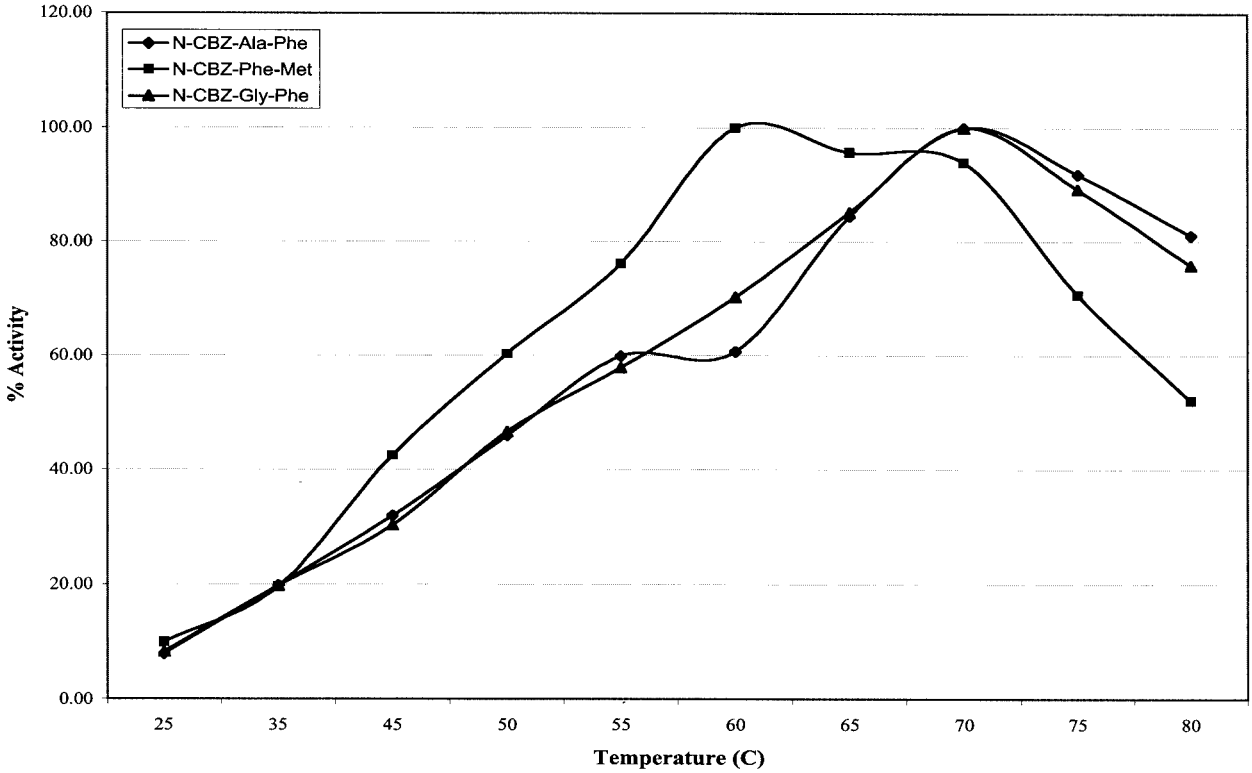

Figure 9. Temperature optimum of squid CP with three *N*-CBZ-dipeptide substrates. CP activity was measured at 25−80 °C and pH 7.5. The experiment was repeated three times, and the data were not significantly different.

Effect of Temperature. The optimum temperature of squid CP-I under the experimental conditions used was 70 °C using *N*-CBZ-Ala-Phe and Gly-Phe as substrates, and it was 60 °C with *N*-CBZ-Phe-Met (Figure 9). About 75−80% of activity remained at 80 °C with *N*-CBZ-Ala-Phe and Gly-Phe, but only 50% was observed with *N*-CBZ-Phe-Met at this temperature. Activity at 35 °C was only 20% of the maximum activity. This

