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Carboxamidopeptidase: Purification and Characterization of a Neurohypophyseal Hormone Inactivating Peptidase from Toad Skin†

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ABSTRACT: Carboxamidopeptidase, an enzyme which inactivates neurohypophyseal hormones, has been purified 3800-fold in an overall yield of 22% from toad skin, a neurohypophyseal hormone target organ, by $(NH_4)_2SO_4$ fractionation, DEAE-Sephadex chromatography, and affinity chromatography on immobilized *p*-aminobenzamidine and concanavalin A-agarose. The purified enzyme is capable of inactivating both [8-arginine]vasopressin (AVP) and oxytocin by hydrolyzing the Arg^8–Gly^9–NH_2 and the Leu^8–Gly^9–NH_2 bonds, respectively, and can hydrolyze the ester substrates, benzoyl-L-arginine ethyl ester (BzArgOEt) and acetyl-L-tyrosine ethyl ester, suggesting that the enzyme has both trypsin-like and chymotrypsin-like activities. Carboxamidopeptidase is maximally active at pH 7.5-8.5 for AVP and BzArgOEt and pH 7.0 for oxytocin. Carboxamidopeptidase is inhibited by ovoinhibitor, ovomucoid, Trasylol, lima bean trypsin inhibitor, concanavalin A, antipain, leupeptin, chymostatin, elastatinal, *p*-nitrophenyl *p*-guanidinobenzoate, and 4-methylumbelliferyl *p*-guanidinobenzoate but not by soybean trypsin inhibitor, α_1-antitrypsin, hirudin, pepstatin, bestatin, phosphoramidon, or cysteine. The enzyme is also inhibited by the serine protease inhibitor, diisopropyl phosphofluoridate (*i*-Pr_2PF), and by the chloromethyl ketone derivatives of tosyllysine, tosylphenylalanine, and (benzyloxycarbonyl)phenylalanine, as well as by the sulfhydryl group reagent, *p*-(chloromercuri)benzoate (PCMB). Inhibition by PCMB is reversed by cysteine. The molecular weight determined by gel filtration in the presence of 1 M NaCl is approximately 100 000. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicates that the enzyme is composed of two identical subunits of 48 000 daltons. Each subunit consists of a heavy chain (28 000 daltons) and a light chain (19 000 daltons) joined by a disulfide bond(s). Labeling experiments using [^{3}H]-*i*-Pr_2PF showed that the enzyme active site is located in the heavy chain.

A neurohypophyseal hormone inactivating peptidase, first detected (Campbell et al., 1965) in extracts of toad urinary bladder, a neurohypophyseal hormone target organ, was originally thought to be trypsin-like in its specificity since it hydrolyzed the Lys^8–Gly^9–NH_2 bond of [8-lysine]vasopressin (LVP)[1] with the release of free glycinamide. Subsequently, it was shown in this laboratory (Glass et al., 1969; Walter et al., 1972; Grzonka et al., 1974) that the toad bladder preparation could hydrolyze not only the Lys^8–Gly^9–NH_2 and Arg^8–Gly^9–NH_2 bonds of LVP and AVP, respectively, which are substrates for trypsin but also the Leu^8–Gly^9–NH_2 bond of oxytocin which is a substrate for chymotrypsin (Walter & Hoffman, 1974). Specificity studies using a variety of neurohypophyseal hormone analogues demonstrated that the en-

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[1] Abbreviations used: LVP, [8-lysine]vasopressin; AVP, [8-arginine]vasopressin; [^{14}C]AVP, [8-arginine,9-glycinamide-*1*-^{14}*C*]vasopressin; [^{14}C]oxytocin, [9-glycinamide-*1*-^{14}*C*]oxytocin; BzArgOEt, *N*-benzoyl-L-arginine ethyl ester; AcTyrOEt, *N*-acetyl-L-tyrosine ethyl ester; CH-Sepharose 4B, Sepharose 4B coupled with 6-aminohexanoic acid; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; MUGB, 4-methylumbelliferyl *p*-guanidinobenzoate; *i*-Pr_2PF, diisopropyl phosphofluoridate; [^{3}H]-*i*-Pr_2PF, [1,3-^{3}H]diisopropyl phosphofluoridate; Tos-Lys-CH_2Cl, tosyllysine chloromethyl ketone; Tos-Phe-CH_2Cl, tosylphenylalanine chloromethyl ketone; Z-Phe-CH_2Cl, (benzyloxycarbonyl)phenylalanine chloromethyl ketone; PCMB, *p*-(chloromercuri)benzoate; D-TrpOMe, D-tryptophan methyl ester; $NaDodSO_4$, sodium dodecyl sulfate; Me_2SO, dimethyl sulfoxide.

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zyme was surprisingly insensitive to amino acid replacements in residue position 8, provided the L configuration was maintained. The ability of the enzyme to release a C-terminal amino acid amide from these peptides suggested the name carboxamidopeptidase. The enzyme apparently did have a high specificity for glycinamide in position 9, since little or no cleavage was seen of analogues in which this moiety was replaced by glycine, glycine methylamide, glycine dimethylamide, prolinamide, or isoglutamine. In view of the interesting specificity of the toad bladder preparation, it was considered important to attempt further purification to determine if the broad specificity for position 8 was due to one or, possibly, several enzymes.

Progress was made in the purification and characterization of carboxamidopeptidase from toad urinary bladder (Simmons & Walter, 1979), but the low level of activity in the bladder and the small amount of tissue available made large-scale purification extremely difficult. It was subsequently found (Simmons, 1979) that toad skin could yield 500–1000 times more enzyme per animal than bladder and give an extract of much higher specific activity. The skin enzyme was found to be identical with the bladder enzyme in terms of substrate specificity, sensitivity to inhibitors, and chromatographic behavior.

In this paper, we report the purification and characterization of carboxamidopeptidase from toad skin and demonstrate that the enzyme retains its unusual ability to hydrolyze both AVP and oxytocin (as well as ester substrates of both trypsin and chymotrypsin) even when purified 3800-fold.

Experimental Section

Materials

Toads (*Bufo marinus*, Mexican) weighing between 200 and 400 g were obtained from Mogul-Ed. Rabbit muscle aldolase, ovalbumin, chymotrypsinogen A, ribonuclease, and Sephadex G-200 were obtained from Pharmacia Fine Chemicals. Bovine neurophysin II was available in this laboratory (Audhya & Walter, 1977). NPGB was purchased from Nutritional Biochemical Corp., Coomassie Brilliant Blue G-250 (for Bradford protein determination) was from Polysciences, Inc., ammonium sulfate, ultra pure, was from Schwarz/Mann, and Trasylol was from Farbenfabriken Bayer A6. Diisopropyl phosphofluoridate was obtained from Aldrich Chemical Co. and [1,3-^{3}H]diisopropyl phosphofluoridate (3.9 Ci/mmol) was from Amersham Corp. Antipain, leupeptin, chymostatin, elastatinal, pepstatin, bestatin, and phosphoramidon were the generous gifts of Dr. Hamao Umezawa, Microbial Chemistry Research Foundation, Tokyo, Japan. [^{14}C]AVP (30 Ci/mol) and [^{14}C]oxytocin (106 Ci/mol) were available from earlier studies (Walter & Havran, 1971). All other biochemicals and reagents were obtained from Sigma Chemical Co.

Methods

The following buffers were used during the course of these studies: 0.025 M sodium phosphate containing 0.001 M EDTA, pH 7.0 (phos–EDTA–7.0); 0.025 M sodium phosphate containing 0.001 M EDTA, pH 7.5 (phos–EDTA–7.5); 0.025 M sodium phosphate containing 0.001 M EDTA and either 1 or 2 M NaCl, pH 7.0 (phos–EDTA–1 M NaCl–7.0 and phos–EDTA–2 M NaCl–7.0, respectively); 0.025 M sodium phosphate containing 0.001 M EDTA and either 0.26 M NaCl or 1 M NaCl, pH 6.0 (phos–EDTA–0.26 M NaCl–6.0 and phos–EDTA–1 M NaCl–6.0, respectively); 0.05 M Tris containing 1 M NaCl, pH 7.5 (Tris–1 M NaCl–7.5).

