Biochimica et Biophysica Acta, 452 (1976) 468—481
© Elsevier/North-Holland Biomedical Press

BBA 67971

HUMAN CARBOXYPEPTIDASE B

II. PURIFICATION OF THE ENZYME FROM PANCREATIC TISSUE AND COMPARISON WITH THE ENZYMES PRESENT IN PANCREATIC SECRETION *

JAMES W. BRODRICK, MICHAEL C. GEOKAS [a] and COREY LARGMAN

[a] *Department of Medicine, Martinez Veterans Administration Hospital, Martinez, Calif. 94553 and the Department of Medicine, School of Medicine, University of California, Davis, Calif. 95616 (U.S.A.)*

(Received May 12th, 1976)

Summary

Carboxypeptidase B (peptidyl-L-lysine(-L-arginine) hydrolase, EC 3.4.12.3) has been isolated and purified to apparent homogeneity from activated extracts of human pancreas tissue. The purified enzyme has been shown to be a single polypeptide of 34 000 daltons. In this respect the enzyme from pancreatic tissue, designated native human carboxypeptidase B, differs from the two forms present in human pancreatic juice (fractions I and II), both of which are composed of two polypeptides of approximately 24 000 and 9000 daltons. In addition, the three forms of human carboxypeptidase B differ in electrophoretic mobility in polyacrylamide gel electrophoresis and in chromatographic behavior on DEAE-cellulose.

Two immunological methods, micro-complement fixation and radioimmunoassay, have shown a high degree of structural similarity between the three forms of human carboxypeptidase B. Micro-complement fixation experiments indicate that the amino acid sequences of the three enzymes differ by less than one percent.

In vitro digestion studies have indicated that trypsin alone is sufficient to convert native carboxypeptidase B to carboxypeptidase B II. However, no combination of trypsin, chymotrypsin, and/or elastase was capable of converting native carboxypeptidase B to carboxypeptidase B I in vitro.

Abbreviations used are: TosArgOMe, *p*-toluenesulfonyl-L-arginine methyl ester; BzTyrOEt, *N*-benzoyl-L-tyrosine ethyl ester; Suc(Ala)$_3$NA, *N*-succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide.

* VA Project 3075-01.

Introduction

Recent work in this laboratory [1] has demonstrated that human pancreatic carboxypeptidase B circulates in blood. This finding has stimulated additional studies concerning the forms of this enzyme in pancreatic tissue as well as in pancreatic secretion.

Carboxypeptidase B (peptidyl-L-lysine-(-L-arginine)hydrolase, E.C. 3.4.12.3) has been isolated from pancreatic extracts derived from several sources [2—5]. In all instances the enzyme has been shown to consist of a single polypeptide of about 34 000 daltons containing one zinc atom per molecule. In some cases carboxypeptidase B was purified directly from activated pancreatic extracts, while other workers prepared crystalline enzyme from partially purified zymogen after activation with trypsin. In addition, Kycia et al. [6] have purified the zymogen from freshly collected pancreatic secretion in which activation was inhibited with diisopropylfluorophosphate.

In attempts to isolate carboxypeptidase B from activated bovine pancreatic juice, Reeck et al. [7,8] isolated two new, fully active, chromatographically distinct forms of the enzyme. Both forms were shown to consist of two polypeptide subunits with molecular weights of about 10 000 and 24 000, which were separable only after complete denaturation.

We have previously reported [9] the isolation of two forms of carboxypeptidase B (I and II) from human pancreatic juice. Human carboxypeptidase B II was purified to homogeneity and characterized with respect to molecular weight, amino acid composition, subunit structure, zinc content, and kinetic parameters for hydrolysis of hippuryl-L-arginine. This enzyme was shown to be similar to the most acidic form of carboxypeptidase B from bovine pancreatic secretion with respect to chromatographic properties and amino acid composition, and was found to consist of two subunits with molecular weights of 24 000 and 9000, respectively.

In order to elucidate further the origin of human carboxypeptidase(s) B present in pancreatic juice and their relationship to the enzyme(s) present in pancreatic tissue, we have purified native carboxypeptidase B from activated extracts of acetone powder of human pancreas. In addition, the human carboxypeptidase B I present in some samples of pancreatic juice has been purified to near homogeneity. In this report, the three forms of human pancreatic carboxypeptidase B (native, I, and II) are compared both in terms of physical and kinetic properties as well as in terms of immunological relatedness measured by radioimmunoassay and microcomplement fixation. Furthermore, a slow proteolytic cleavage of native carboxypeptidase B to form carboxypeptidase B II, catalyzed by trypsin, is reported.

Materials and Methods

Materials

Pure human pancreatic secretion was collected under ice via a polyethylene tube from patients with a pancreatic fistula. Collection of sufficient pancreatic juice for enzyme purification required the storage of some aliquots for up to three years at —10°C. Human pancreatic tissue was obtained at autopsy from

this hospital. Antisera to human pancreatic carboxypeptidase B I or II were produced in rabbits as previously described [1].