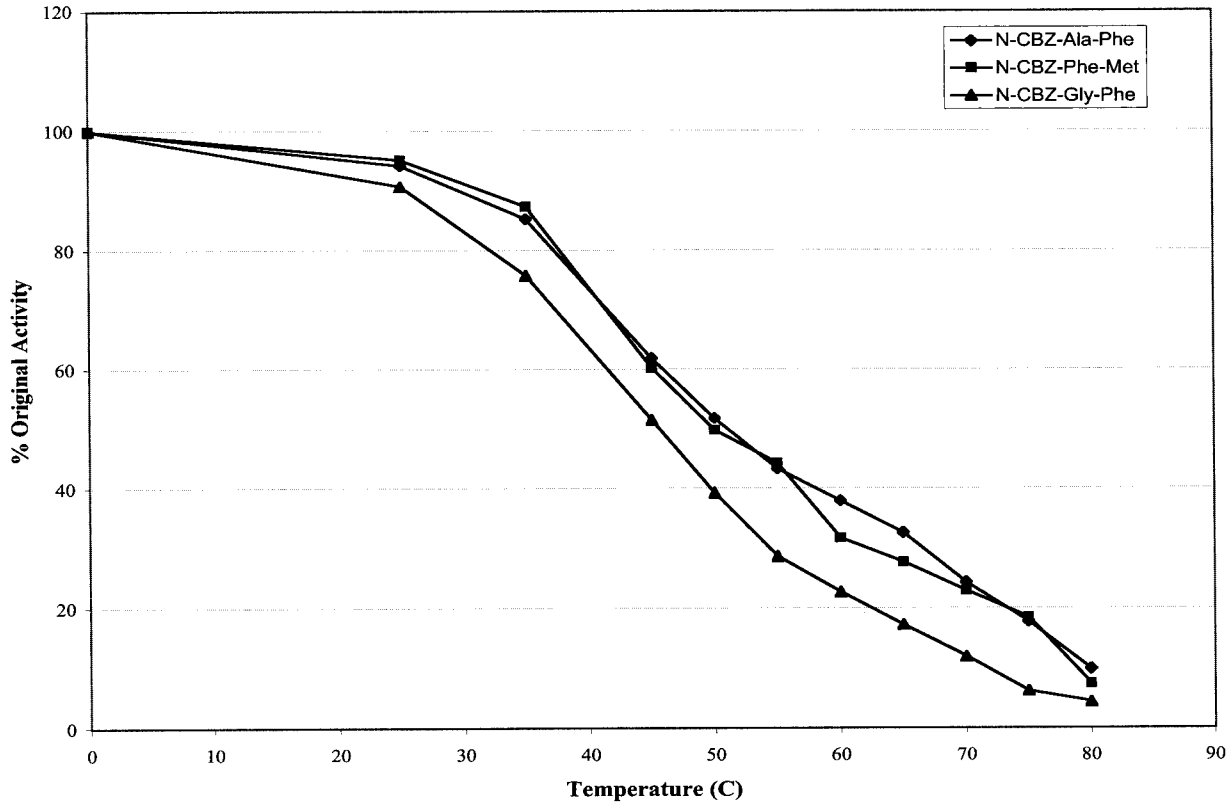

Figure 10. Temperature stability of squid CP at pH 7.5 for 30 min. Residual activity was measured with three *N*-CBZ-dipeptides at 37 °C. The experiment was repeated three times, and the data were not significantly different.

Table 8. Effect of Inhibitors and Thiol Compounds on the Relative Activities of Squid CP-I[a]

inhibitor	concn	Ala-Phe	Phe-Met	Gly-Phe
none		100.0	100.0	100.0
EDTA	1 mM	89.7	77.5	79.2
	17 mM	1.6	4.4	8.8
phenanthroline	1 mM	56.7	58.7	85.9
	5 mM	16.1	11.3	30.2
Pefabloc	1 mM	99.2	54.6	103.0
	5 mM	49.1	30.7	37.2
PCMB	1 mM	98.1	81.8	99.4
	5 mM	94.9	78.0	92.7
E64	10 μM	99.2	82.5	99.8
	50 μM	96.9	94.6	94.4
pepstatin	10 μM	101.7	74.3	100.9
	50 μM	100.2	72.4	94.0
bestatin	200 μM	95.0	69.0	104.0
	500 μM	108.2	72.3	98.4
thiol compounds				
cysteine	1 mM	102.3	82.3	98.8
	5 mM	31.2	22.5	92.4
β-mercaptoethanol	1 mM	115.3	88.1	107.0
	5 mM	106.0	104.7	103.8
dithiothreitol	1 mM	88.2	77.1	92.9
	5 mM	73.3	70.9	84.6

[a] Squid CP was incubated with inhibitors or thiol compounds at 37 °C for 10 min. Substrate mixture (pH 7.5) was added, and activity of the enzyme was measured as described under Materials and Methods. Specific activity of CP-I using *N*-CBZ-Ala-Phe was 7104 units/mg of protein without inhibitor or thiol compound.

