-Rapid Communication-

Purification and Characterization of Carboxypeptidase A from Chicken Pancreas

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Abstract Carboxypeptidase A was purified by about 59-fold with an overall recovery of 3.5% from chicken pancreas. The enzyme occurs as a monomer and has a molecular weight of about 34,000 as estimated by polyacrylamide-gel electrophoresis (PAGE) and sodium dodecyl sulphate (SDS)-PAGE. The enzyme was stable below 55°C, and the optimal temperature was approximately 45°C. The activity of the enzyme was stable in the range of pH 8.0-11.0, and was optimal about pH 9. The Michaelis constant (K_m) value for hippuryl-L-phenylalanine was estimated to be 0.11 mM. The isoelectric point of the enzyme was at pH 6.7. Although the activity of the enzyme was not inhibited by soybean trypsin inhibitor or phenylmethanesulfonyl fluoride, it was inhibited by ethylenediaminetetraacetic acid, o-phenanthroline, 8-hydroxyquinoline and potato carboxypeptidase inhibitor.

Key words : Carboxypeptidase A, Chicken, Pancreas, Digestive enzyme

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Carboxypeptidase A (CPA) is a proteolytic digestive enzyme that preferentially hydrolyzes peptide substrates with COOH-terminal aromatic amino acid residues¹⁸. It is well known that CPA is stored in the pancreas as an inactive proenzyme and is activated by trypsin after secretion into the alimentary tract¹¹. In mammals, a lot of information on CPA has been reported for numerous species (human¹⁴), rat¹⁶), bovine¹³), swine¹¹), goat⁴) and buffalo¹⁷). However, in avian species, the properties of CPA have been studied only in ostrich^{1,12}). There is no information on the characterization of chicken CPA, although the presence of its proenzyme has been suggested¹⁸). In this experiment, we present the purification and characterization of CPA from chicken pancreas.

Materials and Methods

Chemical reagents

Hippuryl-L-phenylalanine and trypsin were purchased from Sigma (MO, USA). The reagents for polyacrylamide-gel electrophoresis were from Bio-Rad Laboratories (CA, USA). The ion exchange columns, Q Sepharose Fast Flow and CM Sepharose CL-6B, were from Pharmacia Biotech (Tokyo, Japan). All other reagents were commercial products of the highest grade available.

Assays of CPA activity and protein determination

Carboxypeptidase A activity was measured with hippuryl-L-phenylalanine as substrate by the method of Folk and Schirmer⁶). The reaction mixture contained 1 mM substrate, 50 mM Tris-HCl buffer (pH 7.5), 0.5 M NaCl and 0.05 ml enzyme solution in a total volume of 1 ml.

The reaction was initiated by adding enzyme solution to the reaction mixture, and changes in absorbance at 254 nm were measured for 10 min at 25°C. One unit of the enzyme is expressed as μ mole of substrate cleaved per minute, and specific activity as the activity per mg protein. During the purification process, the protein concentrations were determined by measuring the absorbance at 280 nm or by the method of Lowry *et al.*¹⁰), with bovine serum albumin as a standard.

Purifications of proenzyme and enzyme

All purification steps were carried out at 4°C. Pancreases obtained from 5-week-old male Single-Comb White Leghorn chicks were washed with cold 0.9% NaCl and rapidly frozen at -80°C. The frozen pancreases were homogenized with 9 volumes of 5 mM Tris-HCl buffer (pH 8.0). The homogenate was centrifuged at 100,000 $\times g$ for 60 min. The supernatant was fractionated with ammonium sulfate at 30 to 50% saturation. The precipitated fraction was dissolved in 5 mM Tris-HCl buffer (pH 8.0), applied to a Q Sepharose Fast Flow column (1.5 cm $\times$ 23 cm) equilibrated with the same buffer, and eluted with the same buffer containing 0.1 M NaCl. The active proCPA fractions were collected and dialyzed against 5 mM sodium phosphate buffer (pH 5.5). The dialysate was applied to a CM Sepharose CL-6B column (1.5 cm $\times$ 23 cm) equilibrated with 5 mM sodium phosphate buffer (pH 5.5) and eluted with the same buffer containing 0.05 M NaCl. The active proCPA fractions were collected, dialyzed against distilled water and concentrated by ultrafiltration with Centriplus (millipore, Tokyo, Japan) as purified proCPA. Purified proCPA was digested with trypsin

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Table 1. Purification and characterization of carboxypeptidase A from chicken pancreas

Purification step	Total activity (units) *	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude extract (100,000xg supernatant)	2740.9	2588.30	1.059	1.000	100.00
Ammonium sulfate (30-50% saturation)	1928.4	918.00	2.101	1.984	70.36
Q Sepharose Fast Flow (stepwise gradient)	868	27.70	31.336	29.59	31.67
CM Sepharose CL-6B (stepwise gradient)	370.6	9.77	37.932	35.819	13.52
Trypsin digestion					
Q Sepharose Fast Flow (linear gradient)	96.8	1.55	62.45	58.971	3.53

*: One unit of the enzyme is expressed as μ mole of substrate cleaved per minute.