Porcine pancreatic elastase (Code ESFF), procine pancreatic carboxypeptidase B (Code COBC), bovine trypsin (Code TRL), and bovine α-chymotrypsin (Code CDI) were obtained from Worthington Biochemical Corporation. Hippuryl-L-arginine was obtained from Schwarz-Mann. *p*-Toluenesulfonyl-L-arginine methyl ester (TosArgOMe) and BzTyrOEt were obtained from Sigma Chemical Corp. Suc(Ala)$_3$NA was obtained from Bachem, Inc. Microgranular diethylaminoethyl cellulose (DE-52) was obtained from the Reeve Angel Company.

Methods

Assays of enzyme activity. Carboxypeptidase B activity was assayed at 25°C using hippuryl-L-arginine as substrate [2]. Protein concentration was determined by the method of Lowry et al. [10] during purification and by absorbance at 280 nm using $\epsilon_{280}^{1\%} = 21.2$ [2] for measuring concentration of purified preparations. Tryptic activity was measured with TosArgOMe according to the procedure described by Walsh [11]. Chymotryptic activity was determined by measuring hydrolysis of BzTyrOEt as described by Walsh and Wilcox [12]. Elastolytic activity was determined using Suc(Ala)$_3$NA by the method of Bieth et al. [13].

Kinetic measurements with hippuryl-L-arginine. Two separate velocity measurements were made at each of eight substrate concentrations. Final enzyme concentrations employed were 0.8 μg/ml and 1.0 μg/ml for native carboxypeptidase B and carboxypeptidase B I, respectively. 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. The data were plotted according to Eadie and Hofstee.

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

Zinc analysis. A dual-element zinc-cadium lamp was employed for zinc analysis in the Perkin-Elmer Model 360 atomic absorption spectrophotometer. All samples were dialyzed versus deionized water in dialysis tubing which had been pretreated to remove impurities.

Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Discontinuous sodium dodecyl sulfate gel electrophoresis was performed using a thin slab of acrylamide gel as described previously [14] according to the general procedures originally described by Studier [15,16]. Samples were completely reduced and denatured prior to electrophoresis by heating for 2 min in a boiling water bath in sample buffer containing 1% (w/v) sodium dodecyl sulfate and 1% (v/v) β-mercaptoethanol. This system was used to determine the molecular weights of native human carboxypeptidase B and the two polypeptides derived from carboxypeptidase B I following the general procedure of Weber and Osborn [17].

Analytical polyacrylamide gel electrophoresis. Proteins were subjected to polyacrylamide disc gel electrophoresis at pH 8.3 following the general method of Davis [18]. Gels were run at 5°C. Proteins were subjected to polyacrylamide disc gel electrophoresis at pH 4.5 following the method of Reisfeld et al. [19].

Separation of the polypeptide chains of carboxypeptidases B I and B II. Carboxypeptidase B I or B II (0.25—2 mg) was reduced with β-mercaptoethanol in 8 M urea and alkylated with iodoacetic acid as described by Crestfield et al. [20]. The reaction mixture was then applied to a 2.0 × 100 cm Sephadex G-75 column equilibrated in 10% acetic acid. The column was developed with the same buffer and two protein peaks were observed. The two peaks of protein obtained in each case were shown to be the heavy and light chains of human carboxypeptidase B I and II, respectively, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Amino acid composition. The amino acid compositions were derived from analysis of protein samples which had been hydrolyzed in 6 N HCl at 110°C for 24 h. The reported values for serine and threonine are uncorrected. Half-cysteine was determined as cysteic acid after performic acid oxidation by the method of Hirs [21].

Micro-complement fixation studies. The immunological relatedness of the three forms of human pancreatic carboxypeptidase B was determined by micro-complement fixation as described by Champion et al. [22]. Anti-carboxypeptidase B II antiserum was diluted 1/11 400 for these studies. The immunological relatedness of native human carboxypeptidase B and porcine carboxypeptidase B was also determined by this method. Anti-carboxypeptidase B II antiserum was diluted by 1/12 500 for experiments with the human enzyme, while a dilution of 1/300 was employed for the porcine enzyme.

Radioimmunoassay measurements of carboxypeptidase B. The three forms of human pancreatic carboxypeptidase B were tested for cross-reactivity in the radioimmunoassay system for human carboxypeptidase B II previously developed in this laboratory [1]. An anti-carboxypeptidase B I antiserum dilution of 1/1 200 000 was employed for these measurements. Carboxypeptidase B concentrations of 0.1—2 ng/ml were employed to construct standard curves for the three enzymes. The percent cross-reactivity was determined by calculating the relative dose levels obtained with known amounts of the three enzymes.