Enzyme Assays. (*1*) *Peptidase Activity.* Enzyme activity was determined by using the substrate [^{14}C]AVP or [^{14}C]-oxytocin specifically labeled in the glycinamide moiety and measuring the release of free [^{14}C]Gly-NH_2. A typical assay mixture consisted of 4 μL of [^{14}C]AVP (0.39 nmol) or [^{14}C]oxytocin (0.13 nmol) in 0.25% acetic acid, 4 μL of 0.14 N NaOH, and 67 μL of enzyme in phos–EDTA–7.0, giving a final volume of 75 μL and a final pH of 7.5. The assay mixture was incubated at 25 °C (or 37 °C) for 15 min. The reaction was stopped by adding 5 μL of 6 N acetic acid or by placing the reaction mixture in a boiling water bath for 5 min. A 50-μL aliquot was removed and spotted on Whatman 3MM paper and subjected to high-voltage electrophoresis (Savant Instruments; 60 V/cm for 15 min; using 0.5% pyridine–5% acetic acid buffer, pH 3.5) in order to separate [^{14}C]Gly-NH_2 from labeled substrate ([^{14}C]AVP or [^{14}C]oxytocin) (Walter, 1973). The relative amounts of labeled product and substrate were determined by scanning the paper strip for radioactivity on a Packard Model 7201 radiochromatogram scanner.

Since the substrate concentration in these assays was low [1.7–5.2 μM; [S] $\ll K_{m(app)}$], the enzyme reaction exhibited first-order kinetics, and the amount of product released as a function of time was not linear. Activity was therefore expressed in terms of the first-order rate constant calculated in decimal logarithms:

$$k_1 = \frac{1}{t} \log \frac{100}{100 - H}$$

where t is the time in minutes and H is the percent hydrolysis at time t (Hill et al., 1958). The first-order rate constant (k_1) was found to be a linear function of enzyme concentration, and for any given concentration of enzyme, log [100/(100 – H)] was a linear function of the time of incubation t. One unit of activity is defined as the amount of enzyme preparation required in the reaction to give a $k_1 = 1$. The specific activity is defined as units of activity per milligram of protein and corresponds to the first-order proteolytic coefficient used by others.

(*2*) *Esterase Activity.* Hydrolysis of BzArgOEt and Ac-TyrOEt was measured at 25 °C by the spectrophotometric method of Schwert & Takenaka (1955) using 1 mM substrate in phos–EDTA–7.5 (also containing 0.22 M NaCl for Ac-TyrOEt). One unit of activity is defined as 1 μmol of substrate hydrolyzed per min and the specific activity is defined as units of activity per milligram of protein. Assay cuvettes were washed with 1 M NaCl solution between assays to remove active enzyme which adsorbed to the surfaces of the quartz cuvettes and accumulated if cuvettes were washed only with distilled water.

Protein Determination. Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. Protein concentrations of benzamidine–CH–Sepharose and concanavalin A–agarose column fractions were determined by the micro dye-binding method of Bradford (1976) using bovine serum albumin as the standard.

Preparation of Affinity Matrices. Benzamidine–CH–Sepharose affinity matrix was prepared by a modification of the method of Schmer (1972). CH–Sepharose 4B (6-aminohexanoic acid covalently attached to Sepharose 4B) (Sigma, lot 17C-0109) (15 g) was swollen and washed in 0.5 M NaCl and then washed with distilled water on a Büchner funnel. The wet packed gel was added to a solution of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (5.4 g) in 53 mL of distilled water, titrated to pH 4.75, and stirred gently at room temperature for 30 min. *p*-Aminobenzamidine hydrochloride (1.1 g) was then added and the mixture was rotated on a solid-phase shaker at room temperature for 20 h,

maintaining the pH at 4.75 during the first 5 h. The gel was then washed extensively on a Büchner funnel with 1 M NaCl and finally with phos–EDTA–2 M NaCl. The capacity of the affinity matrix used in these experiments, as determined by the method of Jesty & Nemerson (1974), was 3 μmol of *p*-aminobenzamidine per mL of wet packed gel. A column of benzamidine–CH–Sepharose could be reused many times by washing the column after use with 2–3 column volumes each of phos–EDTA–2 M NaCl, 8 M urea in phos–EDTA–2 M NaCl, and again phos–EDTA–2 M NaCl.

Concanavalin A–agarose was obtained commercially (Sigma). Before use, the material was packed into a column and washed with a solution of 10 mM $CaCl_2$ and 10 mM $MgCl_2$ in Tris–1 M NaCl–7.5 and finally with Tris–1 M NaCl–7.5.

Purification of Carboxamidopeptidase. All centrifugations were performed at 13700*g* for 15 min.

(*1*) *Tissue Preparation.* Twelve toads (*B. marinus*, Mexican) were decapitated and all of the skin was removed (except for the top of the head and parotid glands). The skin was cleared of any muscle tissue, washed in cold 0.65% NaCl, and then minced by passing it 2 times through a meat chopper. The minced skin (470 g) was then stored refrigerated overnight. All subsequent operations were performed at 4 °C.

(*2*) *Extraction.* Portions of minced skin (40 g) were homogenized in 360 mL of phos–EDTA–1 M NaCl–7.0 in a Waring Blendor (Model PB-5) at high speed for six 20-s intervals with 20-s intermittent rest intervals. The homogenates were pooled, brought to 0.25% (w/v) in sodium deoxycholate, and mechanically stirred for 4 h. The extract was then centrifuged, and the supernatant was collected and diluted 1:1 with phos–EDTA–7.0 buffer.

(*3*) *$(NH_4)_2SO_4$ Fractionation.* The diluted supernatant was slowly brought to 50% $(NH_4)_2SO_4$ saturation (over a 15-min period) and then allowed to stand unstirred for 1 h. The sediment was collected by centrifugation and discarded. The resulting supernatant was rapidly brought to 95% $(NH_4)_2SO_4$, allowed to stand unstirred for 14 h, and then centrifuged. The sediment, containing enzyme activity, was resuspended in 100 mL of phos–EDTA–0.26 M NaCl–6.0, dialyzed against two 6.5-L changes of the same buffer, and centrifuged to remove insoluble material.

(*4*) *DEAE-Sephadex Chromatography.* The 50–95% $(NH_4)_2SO_4$ fraction was applied at the flow rate of 24 mL/h to a 2.5 × 60 cm DEAE-Sephadex A-50-120 column equilibrated with phos–EDTA–0.26 M NaCl–6.0, and 10-mL fractions were collected. After addition of the sample, the column was washed with 1.5 column volumes of the starting buffer. The enzyme was eluted with phos–EDTA–1 M NaCl–6.0.

(*5*) *Ultrafiltration.* The pooled fractions from DEAE-Sephadex were concentrated by ultrafiltration in an Amicon cell with a PM-30 membrane to less than 10 mL. The sample buffer was changed inside the ultrafiltration unit by diluting the sample with 100 mL of phos–EDTA–2 M NaCl–7.0 containing 0.1 M arginine, followed by reconcentration. The sample (12 mL) was then centrifuged to remove insoluble material.

