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HUMAN PANCREATIC CARBOXYPEPTIDASE B

I. ISOLATION, PURIFICATION, AND CHARACTERIZATION OF FRACTION II

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Summary

Human carboxypeptidase B fraction II has been purified from pancreatic juice by DEAE-'Sephadex' chromatography, isoelectric focusing, and 'Sephadex' G-100 gel filtration. The enzyme has been characterized by analytical polyacrylamide disc-gel electrophoresis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, amino acid analysis, K_m determination, molecular weight determination on 'Sephadex' G-100, zinc analysis, and inhibition by metal chelating agents. Human carboxypeptidase B fraction II appeared homogeneous in analytical polyacrylamide disc-gel electrophoresis, but showed two components of 23,500 and 9,200 daltons in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Zinc analysis revealed 0.96 gram atoms of zinc per mole of enzyme, and a K_m of 65 ± 3 μM was determined for hydrolysis of hippuryl-L-arginine.

Introduction

An impressive body of information is available in the literature concerning pancreatic carboxypeptidase B (peptidyl-L-lysine-(L-arginine)hydrolase EC 3.14.12.3) of various animal species [1—6]. However, data concerning the human enzyme are scanty [7,8]. As a first step in the development of radioimmunoassays for human pancreatic proteases in blood, we have undertaken the isolation and purification of human carboxypeptidase B because of lack of immunological cross-reactivity between human carboxypeptidase B and the enzyme from other species [9]. This report describes the purification and

characterization of human carboxypeptidase B fraction II from human pancreatic juice.

Materials and Methods

Pure human pancreatic juice was collected under ice via a polyethylene tube from patients with a pancreatic fistula. Porcine carboxypeptidase B (Code COBC) was obtained from Worthington Biochemical Corporation.

Assay of enzyme activity. Carboxypeptidase B activity was assayed at 25° C using hippuryl-L-arginine as substrate [4]. Protein concentration was determined by the method of Lowry et al. [10] during purification and by absorbance at 280 nm using $\epsilon_{280}^{1\%} = 21.2$ [4] for measuring concentration of the purified preparation.

Kinetic measurements with hippuryl-L-arginine. Two separate velocity measurements were made at eight substrate concentrations. For K_m studies, a final enzyme concentration of 0.6 μg/ml was employed. The solvent for the assays was 25 mM Tris · HCl buffer (pH 7.65) containing 0.1 M NaCl. All measurements were made at 25° C.

Inhibition studies. Human carboxypeptidase B II was preincubated at 25° C for various times with appropriate concentrations of EDTA or 1,10-phenanthroline in 5 mM Tris · HCl buffer (pH 7.65) containing 0.14 M NaCl. Aliquots of the preincubated mixtures were assayed as described above.

Amino acid composition. The amino acid compositions were derived from analyses of protein samples which had been hydrolyzed in 6 M HCl at 110° C for 24, 48 and 72 h. The reported values for serine and threonine are extrapolations to zero time of hydrolysis assuming first order kinetics of destruction.

Analytical polyacrylamide gel electrophoresis. Proteins were subjected to polyacrylamide disc gel electrophoresis at pH 8.3 following the general method of Davis [11]. Gels were run at 5° C in an apparatus obtained from Hoeffer Scientific Instruments. Use of a stacking gel did not improve resolution and was therefore omitted. Proteins were subjected to discontinuous sodium dodecylsulfate polyacrylamide gel electrophoresis according to the method of Laemmli [12].

Isoelectric focusing. Protein samples were subjected to isoelectric focusing in a pH gradient of 7 to 9, according to the method described by Verterberg [13].

Zinc analyses. A dual-element zinc-cadmium lamp was used in the Perkin-Elmer Model 330 atomic absorption spectrophotometer. All samples were dialyzed against deionized water prior to measurement in dialysis tubing which had been pretreated to remove impurities.

Results

Purification of human carboxypeptidase B

Extraction. Lyophilization of 5 l of frozen pancreatic juice yielded a gummy residue, which was extracted with a total of 3 l of ethanol at 0° C to remove a straw-colored impurity. The extracted powder was suspended in approximate-

ly 500 ml of water, dialyzed against 10 mM potassium phosphate buffer (pH 8.0), and applied directly to the first DEAE 'Sephadex' column.