Table 9. Effect of Metal Ion (1 mM) on the Relative Activities of Squid CP[a]

	Ala-Phe	Phe-Met	Gly-Phe
divalent metal			
none	100	100	100
$CoCl_2$	267	201	466
$CaSO_4$	184	131	131
$ZnSO_4$	172	141	186
$MgCl_2$	169	150	189
$CaCl_2$	154	131	118
$ZnCl_2$	152	149	162
$MnSO_4$	150	14	59
$MgSO_4$	149	177	234
$FeSO_4$	120	105	92
$CdSO_4$	19	31	22
$CuSO_4$	14	5	0
monovalent metal			
K_2SO_4	173	136	126
Na_2SO_4	161	138	118
NaCl	159	119	111
KCl	150	127	116
LiCl	150	133	204

[a] Squid CP was incubated with metal salt solution at 37 °C for 10 min. Substrate in 50 mM sodium pyrophosphate buffer (pH 7.5) was added, and activity of the enzyme was measured as described under Materials and Methods. Control activity was 3081 units/mg of protein with *N*-CBZ-Ala-Phe, 1128 units/mg of protein with *N*-CBZ-Gly-Phe, and 916 units/mg of protein with *N*-CBZ-Phe-Met.

temperature optimum is quite high for an enzyme from a cold-water organism, but this is not unprecedented. CP A from eggs of wild salmon (*Salmo salar*) (*37*) and a CP A-like compound from viscera of milk fish (*Chanos chanos*) (*38*) have a temperature optimum of 60 °C. CP-I, a serine CP from *A. oryzae*, has an optimal temperature of ~60 °C, but its pH optimum is only 4 (*35*). CP Tag, from *Thermus aquaticus* YT-1, had the same pH optimum (pH 8) but a higher temperature optimum (80 °C) (*39*). Arrhenius plots of activity with these three substrates were used to determine the effect of temperature on the enzyme. The activation energy (E_a) with *N*-CBZ-Ala-Phe was 54.4 kJ/mol. With *N*-CBZ-Phe-Met, E_a was 56.5 kJ/mol, and with *N*-CBZ-Gly-Phe, it was 50.2 kJ/mol. Ostrich CP A [E_a = 24.7 kJ/mol (*31*)] and

Table 10. Comparison of Amino Acid Composition of CP A from Different Sources[a]

amino acid	human	mol %	bovine 1	mol %	bovine 2	mol %	porcine	mol %	ostrich 1	mol %	ostrich 2	mol %	dog-fish	mol %	cat-fish	mol %	shrimp	mol %	squid[b]	mol %
Lys	17	6.24	15	5.56	15	5.72	13	4.75	13	4.82	20	7.57	14	5.07	15	0.55	4	1.79	8	3.63
His	10	3.91	8	3.16	8	3.25	9	3.50	10	3.95	8	3.23	7	2.70	6	0.17	7	3.33	8	3.55
Arg	10	4.42	11	4.91	10	4.59	12	5.28	16	7.15	11	5.02	14	6.11	13	0.59	8	4.30	8	3.42
Asp	29	9.63	29	9.73	27	9.31	32	10.58	34	11.41	24	8.22	27	8.85	32	3.08	32	12.92	25	11.43
Thr	27	7.95	26	7.74	23	7.03	27	7.92	23	6.85	18	5.47	21	6.10	26	1.69	21	7.52	15	6.72
Ser	26	6.69	32	8.31	30	8.01	28	7.17	24	6.24	29	7.69	25	6.34	29	1.97	25	7.82	19	8.81
Glu	25	9.24	25	9.34	25	9.59	29	10.67	28	10.46	20	7.62	29	10.57	24	1.90	20	8.98	24	10.99
Pro	13	3.69	10	2.87	10	2.95	14	3.96	13	3.73	14	4.10	17	4.76	18	0.66	15	5.18	12	5.42
Gly	23	4.07	23	4.11	23	4.22	26	4.58	21	3.75	25	4.56	30	5.23	25	1.49	24	5.16	24	11.05
Ala	23	4.93	20	4.33	19	4.23	23	4.91	24	5.20	23	5.09	21	4.44	19	1.29	22	5.73	15	6.78
Cys	2	0.60	2	0.61	2	0.62	2	0.60	3	0.91	3	0.93	4	1.18	3	0.03	7	2.55	1	0.28
Val	13	3.76	16	4.68	16	4.80	12	3.45	19	5.55	15	4.48	17	4.85	14	0.75	18	6.33	13	6.07
Met	3	1.13	3	1.14	3	1.17	3	1.12	2	0.76	5	1.94	9	3.33	5	0.03	4	1.82	3	1.21
Ile	23	7.52	20	6.61	20	6.79	19	6.18	16	5.29	20	6.75	20	6.45	19	0.86	13	5.17	10	4.44
Leu	23	7.52	23	7.60	23	7.81	21	6.83	21	6.94	28	9.45	16	5.16	18	1.07	15	5.96	13	6.08
Tyr	17	7.83	19	8.85	19	9.09	17	7.80	17	7.92	17	8.08	19	8.64	18	0.87	16	8.96	8	3.58
Phe	16	6.69	16	6.76	15	6.51	17	7.07	14	5.91	14	6.04	11	4.54	13	0.52	9	4.57	7	2.89
Trp	8	4.18	7	3.69	8	4.34	7	3.64	6	3.17	7	3.77	11	5.67	0	0	3	1.90	8	3.66
total	308	100	305	100	296	100	311	100	304	100	301	100	312	100	297	100	263	100	221	100
MW	34.7		34.5		34.3		34.6		34.2		33.5		34.9		34		30		25	