(0.3 mg/ml) for 30 min at 25°C in 50 mM Tris-HCl buffer (pH 8.0) by the method of Folk and Schirmer⁶⁾. The digested solution was applied to a Q Sepharose Fast Flow column (0.5 cm x 18 cm) equilibrated with 5 mM Tris-HCl buffer (pH 8.0) and eluted with a 0.035 to 0.050 M NaCl gradient in the same buffer. The active CPA frac-

tions were collected, dialyzed against distilled water and concentrated by ultrafiltration with Centriplus (millipore, Tokyo, Japan) as purified CPA.

Determinations of molecular weight, isoelectric point and K_m

Molecular weights of proCPA and CPA were determined by PAGE and SDS-PAGE according to the method of Laemmli⁹⁾ with phosphorylase b (MW 94,000), bovine serum albumin (MW 66,000), ovalbumin (MW 45,000), carbonic anhydrase (MW 29,000) and cytochrome C (MW 12,400) as standard proteins. Purified proCPA and CPA were boiled in Tris-HCl (pH 6.8) containing 20% sucrose, 1% SDS and 1% β -mercaptoethanol⁹⁾ before electrophoresis in 14% acrylamide gel. Protein bands were visualized by staining with Coomassie Brilliant Blue R250. Isoelectric focusing on thin layer polyacrylamide-gel was performed with the Resolmax-IEF (ATTO, Tokyo, Japan) in a pH gradient of 3.0 to 9.0 according to the manufacturer's instructions. K_m was estimated from a Lineweaver-Burk plot. The concentration of substrate varied from 0.1 to 1 mM. Triplicate determinations were made at four different substrate concentrations.

Effects of pH, temperature and inhibitors

The effect of pH was examined over a pH range from 5 to 12. The wide-range buffer used was prepared according to the method of Davies³⁾. The effect of temperature was determined by incubating enzyme solution over a range of 15 to 65°C for 15, 30 and 60 min. After incubation, the remaining CPA activity was measured as described above. The effects of inhibitors were determined by incubating 0.025 ml enzyme solution at 25°C for 30 min with 0.025 ml of 2 mM phenylmethanesulfonyl fluoride (PMSF), soybean trypsin inhibitor (SBTI), ethylenediaminetetraacetic acid (EDTA), 8-hydroxyquinoline, potato carboxypeptidase inhibitor (PCI) or *o*-phenanthroline. After incubation, the remaining CPA activity was measured as described above.

Fig. 1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of purified chicken pancreatic procarboxypeptidase A and carboxypeptidase A. Lane 1, procarboxypeptidase A; lane 2, molecular weight marker proteins; lane 3, carboxypeptidase A.

Results and Discussion

Steps in the purification of CPA from crude extract are shown in Table 1. CPA was purified by about 59-fold with an overall recovery of 3.5% from chicken pancreas by a combination of ammonium sulfate fractionation, anion exchange chromatography and cation exchange chromatography. The purified proCPA and CPA were subjected to PAGE and SDS-PAGE and to assess purity and for determination of the molecular weights. Two single protein bands were seen corresponding to molecular weights 45 KDa and 34 KDa both in PAGE (data was not shown) and SDS-PAGE (Fig.1). Thus the chicken CPA seems to be a monomer protein, and its molecular weight is estimated to be 34,000. This value was in accordance with those reported (33,000 to 35,000) for mammalian

species^{2,5,7,8,14} and ostrich¹⁾.

The activity of purified enzyme was assayed at different temperatures (15 to 65°C). As shown in Fig.2A, the optimal temperature was approximately 45°C. The enzyme was stable below 55°C, and its activity was almost lost at 65°C. The enzyme activity was measured at various pH values (5 to 12). The activity of the enzyme was stable in the range of pH 8.0-11.0. The optimal pH was about 9 (Fig.2B). This value agreed with that reported for ostrich CPA of 8-9¹⁾. The Km value for chicken CPA was 0.11 mM (data was not shown), which is lower than the values of 0.41 mM for ostrich¹⁾ and 1.75 mM for bovine¹⁵⁾. From this finding, substrate specificity of chicken CPA may be different from that of mammals and other avian species. Isoelectric focusing of purified enzyme revealed a single component with a pI value of 6.7 (data was not shown). This value was higher than the values of pH 4.3-5.6 for mammals, but very similar to the value of 6.6 for ostrich¹⁾. Therefore, it is considered that avian CPAs usually present a more alkaline character than mammalian CPAs. Although the activity of the enzyme was not inhibited by PMSF or SBTI, it was completely inhibited by *o*-phenanthroline, PCI, and strongly inhibited by 8-hydroxyquinoline and EDTA (Fig.2C). These findings indicate that chicken CPA might be a metalloprotease like all other CPAs^{1, 11-17)}.

The findings of this study suggest that chicken CPA might have some physical and chemical properties similar to those of other known CPAs, especially that of ostrich¹⁾. Further kinetic studies and primary structure analysis are required to completely characterize chicken CPA.

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Fig. 2. Effects of pH, temperature and inhibitors on chicken pancreatic carboxypeptidase A activity. A: effect of temperature, B: effect of pH, C: effect of inhibitors.

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