First DEAE-Sephadex column. The first DEAE-Sephadex A-50 column (10 × 70 cm) was developed with a non-linear gradient generated by using a closed mixing vessel containing 3.5 l of 10 mM potassium phosphate buffer (pH 8.0) and a reservoir containing 12.0 l of 0.4 M potassium phosphate buffer (pH 6.5). Fractions of 100 ml were collected. The carboxypeptidase B activity was eluted shortly after the pass-through fraction in a total volume of 2200 ml. This material was extensively dialyzed against 10 mM sodium phosphate buffer (pH 8.0), and then applied to the second DEAE-Sephadex column.

Second DEAE-Sephadex column. The second DEAE-Sephadex A-50 column (2.5 × 100 cm) was developed with a linear gradient from 0 to 0.25 M NaCl in a total volume of 2000 ml of 10 mM sodium phosphate buffer (pH 8.0). Fractions of 20 ml were collected. The profile of carboxypeptidase activity and protein concentration is shown in Fig. 1. The carboxypeptidase activity appeared in two peaks, designated human carboxypeptidase B I and human carboxypeptidase B II. Fractions containing human carboxypeptidase B II were pooled and concentrated by ultrafiltration.

Isoelectric focusing of human carboxypeptidase B II. A five ml aliquot of material from the second DEAE-Sephadex column was applied in a 1% solution of pH 7—9 ampholine on a 440 ml isoelectric focusing column. Isoelectric focusing was performed at 4° C over a 137-h period. Human carboxypeptidase - B II activity was resolved into one major peak (pI = 6.93) and several minor peaks. The major peak of human carboxypeptidase B II activity was pooled and dialyzed against 5 mM Tris · HCl buffer (pH 7.3) containing 0.9% NaCl and 0.02% NaN_3, concentrated by ultrafiltration, and applied to a Sephadex G-100 column to remove the ampholines.

Sephadex G-100 gel filtration. The major peak of carboxypeptidase B activity from isoelectric focusing was applied to a 2.5 × 42 cm column of

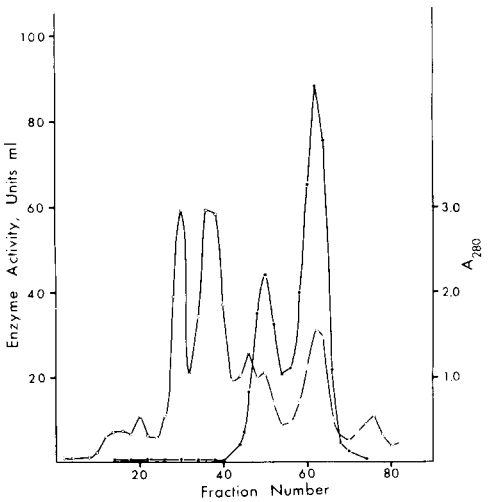

Fig. 1. Chromatography of partially purified carboxypeptidase B from activated human pancreatic juice on DEAE-Sephadex. The procedure is described in Results. ○———○, A_{280}; ●———●, enzyme activity, units/ml.

TABLE I

PURIFICATION OF HUMAN CARBOXYPEPTIDASE BII

Preparative Step	Volume (ml)	Total units	Protein mg/ml	Spec. act. units/mg	Yield (%)	Purification (-fold)
Pancreatic juice	5000	50800	3.2	3.14	100	1.0
Ethanol extract	500	26400	10.3	5.11	52	1.6
First DEAE column	2200	25400	0.92	12.4	50	4.0
Second DEAE column						
Peak I	34*	4870	3.4	42.0	9.5	13.4
Peak II	38*	10620	5.2	54.0	20.0	17.2
Isoelectric Focusing**	32	370	0.29	74.9	3.5***	13.8***
Sephadex G-100	26	568	0.29	74.9	5.3†	23.9

* Material from the secondDEAE column was concentrated by Amicon ultrafiltration.
** A 5 ml portion of the 38 ml of Peak II from DEAE was used for isoelectric focusing.
*** These values reflect interference of Ampholines in the assay of carboxypeptidase B activity.
† Yield of material recovered from isoelectric focusing and Sephadex G-100 gel filtration only was 41%.

