Eur. J. Biochem. 117, 383–388 (1981)

Crystallization and Properties of Carboxypeptidase A_γ from Porcine Pancreas

Atsushi KOIDE, Masayuki YOSHIZAWA, and Kotoku KURACHI

Eisai Research Laboratories, Eisai Co. Ltd, Tokyo

(Received September 17/December 29, 1980)

Carboxypeptidase A_γ from porcine pancreas was purified to homogeneity by ammonium sulfate fractionation, autolysis, batch absorption and elution from DEAE-Sephadex, and crystallization. The overall purification was about 32-fold with a yield of 31% and the specific activity of the purified protein was 108 units/mg protein.

The apparent relative molecular mass determined by gel filtration on a Sephadex G-200 column was 38900. The amino-terminal sequence of the porcine carboxypeptidase A_γ was Asn-Tyr-Ala-Thr-Tyr-His-Thr-Leu-Glu-Glu-Ile-Tyr-Asp-Phe-Met-Asp-Ile-Leu-Val-Ala-Glu-His-Pro-Gln-Leu- which was highly homologous to that of bovine carboxypeptidase A_γ. The purified enzyme was characterized with respect to isoelectric point (4.3). K_m for N^α-carbobenzoxyglycyl-L-phenylalanine (Cbz-Gly-LPhe) (20 mM), amino acid composition, pH optimum, pH stability, stability at different temperatures and effect of drying. The enzyme contained 1.01 mol zinc/mol and was inhibited by chelating agents such as EDTA and *o*-phenanthroline.

Among substrates such as Cbz-Gly-LPhe, N^α-benzoylglycyl-L-arginine, various kinds of amino acid esters, casein and elastin, porcine carboxypeptidase A_γ showed an enzymatic activity only towards Cbz-Gly-LPhe and casein. These data are in good agreement with the substrate specificity of bovine carboxypeptidase A.

As reported by Folk and Schirmer [1] in 1963, porcine pancreatic carboxypeptidase A exists in three electrophoretically distinct forms, carboxypeptidase A_1, A_2 and A_3. These forms are generated by limited proteolysis on the amino-terminal region of procarboxypeptidase A [2]. Carboxypeptidase A_1 and A_2 were well characterized [1]. However, carboxypeptidase A_3 was often contaminated with A_2 and has not yet been fully characterized [1].

Narayanan and Anwar [3] in 1970 reported a porcine carboxypeptidase A, designated as Ae, which was isolated from a twice-crystallized porcine elastase preparation. This species of carboxypeptidase A was different from carboxypeptidase A_1, A_2 and A_3 in terms of solubility and amino acid composition, and was reported as a possible new genetic variant of carboxypeptidase A.

More recently, Kobayashi et al. [4] have reported an isolation of carboxypeptidase A_β from a binary complex preparation of procarboxypeptidase A and a precursor of protease E in porcine secretion. They found that the binary complex consisted of one molecule of carboxypeptidase A_β and one molecule of protease E. The latter enzyme was inhibited by diisopropylfluorophosphate.

During our studies on the development of a method for a large-scale production of carboxypeptidase in high yield from porcine pancreas, we isolated and crystallized porcine carboxypeptidase A_γ. Furthermore, it was found that carboxypeptidase A_γ thus obtained was associated with porcine elastase I in crystalline state.

In this paper, we describe details of the purification method of carboxypeptidase A_γ from porcine pancreas and some properties of the enzyme.

Abbreviations. Cbz-Gly-LPhe, N^α-carbobenzoxyglycyl-L-phenylalanine; Bz-Gly-LArg, N^α-benzoylglycyl-L-arginine.

Enzymes. Carboxypeptidase A (EC 3.4.17.1); chymotrypsin B (EC 3.4.21.1); elastase (EC 3.4.21.11).

EXPERIMENTAL PROCEDURE

Materials

Pancreatic glands were obtained from freshly slaughtered pigs and stored at −20 °C.

N^α-Carbobenzoxyglycyl-L-phenylalanine, N^α-benzoylglycyl-L-arginine, the ethyl esters of N^α-acetyl-L-tyrosine, N^α-benzoyl-L-arginine, glycine, L-leucine, L-isoleucine, and the methyl esters of N^α-benzoyl-L-alanine, L-serine, L-methionine, L-valine, L-tyrosine, L-phenylalanine, L-tryptophan, L-lysine, L-histidine, and L-arginine were obtained from the Peptide Research Foundation (Japan).