[a] Data for CP A amino acid composition: human (*51*), bovine 1 (*52*), bovine 2 (*53*), porcine (*54*), ostrich 1,2 (*31*), dogfish (*24*), catfish (*55*), and shrimp (*56*). [b] Nearest integer residues are based on a molecular weight of 25 kDa.

Table 11. MDI Values of CP A from Different Sources[a]

	human	bovine 1	bovine 2	porcine	ostrich 1	ostrich 2	dogfish	catfish	shrimp
bovine 1	4.46								
bovine 2	4.8	2.12							
porcine	4.87	5.51	6.79						
ostrich 1	8.26	8.73	9.03	6.15					
ostrich 2	8.02	7.80	7.35	9.43	11.44				
dogfish	11.43	10.48	9.65	9.94	10.22	11.11			
catfish	9.81	7.68	8.78	8.46	9.95	10.90	9.92		
shrimp	14.15	12.52	12.97	12.32	11.68	15.01	13.31	10.15	
squid CP-I	18.62	17.63	17.79	15.40	15.15	20.35	17.88	18.05	14.11

[a] Data for CP A amino acid composition: human (*51*), bovine 1 (*52*), bovine 2 (*53*), porcine (*54*), ostrich 1 and 2 (*31*), dogfish (*24*), catfish (*55*), and shrimp (*56*).

CP B [$E_a = 20.1$ kJ/mol (*36*)] were much less responsive to temperature change than squid CP-I.

In the thermal stability study, ~50% of activity remained after 30 min of incubation at 50 °C and the enzyme inactivated >90% after 30 min at 80 °C (Figure 10). The relatively low thermal stability coupled with the temperature optimum of 60−70 °C is unusual.

Effect of Inhibitors and Activators. Seven active site protease inhibitors were individually incubated with squid CP-I to determine their influence on the activity. Data are shown in Table 8. CP-I was proven to be a metalloprotease, as shown by the strong inhibitory effect exerted by metal chelating agents. At 1 mM, both EDTA and *o*-phenanthroline inhibited more effectively than other inhibitors. At 17 mM, EDTA almost completely inhibited CP-I. The cysteine protease inhibitors *p*CMB and E64 and the aspartyl protease inhibitor pepstatin do not have a strong effect on squid CP-I activity. However, 5 mM Pefabloc, a serine protease inhibitor, inhibited more than half the activity with all three substrates. Other CP are inhibited by both metalloprotease and serine protease inhibitors. CP-II, from crab HP, was inhibited 100% by 1 mM *o*-phenanthroline and 65% by 1 mM PMSF, whereas crab HP CP-I was also inhibited 100% by 1 mM *o*-phenanthroline and 30% by 1 mM PMSF (*30*). Both were declared metallopeptidases. Dipeptidyl CP, a 155 kDa metalloprotease from *Bacillus pumilus*, was completely inhibited by 1 mM EDTA but 38% inhibited by 1 mM diisopropyl fluorophosphate (DFP), a serine protease inhibitor (*40*). Like squid CP-I (Table 8), it is activated by Co^{2+} but inhibited by Cu^{2+}. Its optimum pH was 7.5 and optimum temperature 50 °C. DTT (1 mM) inhibited 7−23% squid CP-I activity. Dipeptidyl CP, from *Streptomyces* (*41*), and CP U, from human serum (*42*), are both metallo-CP that are inhibited by 1 mM DTT (63 and 64%, respectively). Squid CP-I was partially inhibited by cysteine and DTT (Table 8).