In vitro conversion studies. Aliquots of native carboxypeptidase B (1.0—1.5 mg) were incubated at a concentration of 0.9 mg/ml with 0.1 mg/ml of the appropriate endopeptidase(s) at 37°C for 6 to 96 h in 25 mM Tris · HCl (pH 8.3) containing 2 mM $CaCl_2$. Samples were taken for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and for assay of carboxypeptidase B activity prior to the addition of the protease used in that experiment. Tryptic, chymotryptic, or elastolytic activity was determined at the beginning and end of the incubation period. In all experiments, 50—75% of the carboxypeptidase activity and at least 50% of the respective protease activity remained at the end of the incubation. The degree of digestion was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by DEAE-cellulose chromatography. Three forms of human pancreatic carboxypeptidase B were resolved by chromatography on a 1.2 × 15 cm column of DEAE-cellulose equilibrated in 10 mM Tris · HCl (pH 8.3). Native carboxypeptidase B was eluted by washing the column with this buffer, while carboxypeptidase B I and II were eluted with a linear gradient from 0 to 60 mM NaCl in a total volume of 600 ml of 10 mM Tris · HCl (pH 8.3).

Results

Purification of human native pancreatic carboxypeptidase B

All purification steps were carried out at 4°C.

1. *Preparation of Acetone Powder.* Acetone powder from a single pancreas was obtained by extracting the diced, semi-frozen tissue with 500 ml of chilled (−20°C) acetone for 1 min in a Waring blender. The suspension was then centrifuged for 10 min at 6000 rev./min and the the supernatant was discarded. This procedure was repeated twice with acetone, once with acetone/ether, (1 : 1, v/v), and once with ether. The resulting powder was dried and stored at −20°C.

2. *Initial extraction of acetone powder.* The acetone powder was suspended in 10 ml of deionized water per gram dry weight by homogenization for 60 s in a Virtis "45" homogenizer at medium speed. The resulting suspension was stirred for 2 to 4 h at 4°C, after adjustment to pH 8.0 with 1 M Tris base, to allow activation to occur. The amount of carboxypeptidase B activity usually reached a maximum after 2 h of incubation and remained constant thereafter, indicating full activation. At this time the suspension was centrifuged for 20 min at 18 000 rev./min to yield the initial extract, which was then dialyzed versus two changes of 10 mM Tris · HCl (pH 8.25).

3. *DEAE-cellulose chromatography.* The dialyzed extract was applied to a 2.0 × 22 cm column of DEAE-cellulose equilibrated with 10 mM Tris · HCl (pH

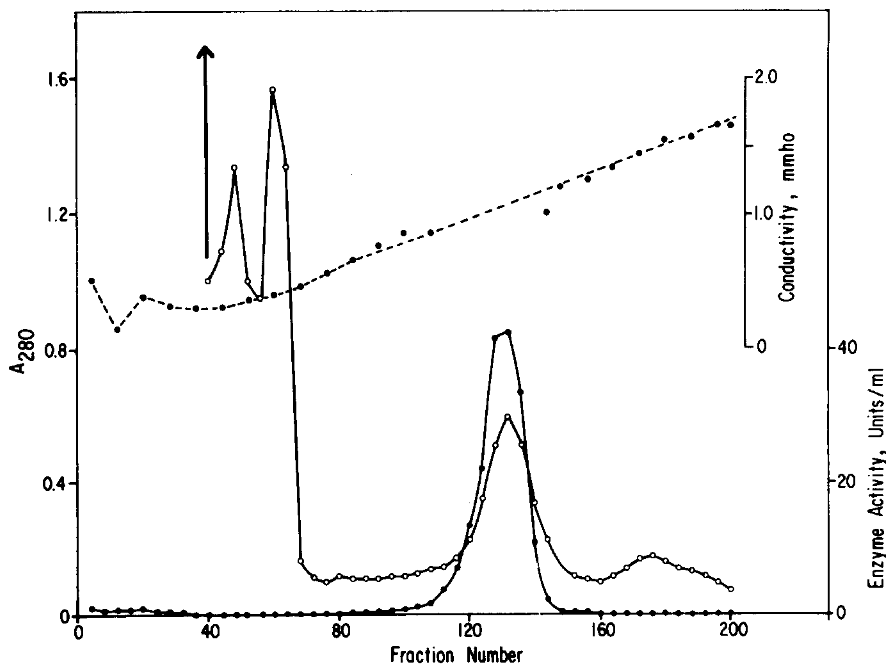

Fig. 1. Chromatography of partially purified carboxypeptidase B from activated human pancreatic tissue extract on CM-Sephadex C-50. The procedure is described in Methods. ○——○, A_{280}; ●——●, enzyme activity; ● - - - - - - ●, conductivity.