Casein was a product of Merck. Elastin was purchased from N.B.C. Chemical Corp. Cellulose acetate membranes (Oxoid) were obtained from Oxoid Ltd, England, and DEAE-Sephadex A-50 was purchased from Pharmacia Fine Chemicals.

Porcine elastase was prepared by the method of Smillie and Hartley [5]. Bovine carboxypeptidase A_γ was purchased from Worthington Biochemical Corp. Soybean trypsin inhibitor was obtained from Miles Laboratories Inc. Hen ovalbumin was obtained from Mann Research Laboratories. Bovine serum albumin was obtained from Seikagaku Kogyo Ltd, Japan. Immunoglobulin G (human) fraction II was purchased from Nutritional Biochemical Corp.

Protein concentration was estimated by the biuret method.

Enzymatic Assays

Esterase activities on the various amino acid esters were determined by pH titration with 0.02 M NaOH of the hydrogen ions released on hydrolysis, using a pH-stat (Radiometer, Copenhagen) with recorder. Assays were performed at 30 °C and pH 8.0 in 10 mM Tris/HCl buffer containing 0.1 M KCl. 5 ml of 20 mM substrate solution were pipetted into a vessel. Then, 0.2 ml (4 mg/ml concentration) of enzyme solution

were added and, under a flux of N_2 gas, the released hydrogen ions were titrated with 0.02 M NaOH solution.

Peptidase activities towards Cbz-Gly-L-Phe and Bz-Gly-L-Arg were assayed by the photometric ninhydrin method [6] at pH 7.5 in 10 mM phosphate buffer, pH 7.5 containing 0.1 M NaCl. 1 ml of 20 mM substrate solution was pipetted into a test tube. After standing for 5 min at 25 °C, 0.2 ml of enzyme solution were added and then incubated for 15 min at 25 °C. The reaction was terminated by the addition of 1 ml of 3 M acetate buffer, pH 5.0 and the color development with ninhydrin reagent was carried out by the method of Yemm and Cocking [6].

Caseinolytic activity was determined according to Kunitz [7].

Elastase activity was determined with elastin as a substrate according to Grant and Robbins [8]. The sample solution, 1 ml, was pipetted into a conical flask. Then, 4 ml of 0.6% elastin were added with vigorous agitation. The conical flasks were incubated with shaking for 30 min at 37 °C. To stop the reaction, 5 ml of 0.05% sodium dodecyl sulfate were added. The sample solution was then centrifuged and the absorbance of the supernatant measured at 275 nm against a blank of water. One unit of elastase activity was defined as the amount of enzyme which yielded 1 µg tyrosine/min in the supernatant of the reaction mixture under those conditions.

Polyacrylamide Electrophoresis

Disc electrophoresis in 7.5% polyacrylamide gels was performed according to Davis [9]. The gels were stained with Coomassie brilliant blue R250. Electrophoresis was performed in 50 mM Tris/HCl pH 9.4 at a constant current of 3 mA/tube, for 80 min at room temperature. Samples of 50 µg of protein were applied to these gels.

Determination of the Apparent Relative Molecular Mass

The M_r of the purified porcine carboxypeptidase A_γ was determined on a Sephadex G-200 column (1.5 × 85 cm) according to Andrews [10]. The column was eluted with 0.1 M phosphate buffer, pH 8.3 containing 1.0 M NaCl at a flow rate of 20 ml/h. The absorbance at 280 nm of the effluent fraction was determined. The standard proteins used were soybean trypsin inhibitor (21500), elastase (25000), ovalbumin (45000), serum albumin (67000) and immunoglobulin G (160000).

Isoelectric Point

The isoelectric point was determined by paper electrophoresis according to Kunkel and Tiselius [11]. Electrophoresis was performed on Whatman no. 1 paper (12.5 × 26 cm) in an acetate buffer (I = 0.3 M) at a constant current, for 5 h at room temperature. The protein was stained with 1% light green in 3% acetic acid. The standard used for neutral position of electrophoretic pattern was D-xylose and this sugar was stained with a mixture of 0.1 M $AgNO_3$ and 6 M NH_3.