Sephadex G-100 equilibrated in 5 mM Tris · HCl buffer (pH 7.3) containing 9.0% NaCl and 0.02% NaN_3. Human carboxypeptidase B II was eluted from the column as a symmetrical peak with no evidence of impurities. The specific activity of this material was higher than before isoelectric focusing, which indicated that the ampholines had been successfully removed. The purification of human carboxypeptidase B II is summarized in Table I. A 24-fold purification was achieved.

Characterization of human carboxypeptidase B fraction II

Polyacrylamide gel electrophoresis. Human carboxypeptidase B II prepared by the above procedure appears to be homogeneous when examined by analytical disc gel electrophoresis at pH 8.3 as can be seen in Fig. 2, left. Examination of human carboxypeptidase B II by sodium dodecylsulfate-polyacrylamide gel electrophoresis revealed that the enzyme was composed of two subunits having molecular weights of approximately 23 500 and 9 200. Use of discontinuous sodium dodecylsulfate-polyacrylamide gel electrophoresis [12] confirmed the existence of only two major polypeptides in purified human carboxypeptidase B II, as shown in Fig. 2, right.

Molecular weight determination by Sephadex G-100 gel filtration. A standard curve of molecular weight versus elution position of the protein on Sephadex G-100 was determined using a set of molecular weight protein standards furnished by Pharmacia Fine Chemicals. Using this method, a molecular weight of 27 700 was determined for human carboxypeptidase B II.

Amino acid composition of human carboxypeptidase B II. The amino acid composition of human carboxypeptidase B II is presented in Table II. The composition was derived from single analyses at 24, 48 and 72 h of hydrolysis as described in Materials and Methods. The amino acid composition is very

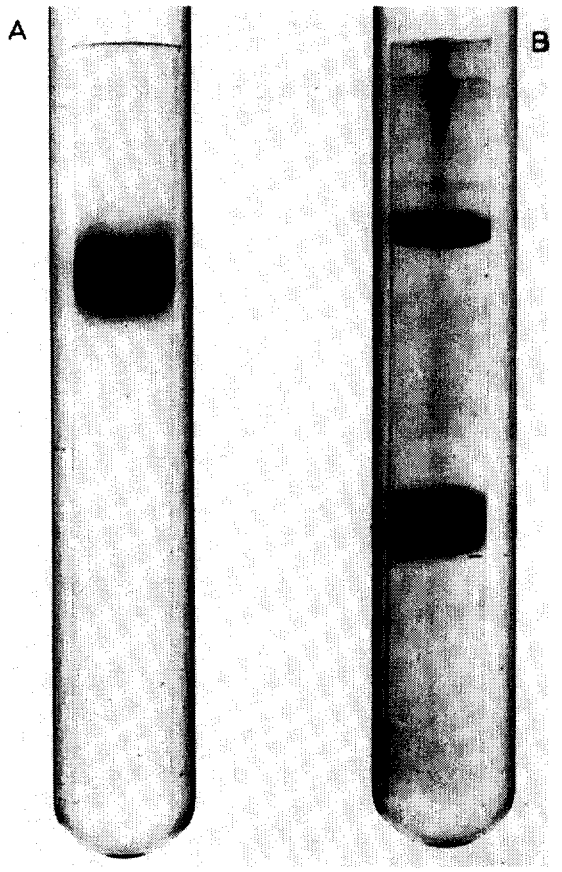

Fig. 2. Gel electrophoresis of purified human carboxypeptidase B II. A, Analytical polyacrylamide disc gel electrophoresis (pH 8.3). Migration was from top to bottom (cathode to anode). Gels were run at 5° C without a stacking gel as described in Materials and Methods. B, Discontinuous sodium dodecylsulfate-polyacrylamide gel electrophoresis. Migration was from top to bottom (cathode to anode). Gels contained 15% acrylamide and 0.7% bis-acrylamide.