(*6*) *Benzamidine–CH–Sepharose Chromatography.* The concentrated enzyme was applied at a flow rate of 32 mL/h to a 1.5 × 16 cm benzamidine–CH–Sepharose affinity column equilibrated with phos–EDTA–2 M NaCl–7.0 containing 0.1 M arginine, and 2-mL fractions were collected. After addition of the sample, the column was washed first with 1 column volume of the equilibration buffer and then with one-third column volume of phos–EDTA–1 M NaCl–7.0. The enzyme was eluted with phos–EDTA–1 M NaCl–7.0 containing 50 mM D-TrpOMe. After 1 column volume of the elution buffer was passed through the column, the eluant flow was directed into a second smaller benzamidine–CH–Sepharose column (1.5 × 5 cm) equilibrated with phos–EDTA–1 M NaCl–7.0, and fractions were then collected from the second column. The enzyme activity which eluted from the second column was pooled and concentrated by ultrafiltration in an Amicon cell with a PM-30 membrane. The D-TrpOMe was removed by ultrafiltration, using repeated dilution of the concentrated sample with Tris–1 M NaCl–7.5, followed by reconcentration.

(*7*) *Concanavalin A–Agarose Chromatography.* The enzyme sample from the previous step (3 mL) was applied at a flow rate of 5.5 mL/h to a 0.5 × 1.5 cm concanavalin A–agarose column equilibrated with Tris–1 M NaCl–7.5. Fractions of 0.5 mL were collected. After addition of the sample, the column was washed with 6 mL of the equilibration buffer. One column volume of a solution of 100 mg/mL α-methyl D-mannoside in Tris–1 M NaCl–7.5 was passed through the column, and the flow was temporarily stopped. Thirty minutes later the same medium was used to elute the enzyme. Active fractions were pooled, concentrated, and converted to phos–EDTA–7.0 by ultrafiltration as described above.

Molecular Weight Determination by Gel Filtration. The molecular weight of carboxamidopeptidase was determined by gel filtration according to the method of Andrews (1965), using a Sephadex G-200 column (1 × 46 cm) equilibrated with phos–EDTA–1 M NaCl–7.0. Rabbit muscle aldolase (M_r 158 000), bovine serum albumin (M_r 68 000), ovalbumin (M_r 45 000), chymotrypsinogen A (M_r 25 000), bovine neurophysin II dimer (M_r 20 000), and ribonuclease (M_r 13 700) were used as standards.

Sodium Dodecyl Sulfate ($NaDodSO_4$)–Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate gel electrophoresis was performed by a modification of the method of Weber & Osborn (1969). Prior to electrophoresis, samples were brought to 5 M urea and 2% $NaDodSO_4$ (and also 2% 2-mercaptoethanol for samples to be electrophoresed under reducing conditions) and heated in a boiling water bath for 4 min. The molecular weight of the enzyme under both reducing and nonreducing conditions was determined from plots of mobility vs. log molecular weight for a standard mixture of proteins [Dalton Mark IV from Sigma, consisting of lysozyme (M_r 14 300), β-lactoglobulin (M_r 18 400), trypsinogen (M_r 24 000), pepsin (M_r 34 700), ovalbumin (M_r 45 000), and albumin (M_r 66 000)].

$NaDodSO_4$ Gel Electrophoresis of [^3H]-i-Pr_2PF-Labeled Enzyme. Carboxamidopeptidase was incubated with 25 μM [^{3}H]-*i*-Pr_2PF at 25 °C in a total volume of 0.2 mL for 2 h until enzyme activity was completely inhibited. The labeled enzyme was exhaustively dialyzed to remove any free [^{3}H]-*i*-Pr_2PF and then subjected to $NaDodSO_4$ gel electrophoresis under both reducing and nonreducing conditions as described above. The gels were cut into 2-mm sections, and each section was placed into 0.3 mL of 1 N NaOH for 12 h. Each NaOH extract was then diluted with 0.35 mL of 1 N HCl and 10 mL of scintillation fluid (3a70B, Research Products International) and counted in a Beckman Model LS-355 liquid scintillation counter.

Results

Purification of Carboxamidopeptidase. A summary of the purification is shown in Table I. Since preliminary experiments had shown that carboxamidopeptidase activity was located in both the dermal and epidermal layers of toad skin,

Table I: Purification of Carboxamidopeptidase

treatment	substrate	total units[a]	total protein (mg)	sp act. (units/mg)	% recovery		purification	
13700*g* supernatant	AVP	1400[b]	4900[b]	0.29	100		1.0	
	oxytocin	350[b]	4900[b]	0.071	100	100	1.0	1.0
	BzArgOEt	210[b]	4900[b]	0.043	100		1.0	
50–95% $(NH_4)_2SO_4$	AVP	1300	2000	0.65	93		2.2	
	oxytocin	350	2000	0.18	100	96	2.5	2.3
	BzArgOEt	200	2000	0.10	95		2.3	
DEAE-Sephadex	AVP	1300	210	6.2	93		21	
	oxytocin	380	210	1.8	109	97	25	22
	BzArgOEt	190	210	0.90	90		21	
benzamidine-CH-Sepharose	AVP	480	1.1	440	34		1500	
	oxytocin	110	1.1	100	31	33	1400	1500
	BzArgOEt	70	1.1	64	33		1500	
concanavalin A-agarose	AVP	310	0.29	1100	22		3800	
	oxytocin	75	0.29	260	21	22	3700	3800
	BzArgOEt	49	0.29	170	23		4000	

[a] All assays were at 25 °C. [b] After dialysis of an aliquot vs. phos-EDTA-7.0.

whole skin was used as a source of the enzyme. After centrifuging the initial enzyme extract, it was necessary to dilute the 13700*g* supernatant prior to $(NH_4)_2SO_4$ fractionation in order to ensure sedimentation of the precipitated protein with resulting higher recovery. The ammonium sulfate fractionation step (50–95%) served to concentrate the enzyme and remove sodium deoxycholate while giving a 2.3-fold purification over the supernatant with 96% recovery.

When the enzyme from the $(NH_4)_2SO_4$ fractionation was applied to a DEAE-Sephadex column equilibrated with phos–EDTA–0.26 M NaCl–6.0, the enzyme remained bound to the column while the bulk of the contaminating protein passed through and eluted in the breakthrough peak. The enzyme was eluted by increasing the NaCl concentration to 1 M in a stepwise fashion. DEAE-Sephadex chromatography resulted in a 9.6-fold purification over the $(NH_4)_2SO_4$ fraction with essentially quantitative recovery and an overall purification of 22-fold.

Figure 1 shows that when the enzyme solution from the DEAE-Sephadex step was applied to the benzamidine–CH–Sepharose column in the presence of 2 M NaCl and 0.1 M arginine, which eliminated most of the nonspecific adsorption, most of the remaining contaminating protein passed through the column. The bulk of the enzyme, however, remained bound to the column (except for some which eluted near the breakthrough peak due to overloading of the column). After application of the enzyme, the column was washed with 1 column volume of the equilibration buffer and then with one-third column volume of phos–EDTA–1 M NaCl–7.0. By decreasing the NaCl concentration of the wash buffer from 2 to 1 M, we suppressed the leaching of bound contaminants from the column just prior to eluting the enzyme. The enzyme was then eluted with 50 mM D-TrpOMe in phos–EDTA–1 M NaCl–7.0. After passing 1 column volume of the elution buffer through the column, the eluant flow was directed to a second smaller benzamidine–CH–Sepharose column. This column served to adsorb contaminating protein which leached from the first column. The enzyme, however, did not bind to the second column since it was in the presence of D-TrpOMe but eluted from this column as a single broad peak. Enzyme activity from the second column was pooled and used for the next step. This dual-column benzamidine–CH–Sepharose affinity system resulted in a 70-fold purification over the previous step and a 1500-fold purification overall and an overall recovery of 33%.

When the enzyme preparation from benzamidine–CH–Sepharose was applied to a concanavalin A–agarose column, the enzyme was retained on the column while inactive protein eluted in the breakthrough peak. When a solution of 100 mg/mL α-methyl D-mannoside in Tris–1 M NaCl–7.5 was passed through the column, the enzyme eluted as a sharp peak. This column gave a 2.5-fold purification over the previous step. The final enzyme preparation was purified 3800-fold overall, with an overall recovery of 22%.