Mobility (U) was calculated from the following equation:

$$U = ld/tV \ (\mathrm{cm^2\ s^{-1}\ V^{-1}}),$$

where l = length of paper, t = migration time, V = voltage, d = migration distance (migration distance of proteins minus migration distance of D-xylose).

Metal Analyses

Zinc, magnesium and calcium contents of porcine carboxypeptidase A_γ were determined by wet-ashing of the protein followed by the estimation in a Hitachi model 303 atomic absorption spectrophotometer using a Westinghouse hollow cathode tube. A sample was prepared by extensive dialysis of lyophilized enzyme. About 50 mg of the lyophilized protein was measured accurately and placed into the flask. Then, 17 ml of concentrated HNO_3 and 3 ml of $HClO_4$ were added and heated gradually. When the preparation became colorless, it was concentrated to about 2 ml by heating for several minutes. The sample solution thus prepared was cooled and then diluted with water to 250 ml followed by estimation in an atomic absorption spectrophotometer.

Amino Acid Analysis

Amino acid analysis was performed in a Durrum model D-500 amino acid analyzer. Cysteine was determined as *S*-carboxymethyl cysteine and methionine was determined as methionine sulfone [12]. Tryptophan was determined by the method of Edelhoch [13]. Carboxymethylation was accomplished by a modification of the method of Crestfield et al. [14] using 7 M guanidinium chloride and dithioerythritol in place of urea and 2-mercaptoethanol. The alkylated protein was separated from excess reagents by dialysis against water in the dark. Duplicate samples were hydrolyzed in 6 M HCl for 24 h at 110 °C.

Amino-Terminal Sequence Analysis

Amino-terminal sequence analyses were performed using Beckman sequencers model 890C according to Edman and Begg [15] with the modification as described by Hermodson et al. [16]. Protein was reduced and carboxymethylated [14] prior to sequence analysis. Repetitive yields of 93–96% were routinely obtained. The degradation products were identified by high-pressure liquid chromatography [17].

pH Optimum

The effect of pH on peptide hydrolysis was tested with 20 mM Cbz-Gly-L-Phe in 50 mM NaCl, at 25 °C. The buffers used were: pH 5.5–8.0, 10 mM phosphate buffer containing 0.1 M NaCl; pH 7.0–9.5, 20 mM barbital buffer containing 0.1 M NaCl. The pH of the reaction mixture was readjusted just before each assay.

Stability

The effect of temperature on the stability of porcine carboxypeptidase A_γ was examined by incubating the enzyme in 20 mM phosphate buffer, pH 7.0 containing 0.3 M NaCl for 24 h at various temperatures. After incubation, aliquots (50 µl) were removed for assay of any proteolytic activity with casein as a substrate. The pH stability of the enzyme was examined with Cbz-Gly-L-Phe as a substrate. The enzyme solution at 1 mM concentration in buffer solutions were incubated for 60 min at 25 °C. Then, aliquots (1 ml) were removed for assay of residual peptidase activity. The 10 mM buffer solutions containing 0.2 M NaCl were: pH 4.0–6.0, acetate; pH 7.0–9.0, barbital; pH 10.0–11.0, carbonate.

Inhibition Studies

The enzyme was incubated with chelating agents for 20 h at 5 °C. 5 ml of porcine carboxypeptidase A_γ solution (130 μg/ml) in 10 mM sodium phosphate, pH 7.5 were dialyzed against 1 l of 10 mM sodium phosphate pH 7.5, containing 1 mM EDTA or *o*-phenanthroline. After dialysis, an aliquot of the sample solution was diluted with 10 mM phosphate buffer, pH 7.5 containing 0.2 M NaCl to 1 l and then used for assays of residual peptidase activity with Cbz-Gly-L-Phe as a substrate. As a control, an aliquot of the enzyme solution without any chelating agents was employed.

Purification of Enzymes

Step I. Extraction and Activation. To fresh porcine pancreas (1 kg) minced with a meat chopper 3 l of 0.1 M sodium acetate were added with mixing at 15–20 °C. Then, 100 g of minced porcine duodenum intestine were directly added to this mixture. The pH was adjusted to 7.3 with 2 M NaOH, and the mixture allowed to stand overnight for 16 h at 10 °C with constant stirring.