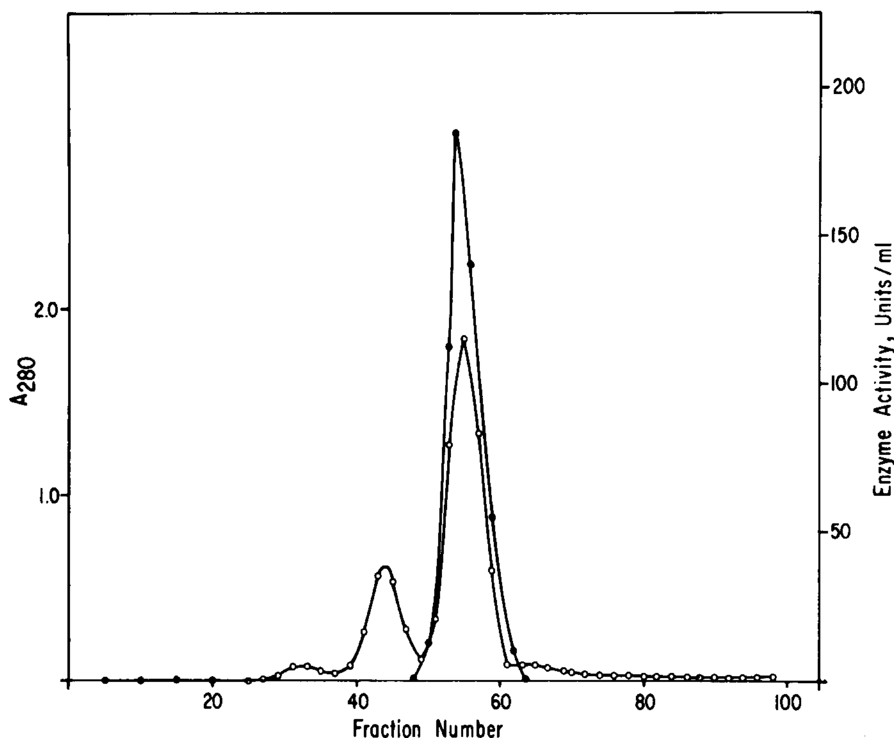

Fig. 2. Gel filtration of partially purified carboxypeptidase B on Sephadex G-100. The procedure is described in Methods. ○———○, A_{280}; ●———●, enzyme activity.

8.25). Fractions containing the carboxypeptidase B activity, which appeared in the void volume, were pooled, adjusted to pH 6.0 with 0.5 M HCl, and applied to a CM-Sephadex column.

4. CM-sephadex chromatography. The carboxypeptidase B fraction from DEAE-Cellulose was applied to a 2.0 × 17 cm column of CM-Sephadex C-50 equilibrated in 10 mM sodium phosphate (pH 6.0) containing 50 mM NaCl. The column was washed with 250 ml of the same buffer, followed by a linear gradient from 50 to 250 mM NaCl in 10 mM sodium phosphate (pH 6.0) in a total volume of 1 liter. The elution profile is shown in Fig. 1. The carboxypeptidase B activity appeared as a single peak which was pooled, concentrated by ultrafiltration with an Amicon PM-10 membrane, and applied to a Sephadex G-100 column.

5. Sephadex G-100 gel filtration. Carboxypeptidase B from the CM-Sephadex column was applied to a 2.0 × 96 cm column of Sephadex G-100 equilibrated in 10 mM Tris · HCl (pH 8.25). The column was then eluted with the same buffer at a flow rate of 10 ml/h. The resulting profile is shown in Fig. 2. The fractions containing carboxypeptidase B activity were pooled and concentrated by ultrafiltration with an Amicon PM-10 membrane. A summary of the purification of human pancreatic carboxypeptidase B is shown in Table I. A 163-fold purification was achieved.

TABLE I

PURIFICATION OF HUMAN PANCREATIC CARBOXYPEPTIDASE B

Fraction	Volume (ml)	Carboxypeptidase B		Protein (mg/ml)	Specific activity (units/mg)	Purification (fold)	Yield (%)
		Units/ml	Total units				
Initial extract	230	25.2	5796	20.8	1.21	1	100
DEAE-Cellulose	387	12.7	4918	2.8	4.53	3.7	85
CM-Sephadex	220	18.8	4139	0.16	117	97	71
Sephadex G-100	41	85	3485	0.43	198	163	60