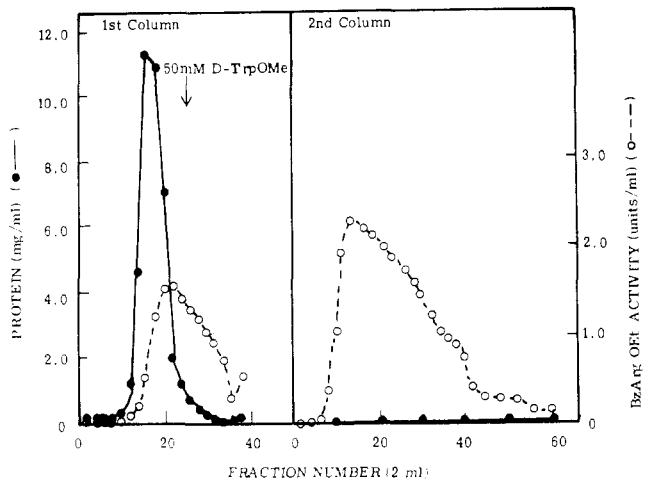

FIGURE 1: Benzamidine–CH–Sepharose chromatography. Enzyme from the DEAE-Sephadex step was chromatographed on benzamidine–CH–Sepharose as described under Methods. Protein (Bradford, 1976) (●); BzArgOEt activity (○).

The purification scheme described above yielded an enzyme preparation which was apparently homogeneous as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (see below) and by electrophoresis in standard Davis polyacrylamide gels (Davis, 1964).[2]

Table I shows that the activity against all three substrates, AVP, oxytocin, and BzArgOEt, copurified in each step.

[2] The enzyme showed a rather diffuse band (R_f = 0.75) in Davis gels (pH 9.4), while in pH 7.5 gels enzyme activity was actually spread out over a large fraction of the gel, suggesting microheterogeneity. Occasionally, a second band was also present after the concanavalin A–agarose step (R_f = 0.61 in Davis gels). This protein, when present, could not be completely separated from the enzyme by a variety of different purification techniques. Partial resolution of the two proteins could be obtained by gel filtration on Sephadex G-200 in the presence of 1 M NaCl. Fractions which contained pure contaminant were devoid of enzyme activity toward BzArgOEt, AVP, and oxytocin.

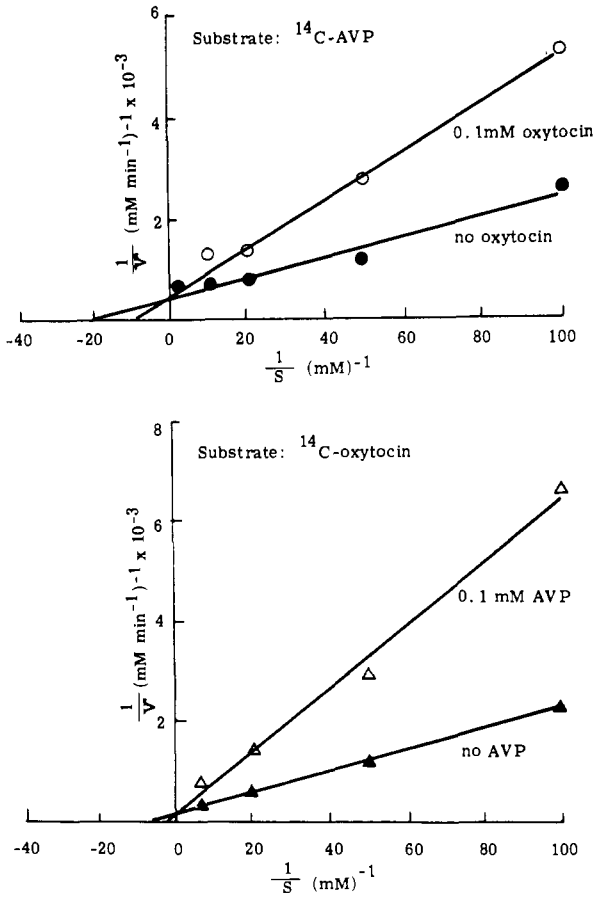

FIGURE 2: Lineweaver–Burk plots for AVP and oxytocin in the presence and absence of each other. The assay was a modification of the standard assay using [^{14}C]AVP or [^{14}C]oxytocin. Substrate concentration was varied by the addition of various amounts of unlabeled peptide and the reaction rate was quantitated from the percent cleavage of the [^{14}C]peptide (with or without 0.1 mM of the other unlabeled peptide). Top panel (40 ng of enzyme): [^{14}C]AVP without oxytocin (●); [^{14}C]AVP with 0.1 mM unlabeled oxytocin (○). Bottom panel (160 ng of enzyme): [^{14}C]oxytocin without AVP (▲); [^{14}C]-oxytocin with 0.1 mM unlabeled AVP (△).

Furthermore, no separation of these activities was seen in a variety of other purification steps utilized in preliminary phases of these studies. These results strongly suggest that these activities are all functions of a single enzyme.

Carboxamidopeptidase appeared to account for all of the BzArgOEt hydrolyzing activity in the 13700*g* supernatant. This suggests that no other trypsin-like enzymes were present in an active form in toad skin extracts under the extraction and assay conditions used.

The purified enzyme could be stored for months at 4 °C in phos–EDTA–7.0 with minimal loss of activity. A 15–20% loss of activity was observed upon freezing and thawing. Dialysis of the purified or partially purified (after DEAE-Sephadex) enzyme often led to severe losses of activity. Any buffer changes, therefore, had to be performed by repeated concentration and dilution in an ultrafiltration unit.

Lineweaver–Burk Plots for AVP and Oxytocin in the Presence and Absence of Each Other. Figure 2 (top) shows a double-reciprocal Lineweaver–Burk plot for the cleavage of [^{14}C]AVP in the presence and absence of unlabeled oxytocin. AVP, assayed without oxytocin, was found to have a $K_{m(app)}$ of 0.05 mM and a V_{max} of 2.2 μM min^{-1} at 23 °C and pH 7.5 (0.53 μg of enzyme per mL). When unlabeled oxytocin was present, the double-reciprocal plot gave the same V_{max} but different $K_{m(app)}$, suggesting that oxytocin was acting as a competitive inhibitor of AVP cleavage. The K_i of oxytocin as an inhibitor was found to be 0.1 mM.

Figure 2 (bottom) shows a double-reciprocal plot for the cleavage of [^{14}C]oxytocin in the presence and absence of unlabeled AVP. Oxytocin, assayed without AVP, was found to have a $K_{m(app)}$ of 0.14 mM and a V_{max} of 1.6 μM min^{-1} (corrected to correspond to the same amount of enzyme used in the AVP experiment, 0.53 μg of enzyme per mL). When unlabeled AVP was present, the double-reciprocal plot gave the same V_{max} but different $K_{m(app)}$, suggesting that AVP was acting as a competitive inhibitor of oxytocin cleavage. The K_i for AVP as an inhibitor was found to be 0.05 mM.

$V_{max}/K_{m(app)}$ for AVP (0.044 min^{-1}) was 4 times larger than for oxytocin (0.011 min^{-1}). Since this parameter is the best measure of substrate specificity (Brot & Bender, 1969), AVP appears to be a better substrate for carboxamidopeptidase than oxytocin under the conditions of the experiment. However, it has been observed that changes in pH and ionic strength can alter the relative activity of the enzyme, since $V_{max}/K_{m(app)}$ decreases for AVP and increases for oxytocin when the pH is lowered or the ionic strength is raised.