Step II. First Ammonium Sulfate Fractionation. The incubated mixture was then adjusted to pH 5.3 with 2 M CH_3-COOH and any material insoluble at this pH was removed by centrifugation after addition of 200 g/l of Celite 545. The supernatant was treated with solid $(NH_4)_2SO_4$ to a final 45% saturation and kept at 3 °C.

Step III. Autolysis of First Ammonium Sulfate Precipitates. The precipitates (183 g) thus obtained were collected by filtering the solution on a filter paper. The precipitate was dissolved in 1.1 l of 0.1 M potassium phosphate, pH 7.0. The dark brown solution was then allowed to stand for 20 h at 20 °C and filtered through a filter paper to obtain a clear filtrate.

Step IV. Second Ammonium Sulfate Fractionation. To the filtrate was added solid $(NH_4)_2SO_4$ to a final 35% saturation, and the solution was adjusted to pH 6.5 and allowed to stand overnight in the cold room.

Step V. First Crystallization of Porcine Carboxypeptidase A_γ. The precipitates (35 g) thus obtained were collected by filtration and dissolved in 0.21 l of 0.1 M carbonate buffer, pH 8.0, and the solution was adjusted to pH 7.0 before leaving it for 3 days at 5 °C under constant stirring. The fine needle crystals were collected by centrifugation, and resuspended in 0.17 l of cold water with constant stirring. The crystals obtained were then resuspended in an appropriate volume of cold water and collected by filtration. The carboxypeptidase A_γ (2.6 g) was obtained from 1 kg of porcine pancreas.

Step VI. Batch Absorption and Elution from DEAE-Sephadex A-50. The crystalline porcine carboxypeptidase A_γ thus obtained was still contaminated with several components including elastoproteinase. Therefore, a further purification step was employed. Carboxypeptidase A_γ crystals were suspended in 65 ml of 50 mM Tris/HCl, pH 9.4 and adjusted to pH 11.0 with 2 M NaOH to dissolve the crystals completely. After the solution was readjusted to pH 9.4, 75 g of wet DEAE-Sephadex A-50, previously equilibrated with 50 mM Tris/HCl, pH 9.4, were added and stirred gently for 4 h at 5 °C. The suspended Sephadex gel was then collected by filtration on a glass filter and washed with 3 l of 50 mM Tris/HCl, pH 9.4, followed with 0.5 l of H_2O. To about 90 g of wet DEAE-Sephadex A-50 cake thus obtained were added 90 ml of 0.2 M sodium citrate (dibasic) until a final concentration of 0.1 M. The slurry was then allowed to stand for 2 h with constant stirring to dissociate the cyrboxypeptidase A_γ from the Sephadex gel. To the pool of fractions containing carboxypeptidase A_γ was added solid $(NH_4)_2SO_4$ to a final concentration of 70% saturation at pH 5.5 and the mixture was allowed to stand overnight in the cold room.

Step VII. Second Crystallization of Carboxypeptidase A_γ. The precipitates (2.8 g) obtained were then dissolved in 5.6 ml of H_2O. After centrifugation of the sample solution to remove any insoluble material, the clear solution was dialyzed extensively against H_2O. The precipitates (2.7 g) produced were collected by centrifugation and dissolved in 40 ml of 5 mM sodium phosphate, pH 5.8 containing $(NH_4)_2SO_4$ at 17% saturation. After removing any insoluble materials by centrifugaton, the solution was then readjusted to pH 5.8 and allowed to stand at 5 °C with occasional stirring. The crystals of carboxypeptidase A_γ appeared in 2 or 3 days and its complete crystallization was achieved in one week. 2 g of carboxypeptidase A_γ crystals were obtained by centrifugation. A carboxypeptidase A_γ sample which was further purified by another recrystallization was essentially identical to that which was obtained after the second crystallization.

RESULTS

Preparation of Porcine Carboxypeptidase A_γ

A summary of a typical purification of porcine carboxypeptidase A_γ is shown in Table 1. The elution pattern from the DEAE-Sephadex A-50 column is shown in Fig. 1. In this chromatography, carboxypeptidase A activity was eluted with 1 M NaCl in carbonate buffer, pH 10.0. The following crystallization steps further purified the protein. The overall recovery of carboxypeptidase A_γ from the tissue was about 30% with a purification of 32-fold. The final specific activity was about 110 units/mg protein for various preparations.