Purification of human pancreatic carboxypeptidase B I and II

We have previously reported on the purification of human carboxypeptidase B II from pancreatic juice by DEAE-Sephadex chromatography, isoelectric focusing, and Sephadex G-100 gel filtration [9]. In more recent experiments, the method employed for purification of native human carboxypeptidase B has been modified for use in the purification of both forms of the enzyme from pancreatic juice. Briefly, pancreatic juice was centrifuged to remove solid materials, treated with ammonium sulfate, chromatographed on CM-Sephadex C-25, and applied to a Sephadex G-100 column as described for the native enzyme. The active fractions were pooled, concentrated by ultrafiltration, and applied to a 2.0 × 20 cm column of DEAE-cellulose equilibrated with 10 mM Tris · HCl (pH 8.3). The column was then washed with 200 ml of the same buffer, followed by a linear gradient from 0 to 60 mM NaCl in 10 mM Tris · HCl (pH 8.3). The elution profile is shown in Fig. 3. Fractions containing carboxypeptidase B I or II were pooled, concentrated by ultrafiltration, and stored at −20°C. The amount of carboxypeptidase B I recovered varied between 0 and 30% of the carboxypeptidase B II isolated from samples of pancreatic juice. Thus the fractions from two or more preparations were stored frozen and later combined and rechromatographed on DEAE-cellulose as described above. The carboxypeptidase B I preparation illustrated in Fig. 3 resulted in a 30% overall yield of carboxypeptidase B. Approximately 15% of the total carboxypeptidase B activity was carboxypeptidase B I.

Criteria of homogeneity and molecular weight studies

Native carboxypeptidase B purified through the Sephadex G-100 step was eluted from a DEAE-cellulose column equilibrated in 10 mM Tris · HCl (pH 8.3) as a symmetrical peak with constant specific activity, appearing several fractions after the void volume. A typical elution position of native carboxypeptidase B is illustrated in the trypsin digestion experiments to be discussed shortly (See Fig. 5). Rechromatography of carboxypeptidase B I on DEAE cellulose with a 0 to 60 mM NaCl gradient usually yielded a single homogenous peak with constant specific activity. In some experiments a shoulder of both protein and activity was observed, suggesting that subfractions of very similar properties might be present.

Polyacrylamide gel electrophoresis. Native carboxypeptidase B and carboxypeptidase B I and II prepared by the above procedures appeared to be homoge-

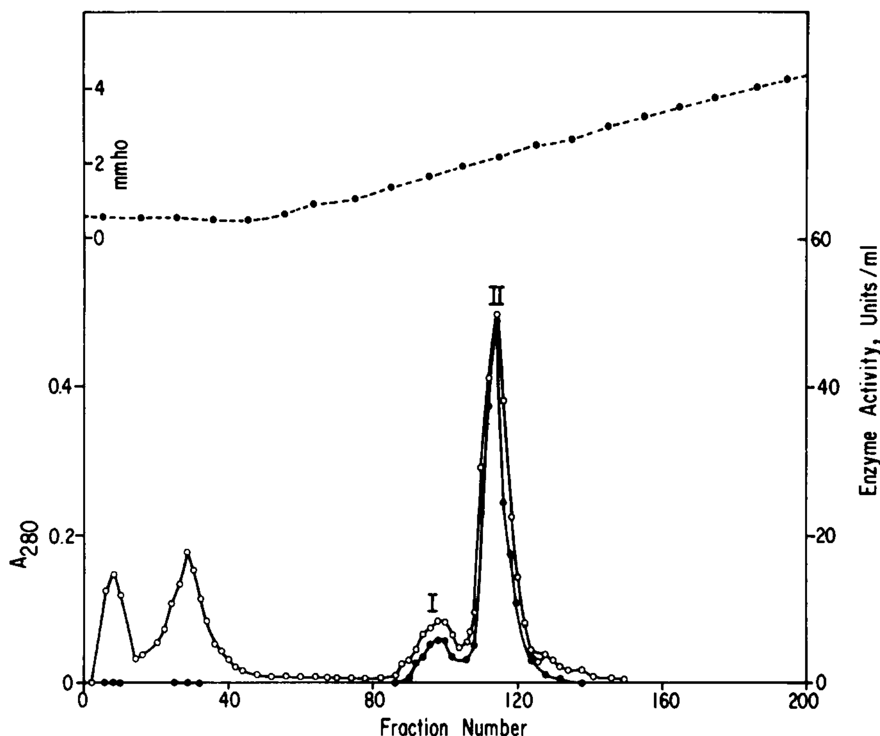

Fig. 3. Chromatography of partially purified carboxypeptidase B from human pancreatic juice on DEAE-Cellulose. The procedure is described in Methods. ○——○, A_{280}; ●——●, enzyme activity; ○ - - - - - - ○, conductivity.

neous when examined by analytical polyacrylamide disc gel electrophoresis at pH 8.3, as can be seen in Fig. 4. Native carboxypeptidase B appeared homogenous after polyacrylamide disc gel electrophoresis at pH 4.5, as shown in Fig. 4. Carboxypeptidase B I and II did not enter the running gel in this system.