BzArgOEt was found to have a $K_{m(app)}$ of 0.09 mM at pH 7.5 and 25 °C.

pH Stability. Figure 3 shows the effect of preincubating the enzyme at various pH values for 2 h at 25 °C. The enzyme demonstrated maximal stability in the pH range of 6.0–7.0 and was at least partially stable over the range of pH 2.5–9.0. No activity remained upon preincubation at pH values above 9.0. The decreased stability between pH 4.0 and 5.0 was apparently associated with precipitation of enzyme in this range since little or no activity was found upon assay of the preincubated enzyme samples without first neutralizing the samples. When stored at various pH values over longer time periods, the enzyme was most stable at pH 6.0, retaining 95% of its activity after 5 days at 25 °C. However, the enzyme could also be stored for months at pH 7.0 with minimal loss of activity when kept at 4 °C.

pH Optimum. The pH optimum for BzArgOEt was 7.5–8.5 (Figure 4, top). Little activity was observed below 6.0 even though the enzyme was stable at low pH values. Failure to see activity above pH 9.0 was due at least in part to the instability of the enzyme in this region (Figure 3).

The pH optimum for AVP was in the range of 7.5–9.0 (Figure 4, middle). Significant buffer effects were noted, the enzyme having higher activity in Tris than in borate at the same pH. Little activity was observed below pH 6.5 or above pH 9.0.

The pH optimum for oxytocin was lower than for BzArgOEt or AVP (Figure 4, bottom). Maximum activity was observed at pH 7.0. The enzyme still possessed 50% maximum activity at pH 5.5–6.0 but little or no activity above pH 9.0. Tris buffer was observed to be inhibitory relative to phosphate, and succinate was inhibitory relative to phosphate and acetate at the same pH.

Temperature Stability at pH 7.5. Carboxamidopeptidase is quite heat labile at pH 7.5 (data not shown). The enzyme irreversibly lost 33% of its maximum activity when preincubated at 35 °C for 30 min and essentially all of its activity at 50 °C for 30 min. The heat lability necessitated doing most routine enzyme assays at 25 °C where no measurable inactivation occurred in the 5–15 min required for assay. It is possible that the enzyme may be more heat stable at pH values lower than 7.5 where the enzyme is more pH stable.

Effect of Organic Solvents on Enzyme Activity and Stability. Carboxamidopeptidase activity (toward BzArgOEt) gradually decreased with increasing concentrations of propylene glycol in the assay (data not shown). The enzyme was

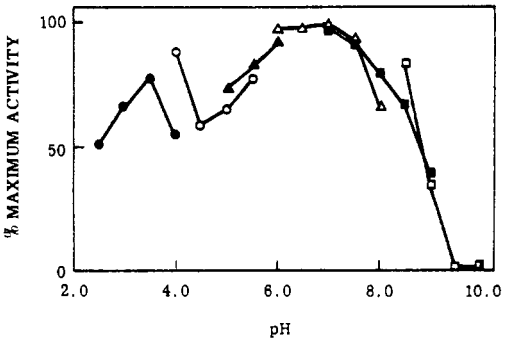

FIGURE 3: pH stability. Enzyme was preincubated in different 0.1 M buffers at 25 °C for 2 h. Aliquots were then removed directly (or after neutralization with 4 volumes of 0.1 M phosphate, pH 7.5, for preincubation mixtures containing citrate and acetate) and assayed for BzArgOEt activity. Citrate (●); acetate (○); succinate (▲); phosphate (△); Tris (■); borate (□).

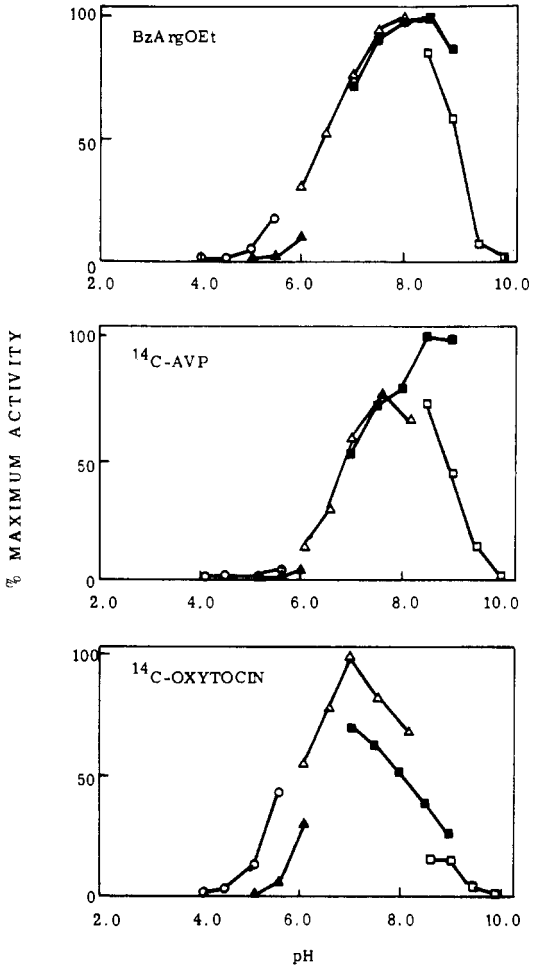

FIGURE 4: pH optimum. Enzyme was incubated with each substrate in various 0.1 M buffers at 25 °C for 15 min by using the standard assays described under Methods. Top panel: BzArgOEt as the substrate. Middle panel: [^{14}C]AVP as the substrate. Bottom panel: [^{14}C]oxytocin as the suubstrate. Acetate (○); succinate (▲); phosphate (△); Tris (■); borate (□).

only 10% active in 20% (v/v) propylene glycol even though the enzyme was completely stable in propylene glycol at concentrations as high as 40%. Above 40%, the enzyme rapidly became irreversibly inactivated, showing little residual activity when preincubated in 55% propylene glycol. The enzyme exhibited similar stability in methanol but was more active at low concentrations of methanol compared to propylene glycol. Carboxamidopeptidase was completely inactive

Table II: Effect of Proteinase Inhibitors on Carboxamidopeptidase Activity

inhibitor	concn	% inhibn
i-Pr_2PF[a]	0.01 mM	41
	0.1 mM	96
Tos-Lys-CH_2Cl[a]	1.0 mM	65
Tos-Phe-CH_2Cl[a,b]	1.0 mM	60
Z-Phe-CH_2Cl[a,c]	0.2 mM	37
PCMB [*p*-(chloromercuri)benzoate][a]	0.005 mM	74
	0.05 mM	97
NPGB (*p*-nitrophenyl *p*-guanidinobenzoate)[d]	1.0 μM	61
	10. μM	100
MUGB (4-methylumbelliferyl *p*-guanidinobenzoate)[d]	1.0 μM	49
	10. μM	82
cysteine[d]	1.0 mM	0
	10. mM	0
ovoinhibitor[d]	0.01 mg/mL	38
	0.1 mg/mL	55
Trasylol (basic pancreatic trypsin inhibitor)[d]	0.01 mg/mL	27
	0.1 mg/mL	50
lima bean trypsin inhibitor[d]	0.1 mg/mL	42
	1.0 mg/mL	60
ovomucoid[d]	0.1 mg/mL	27
	1.0 mg/mL	43
soybean trypsin inhibitor (Kunitz)[d]	1.0 mg/mL	0
α_1-antitrypsin[d]	1.0 mg/mL	0
concanavalin A[d]	0.1 mg/mL	34
hirudin[d]	1.0 unit/mL	0

[a] Enzyme was preincubated with test reagent at the indicated concentration for 10 min at 25 °C and pH 7.5 in a total volume of 40 μL. An aliquot was then removed and assayed for residual activity by using BzArgOEt as the substrate and compared to control enzyme preincubated in the absence of reagent. [b] Preincubation was in 20% methanol (v/v) and pH was 8.0. [c] Preincubation was in 10% Me_2SO (v/v) for 30 min and pH was 7.9. [d] Enzyme was preincubated with or without inhibitor for 5 min at 25 °C and pH 7.5. Reaction was started by the addition of BzArgOEt (to 1 mM) to the preincubation mixture. The indicated inhibitor concentration is the concentration in the final assay mixture.

in 5% dioxane. However, in 10% dimethyl sulfoxide, the V_{max} of the enzyme toward BzArgOEt was actually increased by 70% and the $K_{m(app)}$ was increased 10-fold. Similarly, dimethylformamide at a concentration as low as 0.5% increased BzArgOEt activity in the standard assay by 38%.