TABLE II

AMINO ACID COMPOSITION OF HUMAN CARBOXYPEPTIDASE B FRACTION II*

Asp	8.07	Met**	0.81
Thr	8.85	Ile	6.01
Ser	7.33	Leu	7.48
Glu	10.67	Tyr	7.34
Pro	3.50	Phe	4.23
Gly	7.44	Lys	5.79
Ala	9.95	His	1.84
Cys**	2.38	Arg	5.00
Val	3.30		

* Expressed as Mole %. Bovine and Porcine carboxypeptidase data were recalculated without tryptophan to compare with human carboxypeptidase B.

** Half cystine and methionine were determined after performic acid oxidation as cysteic acid and methione sulfone.

similar to that found for bovine carboxypeptidase B, Fraction II [3], and that of porcine carboxypeptidase B [4].

Enzymatic properties. A K_m of 65 ± 3 μM was determined for hydrolysis of hippuryl-L-arginine as described in Materials and Methods. The activity of human carboxypeptidase B II towards hippuryl-L-phenylalanine was determined using the procedure described by Folk [14]. The specific activity of human carboxypeptidase B II towards hippuryl-L-phenylalanine was approximately 0.4% of that of commercial bovine carboxypeptidase A.

Zinc content and inhibition by metal chelating reagents. An average value of 0.96 g atoms of zinc per mole of protein was determined. EDTA was an effective inhibitor of human carboxypeptidase B II. Pre-incubation with 10 mM EDTA gave 80% inhibition after 2 h and 95% inhibition after 6 h. 1,10-phenanthroline was less effective as an inhibitor of human carboxypeptidase B II. Pre-incubation with 1 mM 1,10-phenanthroline caused 35% inhibition after 6 h and 87% inhibition after 24 h. Pre-incubation with 0.1 mM 1,10-phenanthroline caused 30% inhibition after 24 h.

Discussion

Two forms of carboxypeptidase B have been observed in human pancreatic juice. These forms probably represent degrees of modification of the intact polypeptide chain by internal cleavage as has been shown for the bovine enzyme [3,15].

We have purified to homogeneity one of the forms of carboxypeptidase B by DEAE-Sephadex chromatography and isoelectric focusing. The properties of this enzyme are similar to those of bovine carboxypeptidase B, fraction II, isolated by Reeck et al. from pancreatic juice. Both enzymes are eluted from anion exchange resins by increasing salt gradients as the second peak of carboxypeptidase B activity, are composed of two subunits with molecular weights of approximately 23 000 and 9 000, and have similar amino acid compositions. The molecular weight value of 27 700 for human carboxypeptidase B II obtained by Sephadex gel filtration, however, is not consistent with either our value determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis (mol. wt of sum of polypeptides 33 000) or the result obtained by Reeck and Neurath [3] for bovine carboxypeptidase B, fraction II (34 000). It should be noted that porcine carboxypeptidase B with molecular weight 34 000 as determined by Folk et al. [4] gave a value of 30 000 in our system. It may be that molecular weight determination by Sephadex G-100 gives consistently low values for carboxypeptidase B.

Isoelectric focusing of human carboxypeptidase B II gave one major peak and two smaller peaks of activity. These peaks may represent varying degrees of deamidation, since we could find no differences between the subfractions from isoelectric focusing. Due to the fact that ampholine interferes in the carboxypeptidase B assay of fractions from isoelectric focusing, specific activities of these fractions were lower than that of the material applied to the column. Furthermore, dialysis against 10 mM Tris buffer (pH 7.8) did not significantly increase specific activity. On the other hand, addition of 50 μM $ZnCl_2$ to undialyzed samples maximally enhanced specific activity. Removal of ampho-

line by Sephadex G-100 gel filtration of the pooled peak fractions raised the specific activity to the level attainable by addition of zinc. However, further addition of zinc did not increase specific activity, and zinc was inhibitory above 0.1 mM. The fact that human carboxypeptidase B is inhibited by EDTA and by 1,10-phenathroline demonstrates that the zinc atom found in this enzyme is necessary for activity.

Reeck et al. [3] have observed the uncleaved form of bovine carboxypeptidase B in some instances during fractionation of the enzyme from pancreatic juice on DEAE-cellulose columns. A similar form of uncleaved human carboxypeptidase B has been noted in varying concentrations in our preparations of human carboxypeptidase B fraction I from DEAE-Sephadex chromatography. Work is now in progress concerning the purification of these two other forms of carboxypeptidase B in human pancreatic juice.

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