Porcine carboxypeptidase A_γ thus purified was readily denatured and lost its activity upon lyophilization. Carboxypeptidase A_γ crystals were best stored in 5 mM sodium phosphate pH 5.8 containing ammonium sulfate at 17% saturation, and a few drops of toluene in the cold room.

Homogeneity of the Purified Enzyme

A single, sharp protein band was observed for carboxypeptidase A_γ when it was examined by disc polyacrylamide gel electrophoresis at pH 9.4 (Fig. 2). Chemical purity, which was examined by sequence analysis, gave asparagine as the sole amino-terminal residue. The further sequence analysis showed only a single sequence for this protein. Crystals of the protein in a needle form are shown in Fig. 3.

Apparent Relative Molecular Mass

The M_r of porcine carboxypeptidase A_γ has been estimated as 38900 on Sephadex G-200 column.

Isoelectric Point

The isoelectric point of porcine carboxypeptidase A_γ was determined to be 4.3 which was more acidic than the value of 5.6 for bovine carboxypeptidase A_γ determined under the same conditions.

Table 1. *Purification of carboxypeptidase A_γ from porcine pancreas*
One unit of activity is defined as 1 μmol of phenylalanine liberated/min from Cbz-Gly-LPhe under conditions described in Experimental Procedure

Purification step	Total volume	Total protein	Total activity	Specific activity	Yield	Purity
	ml	g	units	units/mg	%	-fold
Activation extract	3490	115	379000	3.3	100	1
45%-saturated ammonium sulfate fractionation	1408	69.8	370000	5.3	97.6	1.6
Autolyzation	1408	57.7	349000	6.1	92.1	1.8
35%-saturated ammonium sulfate fractionation	350	14.8	317000	21	83.6	6.4
First crystallization	180	3.0	202000	67	53.3	20
DEAE-Sephadex A-50	384	1.7	168000	99	44.3	30
Second crystallization	40	1.1	119000	108	31.4	32

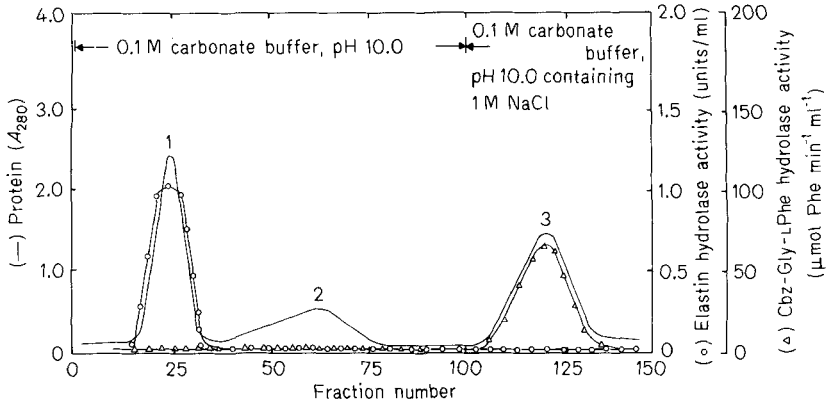


Fig. 1. *Elution profile of carboxypeptidase A_γ from a DEAE-Sephadex A-50 column.* The crystalline enzyme (200 mg) was dissolved in 15 ml of 0.1 M carbonate buffer, pH 10.0 and the pH was then adjusted to 11.0. After removing any insoluble material, the clear solution was applied to a column of DEAE-Sephadex A-50 (2 × 50 cm) equilibrated with 0.1 M carbonate buffer, pH 10.0. Fractions of 6.6 ml were collected. After eluting peak 1 and peak 2 with the same buffer, peak 3 was eluted with 0.1 M carbonate buffer, pH 10.0 containing 1 M NaCl. All operations were performed at 4 °C

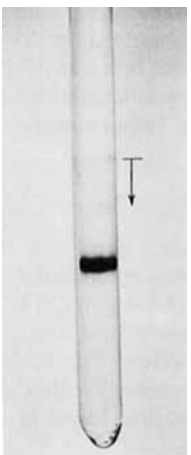


Fig. 2. *Disc polyacrylamide gel electrophoresis of porcine carboxypeptidase A_γ.* Electrophoresis was carried out in 7.5% standard polyacrylamide gel, pH 9.4, at 3 mA per tube, for 80 min. The sample contains 50 μg of protein

Amino Acid Composition

The amino acid composition of porcine carboxypeptidase A_γ is shown in Table 2 along with the compositions of porcine carboxypeptidase A_e and A_2, and bovine carboxypeptidase A_γ. Porcine carboxypeptidase A preparations have a little higher content of aspartic acid or asparagine (undefined), glutamic acid or glutamine (undefined) and proline, whereas bovine carboxypeptidase A_γ has a little higher content of serine. Porcine carboxypeptidase A_γ and A_e have a little higher content of isoleucine.