Effect of Proteinase Inhibitors on Carboxamidopeptidase Activity. Table II shows that various protease inhibitors were capable of inhibiting carboxamidopeptidase. The serine protease inhibitor, diisopropyl phosphofluoridate (*i*-Pr_2PF), essentially completely and irreversibly inhibited carboxamidopeptidase at 0.1 mM when preincubated with the enzyme for 10 min at 25 °C and pH 7.5. Reagents which alkylate the active-site histidine residue of trypsin (Tos-Lys-CH_2Cl) and chymotrypsin (Tos-Phe-CH_2Cl and Z-Phe-CH_2Cl) also caused significant inhibition of carboxamidopeptidase. The most effective chemical modification reagent was *p*-(chloromercuri)benzoate (PCMB) which covalently modifies free sulfhydryl groups. This reagent was completely inhibitory at 0.05 mM. PCMB inhibition could be reversed by the addition of 1 mM cysteine, the enzyme regaining 80% of its activity within 15 min. Cysteine alone, at concentrations as high as 10 mM, had no effect on carboxamidopeptidase activity.

Synthetic active-site titrants of trypsin, *p*-nitrophenyl *p*-guanidinobenzoate (NPGB) and 4-methylumbelliferyl *p*-guanidinobenzoate (MUGB), were effective inhibitors of carboxamidopeptidase, causing essentially complete inhibition at 10 μM. The rate of turnover of 50 μM NPGB by carboxamidopeptidase at pH 8.0 was measured spectrophotometrically at 403 nm and was found to be at most only 1/3000 the rate of turnover of BzArgOEt in the standard assay.

Table III: Effect of Microbial Proteinase Inhibitors[a]

inhibitor	IC_{50} (μg/mL)[b]	K_i (M)	type of inhibn
antipain	4.6	1.6×10^{-6}	competitive
leupeptin	44	1.4×10^{-5}	competitive
chymostatin[c]	65	4.0×10^{-5}	competitive
elastatinal	170	6.3×10^{-5}	competitive
pepstatin[c]	>200		
bestatin	>200		
phosphoramidon	>200		

[a] Inhibition measured at pH 7.5 by using BzArgOEt as the substrate. [b] BzArgOEt concentration was 1 mM. [c] Assayed at pH 7.8 in the presence of 10% Me_2SO.

Therefore, NPGB was an effective inhibitor but a very poor substrate.

Various natural protein proteinase inhibitors were also capable of inhibiting carboxamidopeptidase. Ovoinhibitor from chicken egg white was the best inhibitor of this type, giving 38% inhibition at 0.01 mg/mL. Trasylol (basic pancreatic trypsin inhibitor, bovine), lima bean trypsin inhibitor, and ovomucoid from chicken egg white also served as inhibitors. However, soybean trypsin inhibitor and α_1-antitrypsin from human plasma had no effect on carboxamidopeptidase at concentrations as high as 1 mg/mL. Concanavalin A, a lectin from jack beans which we found to bind to carboxamidopeptidase, inhibited the enzyme at 0.1 mg/mL. Hirudin, an inhibitor of thrombin, had no effect on the enzyme at 1 unit/mL.

Table III shows the effect of various microbial proteinase inhibitors (Umezawa & Aoyagi, 1977) on carboxamidopeptidase. IC_{50} values listed in the table (inhibitor concentrations necessary to give 50% inhibition of the cleavage of 1 mM BzArgOEt) were calculated from plots of percent inhibition vs. log inhibitor concentration. K_i values were determined from Dixon (1953) plots. Antipain, leupeptin, chymostatin, and elastatinal were all competitive inhibitors of carboxamidopeptidase. Antipain was the best inhibitor, causing 50% inhibition at 4.6 μg/mL and giving a K_i value (1.6×10^{-6} M) at least 1 order of magnitude lower than the K_i values of the other inhibitors. Pepstatin, bestatin, and phosphoramidon failed to inhibit the enzyme at concentrations as high as 200 μg/mL.

Time Course of Inactivation of Carboxamidopeptidase by i-Pr_2PF. Figure 5 shows that carboxamidopeptidase activity continuously decreased with increasing time of preincubation with *i*-Pr_2PF. The BzArgOEt activity of carboxamidopeptidase decreased at the same rate as did the AcTyrOEt activity, suggesting that both activities were being inhibited at the same rate by *i*-Pr_2PF. The pseudo-first-order rate constant for the reaction of the enzyme with *i*-Pr_2PF (k_{obsd}), calculated from

$$k_{obsd} = \frac{\ln \frac{e_1}{e_2}}{t_2 - t_1}$$

where e_1 is the enzyme activity at time t_1 and e_2 is the enzyme activity at time t_2, was 7.4×10^{-4} s^{-1}. The second-order rate constant (Powers, 1977), $k_{obsd}/[i\text{-}Pr_2PF]$, was 74 M^{-1} s^{-1}. Trypsin, on the other hand, was inhibited at a much slower rate by *i*-Pr_2PF while chymotrypsin was inhibited at a much faster rate than carboxamidopeptidase (Figure 5).

Molecular Weight Determination by Gel Filtration. Carboxamidopeptidase eluted from a Sephadex G-200 column as a single sharp peak when the column was equilibrated with buffer containing 1 M NaCl. The molecular weight, estimated by the method of Andrews (1965), was 100 000.

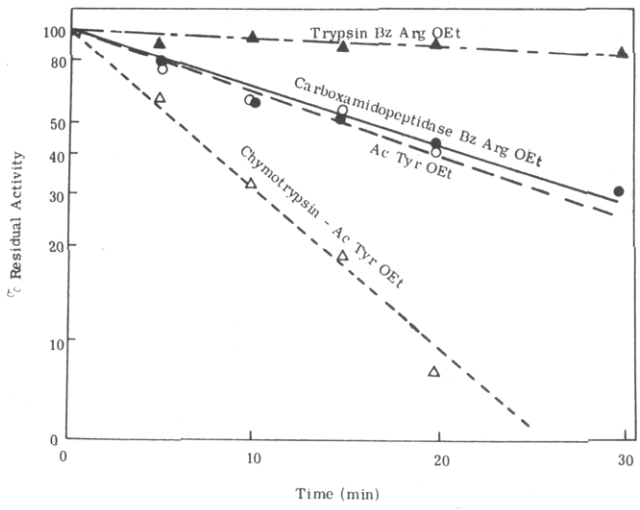

FIGURE 5: Time course of inactivation of carboxamidopeptidase, trypsin, and chymotrypsin by *i*-Pr_2PF. Enzyme was preincubated at 26 °C with 10 μM *i*-Pr_2PF in phos–EDTA–7.5. Aliquots were removed at 0, 5, 10, 15, and 30 min and assayed for residual activity at pH 7.5 by using BzArgOEt (carboxamidopeptidase and trypsin) or AcTyrOEt (carboxamidopeptidase and chymotrypsin) as the substrate. Enzyme preincubated in the absence of *i*-Pr_2PF served as a control. Log percent residual activity was plotted vs. time of preincubation. Carboxamidopeptidase, BzArgOEt activity (●); carboxamidopeptidase, AcTyrOEt activity (○); trypsin (▲); chymotrypsin (△).