Molecular weight determination by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. When native carboxypeptidase B was subjected to discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis, a single component was detected. The molecular weight of this polypeptide was estimated to be 34 000, using reference proteins of known molecular weight. Discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis of carboxypeptidase B I after reduction with β-mercaptoethanol revealed two bands with molecular weights of 23 500 and 9200, respectively. In some preparations an additional minor band was observed, with a mobility very similar to that of native carboxypeptidase B. Rechromatography on either DEAE-cellulose or CM-Sephadex failed to separate this component.

Characterization of the forms of human pancreatic carboxypeptidase B

Kinetic properties, zinc content, and inhibition studies. K_m values of 0.19 mM and 0.18 mM were determined for native carboxypeptidase B and for carboxypeptidase B I, respectively, for hydrolysis of hippuryl-L-arginine, as described in Methods. By comparison, a K_m of 0.07 mM for hydrolysis of the

Fig. 4. Gel electrophoresis of purified human carboxypeptidase B. A. Native carboxypeptidase B in a 10% acrylamide gel at pH 8.3 B. Carboxypeptidase B I in a 10% gel at pH 8.3 C. Carboxypeptidase B II in a 10% gel at pH 8.3. Migration was from top to bottom (cathode to anode). D. Native carboxypeptidase B in a 15% gel at pH 4.5. Migration was from top to bottom (anode to cathode).

same substrate by carboxypeptidase B II was previously determined [9].

The specific activities of native carboxypeptidase B and carboxypeptidase B I for hydrolysis of hippuryl-L-phenylalanine were approximately 0.6% and 0.3% that of commercially obtained carboxypeptidase A. Values of 1.11 and 0.73 gatoms of zinc per mol were determined for native carboxypeptidase B and carboxypeptidase B I, respectively. Both enzymes were inhibited approximately 35% by incubation for 1 h in 1 mM EDTA, while incubation in 0.1 mM, 1,10-phenanthroline for 1 h resulted in 45% and 30% inhibition of native carboxypeptidase B and carboxypeptidase B I, respectively.

Immunological cross-reactivity of carboxypeptidase B. Native carboxypeptidase B was indistinguishable from carboxypeptidase B II in the radioimmunoassay system developed for carboxypeptidase B II [1]. In this assay, carboxypeptidase B I was 90% cross-reactive with carboxypeptidase B II, while porcine carboxypeptidase B did not cross-react to a measurable extent. The Immunological Distance of the human enzymes from each other and from porcine carboxypeptidase B were determined using a micro-complement fixation

TABLE II

AMINO ACID COMPOSITION OF HUMAN PANCREATIC CARBOXYPEPTIDASES B *

Residue	Native Carboxypeptidase B	Carboxypeptidase B I	Carboxypeptidase B II
Asp	24.8	25.8	24.7
Thr	24.9	23.8	27.1
Ser	17.8	21.4	22.4
Glu	32.6	32.4	32.6
Pro	13.1	12.0	10.7
Gly	22.9	23.1	22.8
Ala	30.3	30.5	30.4
Cys/2 **	8.8	7.5	7.3
Val	10.8	12.1	10.1
Met **	3.1	2.7	2.5
Ile	18.6	17.5	18.4
Leu	22.9	23.5	22.9
Tyr	21.3	22.0	22.5
Phe	12.9	13.5	12.9
His	5.8	4.7	5.6
Lys	17.8	17.5	17.7
Arg	16.8	16.0	15.3

* Residues per 34 000 daltons.

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

assay [22,23]. In this system, the Immunological Distance between native carboxypeptidase B and carboxypeptidase B I was 1.79; between carboxypeptidases B I and II was 0.30; and between native carboxypeptidase B and carboxypeptidase B II was 2.07. Using the same technique, an Immunological Distance of 156 was determined between native human carboxypeptidase B and porcine carboxypeptidase B.

Amino acid compositions of native carboxypeptidase B and carboxypeptidase B I. The amino acid compositions of native carboxypeptidase B and carboxypeptidase B I are presented in Table II. The amino acid composition of carboxypeptidase B II [9] is also presented for comparison.

Separation of the light and heavy chains of human carboxypeptidases B I and II. The carboxymethylated light and heavy polypeptide chains of carboxypeptidases B I and II were separated by Sephadex G-75 gel filtration in 10% acetic acid. The amino acid composition of the two polypeptides from each carboxypeptidase B are shown in Table III. Summation of the compositions of the light and heavy chains of each protein agrees well with the composition of intact human carboxypeptidases B I and II, respectively (Table II).