The amino acid sequence was then determined on reduced and carboxymethylated porcine carboxypeptidase A_γ. The amino acid sequence of the first 25 residues is given in Table 3. The yield for residues 1–25 was 0.6, 0.7, 0.6, not determined, 0.7, 0.6, not determined, 0.6, 0.4, 0.5, 0.5, 0.6, 0.4, 0.7, 0.6, 0.3, 0.3, 0.4, 0.3, 0.3, 0.2, 0.2, 0.1, 0.2 and 0.2 mol/38900 g of protein, respectively.

Metal Content and Inhibition with Chelating Agents

The peptidase activity of the purified preparation of porcine carboxypeptidase A_γ was inhibited upon dialysis against 1 mM EDTA or 1 mM *o*-phenanthroline by respectively 34% and 87% of the activity at pH 7.5. This indicated that it was metalloenzyme. Since the most common metals found in mammalian carboxypeptidases are zinc, magnesium and calcium, the analyses for these elements were performed. The protein contained 1.01 mol zinc/mol, whereas no magnesium or calcium were found.

pH Optimum

The enzyme showed an optimum pH at about 7.5 in phosphate or in barbital buffer (Fig. 4).

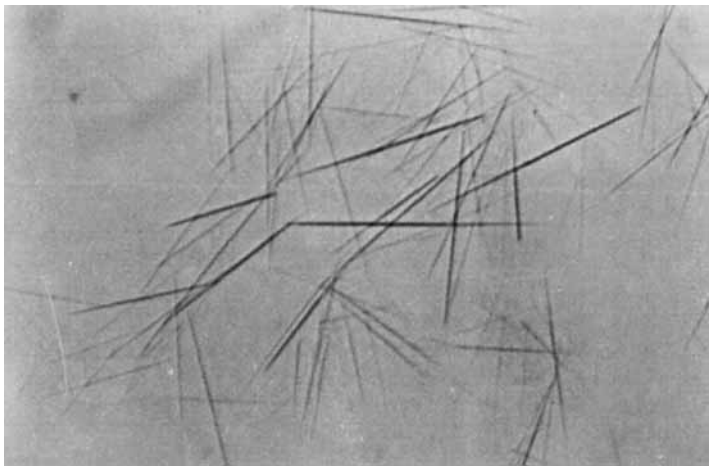


Fig. 3. *Photograph of porcine carboxypeptidase A_γ crystals.* (Magnification ×200)

Table 2. *Amino acid composition of porcine carboxypeptidase A_γ, A_ϵ and A_2 and bovine carboxypeptidase A_γ*

Amino acid	Porcine A_γ		A_ϵ [3]	A_2 [2]	Bovine A_γ [18]
	measured	integer			
	mol/mol protein				
Lys	15.1	15	13	13	15
His	10.4	10	8	9	8
Arg	12.0	12	10	11	10
Asx	36.3	36	31	32	28
Thr[a]	27.4	27	24	27	24
Ser[a]	28.4	28	25	28	31
Glx	31.5	32	28	29	25
Pro	15.6	16	14	14	10
Gly	27.8	28	25	26	23
Ala	24.4	24	21	22	19
Cys[b]	2.4	2	2	2	2
Val[c]	14.9	15	14	12	16
Met[d]	3.6	4	3	3	3
Ile	26.6	27	24	19	20
Leu	24.9	25	21	21	23
Tyr	18.0	18	16	17	19
Phe	18.8	19	16	17	15
Trp[e]	9.0	9	8	7	7
Sum		347	303	309	300
M_r		38900	34000	34600	34600

[a] Values extrapolated to zero-hour hydrolysis.
[b] Determined as carboxymethyl-cysteine after reduction and alkylation.
[c] Values after 96-h hydrolysis.
[d] Determined as methionine sulfone after performic acid oxidation.
[e] Determined by the method of Edelhoch [13].