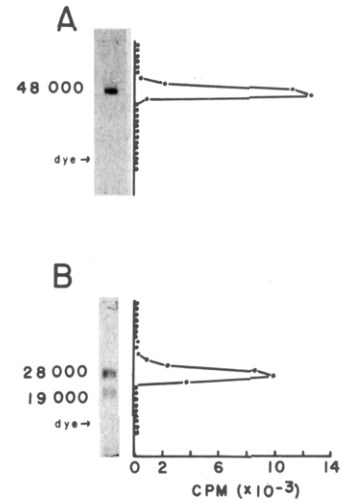

FIGURE 6: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of carboxamidopeptidase. $NaDodSO_4$ gel electrophoresis was performed as described under Methods using either (A) nonreducing conditions or (B) reducing conditions (presence of 2-mercaptoethanol). Gels were stained for protein by using Coomassie brilliant blue. Companion gels containing [^{3}H]-*i*-Pr_2PF-labeled carboxamidopeptidase were cut into 2-mm sections, extracted, and counted for radioactivity as described under Methods.

In the absence of 1 M NaCl, the enzyme (partially purified) eluted from Sephadex G-200 as a broad peak near the void volume. When chromatographed on Sepharose 4B in the absence of NaCl, the enzyme also eluted as a broad peak with an average molecular weight of 320 000, suggesting that the enzyme may form higher molecular weight aggregates in the absence of high salt.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Figure 6A shows that purified carboxamidopeptidase gave only one band in $NaDodSO_4$ gels under nonreducing conditions corresponding to a molecular weight of 48 000. All of the incorporated [^{3}H]-*i*-Pr_2PF was associated with this band.

Figure 6B shows that carboxamidopeptidase gave two bands in $NaDodSO_4$ gels under reducing conditions corresponding to molecular weights of 28 000 and 19 000. Incorporated [^{3}H]-*i*-Pr_2PF was associated with the 28 000-dalton band.

Since carboxamidopeptidase is a glycoprotein (see below), it is possible that the molecular weight determinations by both gel filtration and $NaDodSO_4$ gel electrophoresis may be somewhat overestimated.

Discussion

The extraction of carboxamidopeptidase from toad skin was most effective when 1 M NaCl was included in the homogenizing media, yielding 2.5 times more activity than when dilute buffer alone was used. High salt concentration has been shown to be effective in extracting other proteases from skin (Hopsu-Havu et al., 1977) and may involve disruption of the proteoglycan matrix. The amount of carboxamidopeptidase activity in the extract was further doubled by subsequent addition of 0.25% sodium deoxycholate to the homogenate, followed by stirring for 4 h, while stirring for periods longer than 4–6 h led to a gradual loss of activity.

Initial attempts to purify the enzyme on a DEAE-Sephadex column at pH 7.0 using a linear chloride gradient revealed that a major peak of activity accounting for about 30% of the applied enzyme eluted at 0.32 M NaCl while additional activity continued to gradually elute from the column throughout the gradient up to 1 M NaCl. Since the relative AVP, oxytocin, and BzArgOEt activities were the same for any fraction tested, the behavior suggested the possibility of multiple charged forms of one enzyme. (A diffuse band in standard gel electrophoresis supports this contention.) In order to elute as much enzyme activity as possible from the column in a minimal volume, we utilized a stepwise rather than gradient elution.

It was noted that since carboxamidopeptidase could cleave the Arg–Gly bond of AVP, the enzyme must have a binding site for arginine and therefore might be inhibited by trypsin inhibitors such as benzamidine and *p*-aminobenzamidine which possess an amidino group similar to arginine (Mares-Guia & Shaw, 1965). Both benzamidine and *p*-aminobenzamidine were found to inhibit carboxamidopeptidase, having K_i values of about 5 mM and 1 mM, respectively. This observation was used as a basis for the development of an affinity chromatographic column in which *p*-aminobenzamidine was covalently attached to Sepharose 4B through a 6-aminohexanoic acid spacer arm. Carboxamidopeptidase effectively adsorbed to the benzamidine–CH–Sepharose affinity material and, in early studies, was eluted from the affinity matrix with 0.2 M benzamidine, resulting in a sevenfold purification over the DEAE-Sephadex eluent. However, it was realized that this affinity column might be made more efficient by taking advantage of the dual specificity of carboxamidopeptidase, i.e., its chymotrypsin- as well as trypsin-like specificity. A chymotrypsin inhibitor, D-TrpOMe (Kezdy et al., 1972), was found to inhibit carboxamidopeptidase (K_i = 0.1 mM) and was therefore used to selectively elute the enzyme from the benzamidine–CH–Sepharose column. Elution with 50 mM D-TrpOMe increased the effectiveness of the affinity column, giving a 30-fold purification in one step. When a second smaller affinity column was placed in series with the first column to adsorb contaminants which leached along with the eluted enzyme from the first column, the purification was increased to 70-fold. Recoveries from this dual benzamidine–CH–Sepharose step ranged from 35 to 60%.

Carboxamidopeptidase was observed to bind to concanavalin A–agarose and to elute with a solution containing 100 mg/mL α-methyl D-mannoside, suggesting that the enzyme is a glycoprotein and that glucose or mannose residues on the carbohydrate moieties bind to the immobilized concanvalin A lectin. The fact that carboxamidopeptidase is a glycoprotein could explain the presence of multiple charged forms since different populations of enzyme molecules could possess different amounts of negatively charged sialic acid residues.

The pH optimum studies using [^{14}C]AVP and [^{14}C]oxytocin were done at very low substrate concentrations [[S] << $K_{m(app)}$] so that the results reflect the pH dependence of both $K_{m(app)}$ and k_{cat}. The difference in the pH optimum seen for AVP and oxytocin can therefore possibly be explained by differences in the pH dependence of binding of the positively charged arginine residue of AVP and the neutral leucine residue of oxytocin to the S_1 binding site (Schechter & Berger, 1967) of the enzyme. The loss of carboxamidopeptidase activity above pH 9.0 is apparently due to deprotonation of a residue(s) responsible for stabilizing the enzyme structure since the enzyme is both inactive and unstable above pH 9.0.

Carboxamidopeptidase was essentially completely inhibited by preincubation with 0.1 mM *i*-Pr_2PF (diisopropyl phosphofluoridate) for 10 min. *i*-Pr_2PF characteristically inhibits most proteases and peptidases containing a reactive serine as part of its active site and is generally considered to be a group-specific inhibitor for these types of enzymes (Barrett, 1977). On the basis of inhibition by *i*-Pr_2PF, carboxamidopeptidase can therefore tentatively be classified as a serine protease (peptidase). When compared to two other serine proteases, carboxamidopeptidase was found to be more reactive with *i*-Pr_2PF than trypsin but less reactive than chymotrypsin. Carboxamidopeptidase was also inhibited by the trypsin inhibitor, Tos-Lys-CH_2Cl, and the chymotrypsin inhibitors, Tos-Phe-CH_2Cl and Z-Phe-CH_2Cl, an observation that is in keeping with the dual trypsin–chymotrypsin-like specificity of this enzyme.

p-(Chloromercuri)benzoate (PCMB) is known to specifically react with free sulfhydryl groups of proteins to form mercaptides (Barrett, 1977). PCMB was found to completely inhibit carboxamidopeptidase at concentrations as low as 50 μM. Carboxamidopeptidase, therefore, appears to contain a free sulfhydryl group whose modification by PCMB results in loss of activity. It is not known whether the sulfhydryl group is located at the active site or at some distant point which is critical to enzyme conformation. The inhibition by PCMB was reversed by the addition of cysteine which presumably displaces the mercurial from the enzyme sulfhydryl group. We observed that carboxamidopeptidase could be partially purified by covalently binding the enzyme to PCMB–Sepharose (Cuatrecasas, 1970) and then eluting the enzyme with 0.01 M cysteine.

The trypsin active-site titrants, NPGB and MUGB, were good inhibitors of carboxamidopeptidase. These synthetic esters were presumably cleaved by the enzyme, resulting in a stable guanidinobenzoyl–enzyme intermediate whose deacylation rate is extremely slow (Chase & Shaw, 1970). Indeed, these compounds caused instantaneous inhibition of the enzyme but were turned over by the enzyme only slowly.