Enzymatic conversion of native carboxypeptidase B to carboxypeptidase B II. Initial experiments on digestion of native carboxypeptidase B were performed by mixing 20 μg of the enzyme with 2 μg of trypsin, chymotrypsin, or elastase, or combinations of the endopeptidases. After incubation overnight and analysis of the reaction mixtures by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, it was found that only trypsin produced observable

TABLE III

AMINO ACID COMPOSITIONS OF THE SEPARATED CHAINS OF CARBOXYPEPTIDASES B I AND II

	Carboxypeptidase B I				Carboxypeptidase B II			
	Light * chain	Heavy ** chain	Sum	Intact *** protein	Light * chain	Heavy ** chain	Sum	Intact *** protein
Asp	5.3	20.3	25.6	25.8	5.7	20.4	26.1	24.7
Thr	6.2	17.6	23.8	23.8	6.6	18.2	24.8	27.1
Ser	5.9	17.5	23.4	21.4	4.1	15.5	19.6	22.4
Glu	13.0	20.7	33.7	32.4	13.0	21.2	34.2	32.6
Pro	3.4	9.1	12.5	12.0	3.5	8.9	12.4	10.7
Gly	7.9	19.4	27.3	23.1	7.5	16.4	23.9	22.8
Ala	11.0	21.9	32.9	30.5	10.0	21.0	31.0	30.4
Cys/2	2.1 †	4.4 †	6.5	7.5 ††	1.7 †	4.4 †	6.1	7.3 ††
Val	5.4	7.5	12.9	12.1	5.5	6.4	11.9	10.1
Met	0.5	1.6	2.1	2.7	0.9	0.7	1.6	2.5
Ile	6.0	10.7	16.7	17.5	6.1	12.3	18.4	18.4
Leu	3.9	17.9	21.8	23.5	3.8	19.3	23.1	22.9
Tyr	3.6	14.5	18.1	22.0	4.0	17.7	21.7	22.5
Phe	5.2	7.9	13.1	13.5	5.5	8.3	13.8	12.9
His	1.8	5.2	7.0	4.7	1.1	3.5	4.6	5.6
Lys	5.6	13.0	18.6	17.5	5.8	11.6	17.4	17.7
Arg	4.7	9.6	14.3	16.0	5.4	10.3	15.7	15.3

* Residues per 10 000 daltons.
** Residues per 24 000 daltons.
*** Residues per 34 000 daltons.
† Determined as carboxymethylcystine.
†† Determined as cysteic acid after performic acid oxidation.

digestion, and this was not augmented by addition of chymotrypsin, elastase, or both of these enzymes. When native carboxypeptidase B was incubated with bovine trypsin for 6, 24, or 96 h as described in Methods, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of aliquots of the resulting mixtures showed reduction in intensity of the 34 000 dalton band corresponding to native carboxypeptidase B and appearance and enhancement with time of bands corresponding to 24 000 and 10 000 daltons. Since these bands might represent the large and small polypeptides, respectively, of either carboxypeptidase B I or II, DEAE-cellulose chromatography of the digestion mixtures was employed in order to resolve native carboxypeptidase B and carboxypeptidase B I and II. Results for a typical set of tryptic digestions are shown in Fig. 5. The peak of activity which was eluted prior to the NaCl gradient corresponds to native carboxypeptidase B. This peak diminished with increasing digestion time. A second peak of activity eluting at the NaCl concentration corresponding to authentic carboxypeptidase B II, was observed in all digestion conditions tested. Since this system reproducibly resolves carboxypeptidase B I and II (Fig. 3), it appears that trypsin converts native carboxypeptidase B to carboxypeptidase B II only. In other experiments, bovine chymotrypsin and porcine elastase were shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be incapable of converting native carboxypeptidase B to either carboxypeptidase B I or II. Addition of either enzyme to digestion experi-