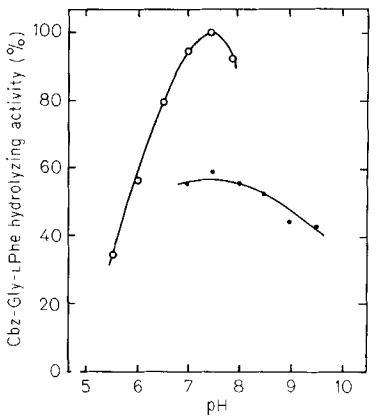


Fig. 4. *pH dependence of peptidase activity of porcine carboxypeptidase A_γ.* The effect of pH on peptide hydrolysis was tested with 20 mM Cbz-Gly-L-Phe in 50 mM NaCl, at 25 °C. The buffers containing 0.1 M NaCl were: (○– –○) 10 mM phosphate; (●—— ●) 10 mM barbital

Stability

Porcine carboxypeptidase Aγ was stable below 40 °C. Above this temperature a marked decrease of the activity was observed. The enzyme was stable in the pH range of 5–9 at 25 °C, maintaining its full activity. At same temperature a marked decrease of the activity was observed below pH 5.0. The lyophilized enzyme showed only 22% residual activity when tested with Cbz-Gly-L-Phe as a substrate. The denaturation of porcine carboxypeptidase A_γ by lyophilization is independent of the pH of the solution. A lyophilization, vacuum-drying and acetone-drying of the enzyme caused about the same loss in activity. Freezing of the enzyme preparation in 0.72 M $(NH_4)_2SO_4$ caused a marked loss of activity. However, no loss of activity was observed when it was frozen in 2.1 M $(NH_4)_2SO_4$.

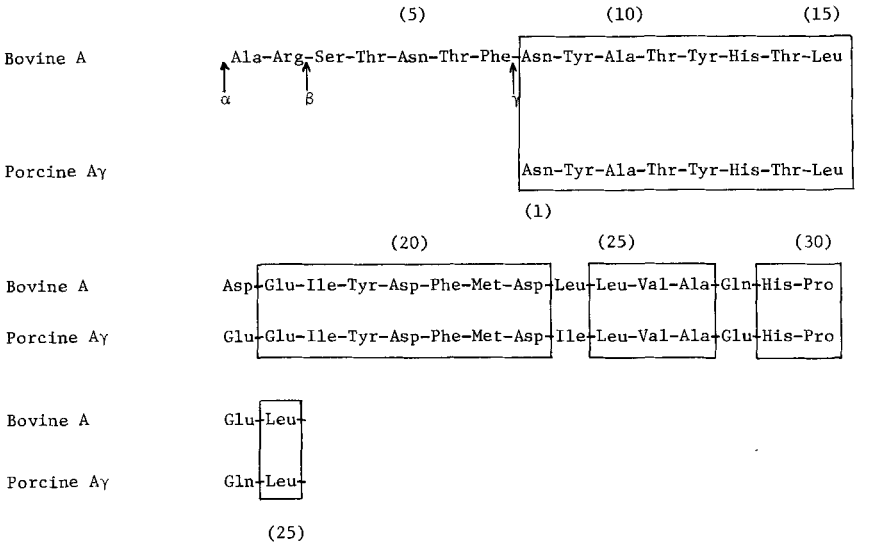
Kinetic Parameters

The kinetic parameters K_m and V of porcine carboxypeptidase A_γ were determined from activity assays at different substrate concentrations (0.02–0.1 M). The K_m for both porcine carboxypeptidase A_γ and bovine carboxypeptidase A_γ toward Cbz-Gly-L-Phe was 20 mM. Porcine carboxypeptidase A_γ, however, had a V of 235.5 µmol Cbz-Gly-L-Phe min^{-1} mg^{-1} which is smaller than that of bovine carboxypeptidase A_γ by 23%.

Substrate Specificity

The substrate specificity of the enzyme was studied using substrates such as Cbz-Gly-L-Phe, Bz-Gly-L-Arg, various kinds

Table 3. *Amino-terminal sequences of porcine and bovine carboxypeptidase A_γ*



of amino acid esters (see Experimental Procedure), casein and elastin. Porcine carboxypeptidase A_γ showed on enzymatic activity only towards Cbz-Gly-L-Phe with a specific activity of 108 μmol phenylalanine released min^{-1} (mg protein)$^{-1}$ and casein with a specific activity of 8.8 units/mg protein at 35 °C. These data are in good agreement with the substrate specificity of bovine carboxypeptidase A.