Effect of Metal Ions. Co^{2+} activated CP-I the best of ions tested with all three substrates (200−465% of original activity, Table 9) with $MgSO_4$, $MgCl_2$, and $ZnSO_4$ giving similar activities. Cu^{2+} and Cd^{2+} inhibited squid CP-I. In both monovalent and divalent metal salts, sulfate anions gave better activation than chloride anions, especially with *N*-CBZ-Ala-Phe and Gly-Phe as substrates. All monovalent metal ions (K^+, Na^+, and Li^+) activated squid CP-I. Most metalloexopeptidases require zinc for enzyme activity to occur (*43*). CP A, a metal-containing exopeptidase, has Zn^{2+} tightly bound in the active site. When Zn^{2+} is removed from CP A, the metal-free protein has no enzymatic activity but it still binds peptide substrate (*44*). The assignment of squid CP-I to the metalloprotease class was confirmed by the fact that the activity of enzyme was restored by dialysis with Zn^{2+} after chromatofocusing (Table 3). In the absence of divalent cations, almost no activity of CP Tag was detected (*39*). The order of activation by metal ions of CP Tag was $Co^{2+} > Ca^{2+} > Mg^{2+} > Cu^{2+} > Zn^{2+}$. CP-D, from bovine pituitary, was activated similarly by metal ions as squid CP-I. It was most activated by Co^{2+}, followed by Zn^{2+} and Ca^{2+}, but it was inhibited by Cu^{2+} and Hg^{2+} (*27*).

Squid CP-I was strongly inhibited by metal chelating agents, but it was also inhibited by Pefabloc, which is a

serine protease inhibitor. On the basis of preferences for hydrophobic amino acids at the P1′ position, two subclasses were identified. Carboxypeptidase A (EC 3.4.17.1) is a metalloprotease (30−40 kDa), and carboxypeptidase C (EC 3.4.16.5) is a serine carboxypeptidase (60−175 kDa) (*45*, *46*). CP A rapidly releases the C terminus of Tyr, Phe, Trp, Leu, Ile, Thr, Gln, His, Val, and Ala. It slowly releases Asn, Ser, Lys, and Met and very slowly releases Gly, Asp, and Glu from the C terminus (*47*). CP A does not hydrolyze peptide with Pro and Arg as C-terminal amino acids. CP A, with a pH optimum of 7−8.5, contains Zn^{2+} in the active site. Its active pH range for hydrolysis is 5−10, whereas the active range for CP C is pH 4−6. For pH stability, CP A is stable in the pH range 5−10, whereas CP C is stable at pH 3−8. CP C has broad substrate specificity with an optimum pH of 4.5−6.0 (*28*, *48*, *49*), is inhibited by the action of DFP, and is sensitive to thiol-blocking reagents. Even though CP C is a serine CP, it may contain Zn^{2+} in the active site (*50*). CP C, from orange leaves, was not inhibited by EDTA or DFP, a serine protease inhibitor (*50*). Because squid CP-I shares common characteristics (broad substrate specificity, pH optimum, and active site classification) with CP A, its amino acid composition was compared with that of CP A from other sources.

Amino Acid Composition of CP-I. The amino acid composition of squid CP-I is shown in Table 10, along with the composition of CP A from other sources with the comparison in mole percent. Compared to other CP A, squid CP-I had a higher content of Gly but lower contents of Cys, Tyr, and Phe. Comparing the MDI of CP A from different sources, squid CP-I was more homologous with white shrimp CP A (MDI = 14.11, Table 11). Shrimps and squids are both marine invertebrates, whereas the other species are vertebrate organisms (phylum Chordata). These data show that CP-I has homology in amino acid composition with other sources of CP A.

In summary, squid CP-I has many characteristics in common with CP A isolated from other sources. These characteristics include metalloprotease classification, amino acid composition, broad substrate specificity, optimum pH, and pH stability. On the contrary, some properties of squid CP-I are quite different from data previously reported for CP A from other sources such as molecular weight (23−25 kDa compared to 30−40 kDa) and p*I*. Squid CP-I, with its broad specificity for hydrophobic amino acids, is a promising processing aid for protein hydrolysis to improve flavor and reduce bitterness in food protein hydrolysates.

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Received for review March 8, 2001. Revised manuscript received July 16, 2001. Accepted August 2, 2001. Funding provided by the California Dairy Research Foundation for this research is appreciated.

JF010320H