Carboxamidopeptidase was observed to be inhibited by naturally occurring protein proteinase inhibitors such as ovoinhibitor, ovomucoid, Trasylol, and lima bean trypsin inhibitor. However, the inhibition was relatively much weaker than that seen for enzymes like trypsin and chymotrypsin where inhibition is seen at a 1:1 molar ratio (Kassel, 1970a–c). By comparison, inhibition experiments in Table II were performed by using a 10^2–10^5-fold molar excess of these inhibitors.

Four microbial proteinase inhibitors isolated from actinomyces preparations by Umezawa and co-workers (Umezawa & Aoyagi, 1977) were found to be inhibitors of carboxamidopeptidase: antipain, leupeptin, chymostatin, and elastatinal. Antipain and leupeptin both contain a C-terminal argininal residue and inhibit trypsin but not chymotrypsin. Chymostatin, on the other hand, contains a C-terminal phenylalaninal residue and inhibits chymotrypsin but not trypsin. Elastatinal, an inhibitor of elastase, possesses a C-terminal alaninal. Other microbial inhibitors such as pepstatin (an inhibitor of acid proteases such as pepsin and cathepsin D), bestatin (an inhibitor of aminopeptidases), and phosphoramidon (an inhibitor of metalloproteases such as thermolysin) were without effect on carboxamidopeptidase.

Carboxamidopeptidase was assayed and purified in buffers containing 1 mM EDTA, suggesting that the enzyme is both active and stable in the absence of metal ions and therefore is not a metalloprotease. Calcium ions (1 or 10 mM) have little or no effect on the enzyme activity (BzArgOEt), but at 10 mM make the enzyme more temperature labile (results not shown).

The molecular weight of carboxamidopeptidase was estimated by gel filtration in the presence of 1 M NaCl to be 100 000, although higher molecular weight aggregates can apparently form in the absence of high salt. When carboxamidopeptidase was subjected to $NaDodSO_4$ gel electrophoresis under nonreducing conditions (Figure 6A), only one major band was observed when stained for protein. In a companion gel containing carboxamidopeptidase labeled with [^{3}H]-*i*-Pr_2PF at the active site, only one peak of radioactivity was observed whose mobility corresponded to the single protein band. Thus, only one *i*-Pr_2PF-sensitive enzyme was present and this enzyme corresponded to the bulk of the protein in the preparation. The molecular weight of this protein was estimated to be 48 000, indicating that carboxamidopeptidase consists of two identical noncovalently bound subunits.

When $NaDodSO_4$ gel electrophoresis was performed under reducing conditions (in the presence of 2-mercaptoethanol) (Figure 6B), two major bands were observed which stained for protein. The two bands had about equal staining intensity and their mobilities corresponded to molecular weights of 28 000 and 19 000, suggesting that each subunit of carboxamidopeptidase is actually composed of two polypeptide chains which are joined by a disulfide bond(s) and dissociate in the presence of 2-mercaptoethanol. Figure 6B also shows that when a companion gel containing carboxamidopeptidase labeled at the active site with [^{3}H]-*i*-Pr_2PF was subjected to electrophoresis under reducing conditions, a single peak of radioactivity was observed which had the same mobility as the 28 000-dalton chain. This suggests that the active site of carboxamidopeptidase is located in the heavy chain.

Carboxamidopeptidase is characterized by its unusual ability to hydrolyze both the Arg^8–Gly^9–NH_2 bond of AVP and the Leu^8–Gly^9–NH_2 bond of oxytocin and to hydrolyze the esters, BzArgOEt and AcTyrOEt, which are substrates for trypsin and chymotrypsin, respectively. This dual trypsin–chymotrypsin-like specificity was first noted in studies of crude toad urinary bladder enzyme preparations and is confirmed here for purified skin carboxamidopeptidase. Several lines of evidence strongly suggest that these activities are all due to a single enzyme. (1) Trypsin-like activity (AVP and BzArgOEt) and chymotrypsin-like activity (oxytocin) copurified at each step of the isolation procedure (Table I). No separation of these activities was ever observed in any of the many separation techniques used. (2) Preparations which give a single band of protein on standard as well as $NaDodSO_4$–polyacrylamide gels contain all of the activities (AVP, BzArgOEt, oxytocin, and AcTyrOEt). (3) [^{3}H]-*i*-Pr_2PF, which inhibits all of the activities, was found to be incorporated only into a single band on $NaDodSO_4$ gels. (4) The time course of inactivtion of trypsin-like activity (BzArgOEt) by *i*-Pr_2PF was identical with that of chymotrypsin-like activity (AcTyrOEt). (5) Cleavage of a trypsin substrate (BzArgOEt) was inhibited by several chymotrypsin inhibitors (D-TrpOMe, chymostatin, Tos-Phe-CH_2Cl, and Z-Phe-CH_2Cl). (6) Enzyme which was bound to a trypsin inhibitor affinity column (benzamidine–CH–Sepharose) was eluted by a chymotrypsin inhibitor (D-TrpOMe). (7) Cleavage of AVP was competitively inhibited by oxytocin while cleavage of oxytocin was competitively inhibited by AVP. The K_i values of these peptides as inhibitors were identical with their $K_{m(app)}$ values as substrates.

The ability to cleave ester substrates of both trypsin and chymotrypsin is shared by certain other serine proteases such as subtilisin (Ottesen & Svendsen, 1970), *Streptomyces griseus* proteases A and B (Jurasek et al., 1976), pig pancreatic kallikrein (Fiedler, 1976), C1s esterase (Cooper & Ziccardi, 1976), and crab hepatopancreas collagenase (Eisen et al., 1973), but the properties of these enzymes differ in many significant respects from those of carboxamidopeptidase. Another serine esterase which hydrolyzes both BzArgOEt and AcTyrOEt has been partially purified (54-fold) from rat skin (Seppa & Jarvinen, 1976). This enzyme, like carboxamidopeptidase, is inhibited by both *i*-Pr_2PF and PCMB and has a molecular weight of 125 000. It differs from carboxamidopeptidase in having a lower pH optimum (6.0–6.5), requiring KCl for activity and stability, and by its inability to be inhibited by Tos-Lys-CH_2Cl (1 mM), lima bean trypsin inhibitor, ovomucoid, or Trasylol (1 mg/mL). The peptidase activity of this enzyme was not examined.

The purification scheme developed for carboxamidopeptidase using affinity chromatography is based on the functional properties of the enzyme rather than on its physicochemical characteristics. The affinity column should therefore facilitate the identification and purification of functionally similar enzymes from other sources even though these enzymes may differ from toad skin carboxamidopeptidase in terms of charge, size, etc.

The significance of carboxamidopeptidase in the metabolism of neurohypophyseal hormones is unknown (Walter & Simmons, 1977). Toad skin, like bladder, is a target organ for neurohypophyseal hormones (Bentley, 1974). Earlier studies showed that intact toad urinary bladder can produce traces of [^{14}C]Gly-NH_2 from [8-lysine,9-glycinamide-*1*-^{14}*C*]vasopressin when incubated in a bath containing the hormone (Havran et al., 1971). This observation is in accord with the presence of carboxamidopeptidase in the urinary bladder and suggests that the enzyme may have access to the circulating hormone in the intact tissue. Similar experiments have not yet been done with toad skin.

The presence of relatively high concentrations of carboxamidopeptidase in skin, which is primarily connective tissue, is interesting in view of the fact that this enzyme is also localized almost exclusively in the connective tissue of the urinary bladder rather than in the epithelial cells (Simmons & Walter, 1979). It is possible that carboxamidopeptidase has a role in connective tissue turnover. Preliminary experiments have suggested that the specificity of carboxamidopeptidase is not limited to intact neurohypophyseal hormones or to ester substrates of trypsin and chymotrypsin. A more detailed examination of the substrate specificity of carbox-

amidopeptidase is in progress.

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