DISCUSSION

Porcine pancreatic carboxypeptidase A_γ has been isolated and characterized as to its enzymatic properties. The autolysis step employed for the purification of the enzyme was most critical for its purification. The autolysis process digested most contaminating proteins to small peptide fragments, which could be removed by a subsequent ammonium sulfate fractionation. This unique procedure made a large-scale preparation possible.

Porcine carboxypeptidase A_γ is composed of a single polypeptide chain with an apparent M_r of 38900. The amino-terminal sequence of this porcine carboxypeptidase A_γ had a very high homology to bovine carboxypeptidase A_γ (Table 3). Furthermore, the substrate specificity of this enzyme shows that it belongs to the family of carboxypeptidase A.

This enzyme seems to correspond to porcine carboxypeptidase A_3, reported by Folk and Schirmer [1] and by Folk [2], based on the amino acid composition and the amino-terminal sequence data.

Narayanan and Anwar [3] reported on porcine carboxypeptidase A_e which had an amino acid composition very similar to that of porcine carboxypeptidase A_γ described here. An amino-terminal residue of aspartic acid was reported for porcine carboxypeptidase A_e. But this residue could be asparagine as described in this report. These data indicate that carboxypeptidase A_e might be identical to our carboxypeptidase A_γ, although there are some differences in pH optimum and estimated M_r value.

The authors wish to thank Mr K. Ogawa (Division of Production) and Dr K. Araki, Mr S. Yoshida, and Mr M. Aoyama (Division of Research and Development, Eisai Co. Ltd) for their continuous interest and valuable discussion throughout this work.

REFERENCES

1. Folk, J. E. & Schirmer, E. W. (1963) *J. Biol. Chem. 238*, 3884–3894.
2. Folk, J. E. (1963) *J. Biol. Chem. 238*, 3895–3898.
3. Narayanan, A. S. & Anwar, R. A. (1970) *Can. J. Biochem. 48*, 7–11.
4. Kobayashi, R., Kobayashi, Y. & Hirs, C. H. W. (1978) *J. Biol. Chem. 253*, 5526–5530.
5. Smillie, L. B. & Hartley, B. S. (1966) *Biochem. J. 101*, 232–241.
6. Yemm, E. W. & Cocking, E. C. (1955) *Analyst 80*, 209–213.
7. Kunitz, M. (1947) *J. Gen. Physiol. 30*, 291–310.
8. Grant, N. H. & Robbins, K. C. (1957) *Arch. Biochem. Biophys. 66*, 396–403.
9. Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404–427.
10. Andrews, P. (1964) *Biochem. J. 91*, 222–233.
11. Kunkel, H. G. & Tiselius, A. (1951) *J. Gen. Physiol. 35*, 89–118.
12. Hirs, C. H. W. (1967 *Methods Enzymol. 11*, 197–199.
13. Edelhoch, H. (1967) *Biochemistry*, 6, 1948–1954.
14. Crestfield, A. M., Moore, S. & Stein, W. H. (1963) *J. Biol. Chem. 238*, 622–627.
15. Edman, P. & Begg, G. (1967) *Eur. J. Biochem. 1*, 80–91.
16. Hermodson, M. A., Ericsson, L. H., Titani, K., Neurath, H. & Walsh, K. A. (1972) *Biochemistry, 11*, 4493–4501.
17. Ericsson, L. H., Wade, R. D., Gagnon, J., McDonald, R. M., Granberg, R. & Walsh, K. A. (1977) in *Solid-Phase Methods in Protein Sequence Analysis* (Previero, A. & Coletti-Previero, M. A., eds) pp. 137–142, Elsevier/North-Holland, Amsterdam, New York.
18. Bradshaw, R. A., Ericsson, L. H., Walsh, K. A. & Neurath, H. (1969) *Proc. Natl Acad. Sci. USA, 63*, 1389–1394.

A. Koide and M. Yoshizawa, Eisai Research Laboratories, Eisai Co. Ltd, Koishikawa 4, Bunkyo-ku, Tokyo, Japan

K. Kurachi, Department of Biochemistry, University of Washington, School of Medicine, Seattle, Washington, USA 98195