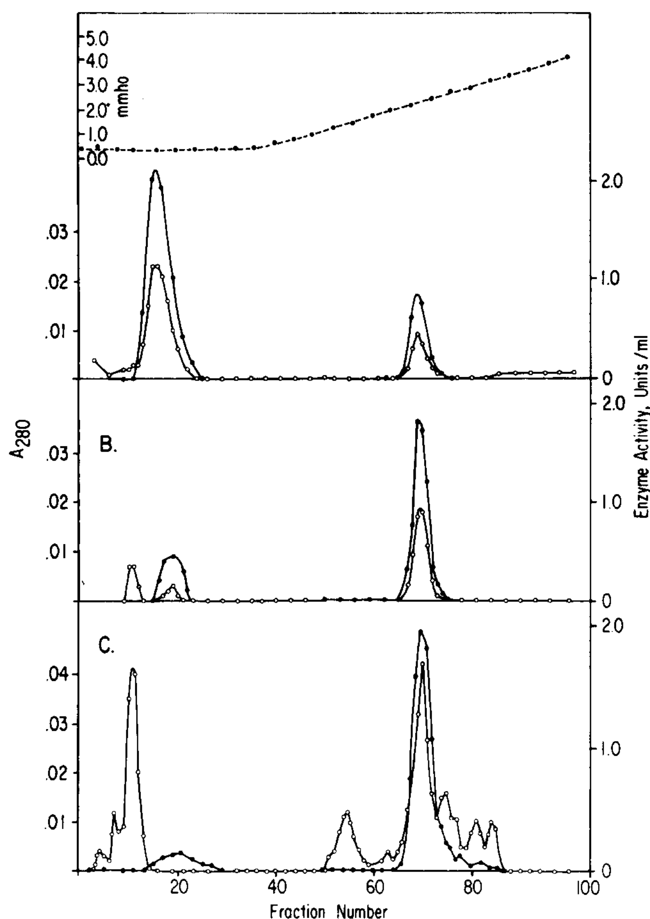

Fig. 5. Chromatography of tryptic digests of native carboxypeptidase B on DEAE-Cellulose A. 6 h digest. B. 24 h digest. C. 96 h digest. All columns were eluted with identical salt concentrations as described in Methods. ○——○, A_{280}; ●——●, enzyme activity; ● - - - - - - ●, conductivity.

ments did not result in enhancement of the degree of digestion observed with trypsin alone. Furthermore, DEAE-cellulose chromatography experiments demonstrated that no combination of trypsin, chymotrypsin, and/or elastase converted native carboxypeptidase B to a measurable amount of carboxypeptidase B I.

Discussion

Purification of carboxypeptidase B from activated extracts of human pancreas tissue yields a protein of 34 000 molecular weight, referred to as native carboxypeptidase B. On the other hand, purification of carboxypeptidase B from pancreatic juice which has been held at −10°C for up to three years yielded two forms of the enzyme; each of which is composed of two polypeptide chains, of 24 000 and 10 000 daltons, respectively. Carboxypeptidase B II has been purified to homogeneity and was the subject of an earlier communica-

tion [9]. Carboxypeptidase B I has now been purified to near homogeneity.

The three forms of human pancreatic carboxypeptidase B are very similar in terms of K_m for hydrilysis of hippuryl-L-arginine, amino acid composition, zinc content, and inhibition by metal chelating reagents. The human enzymes approach 100% cross-reactivity in a previously described [1] radioimmunoassay system. In comparison, porcine carboxypeptidase B does not cross-react with the human enzymes in this system. Furthermore, we have employed the quantitative microcomplement fixation system described by Champion et al. [22], in order to determine the Immunological Distances between the human enzymes as well as between native carboxypeptidase B and the porcine enzyme. Prager and Wilson [23,24] have shown for a number of proteins, that the Immunological Distance between homologous proteins from different species is proportional to their degree of amino acid sequence difference. Since the human carboxypeptidases B differ by small Immunological Distances, they probably differ by no more than 1% in amino acid sequence. On the other hand, the Immunological Distance between human native carboxypeptidase B and the porcine enzyme suggests that they differ by apporximately 30% in amino acid sequence. Thus the three forms of human carboxypeptidase B appear to be very similar in structure, on the basis of comparison by two sensitive immunological techniques.

The three forms of human carboxypeptidase B differ in ionic character. Native carboxypeptidase B appears to be more basic than carboxypeptidase B I or II on the basis of elution from DEAE-cellulose or migration in polyacrylamide gel electrophoresis at both pH 8.3 and at pH 4.5. Furthermore, carboxypeptidase B I is slightly more basic than carboxypeptidase B II by the criterion of elution from DEAE-cellulose. This finding suggests that carboxypeptidase B I is not formed from carboxypeptidase B II by chemical deamidation due to prolonged storage.

Enzyme digestion studies were performed to clarify the relationship between human native carboxypeptidase B and carboxypeptidase B I and II. Digestion with bovine trypsin has been shown to be sufficient to convert native carboxypeptidase B to carboxypeptidase B II. However. However, no combination of trypsin, chymotrypsin, and/or elastase is capable of producing carboxypeptidase B I from native carboxypeptidase B. In fact, carboxypeptidase B I has only been found in pancreatic juice which has been stored for a number of years. A fresh sample of human pancreatic juice recently obtained in this laboratory was shown to contain only native carboxypeptidase B. This fact, taken in conjunction with the relative resistance of native carboxypeptidase B to proteolytic digestion at 37°C suggests that native carboxypeptidase B is the major form of this enzyme in normal pancreatic secretion and is probably the form of carboxypeptidase B previously determined in human serum [1].

Acknowledgements

We wish to thank Calvin Lee and Nancy Waldeck for technical assistance. We also wish to thank Dr. Allan Wilson for microcomplement fixation facilities and for the use of an amino acid analyzer and Dr. Ronald Tompkins for a sample of fresh pancreatic juice.

References

1 Geokas, M.C., Wollesen, F. and Rinderknecht, H. (1974) J. Lab. Clin. Med. 84, 574—586
2 Folk, J.E., Piez, K.A., Carroll, W.R. and Gladner, J.A. (1960) J. Biol. Chem. 235, 2272—2277
3 Wintersberger, E., Cox, D.J. and Neurath, H. (1962) Biochemistry 1, 1069—1077
4 Prahl, J.W. and Neurath, H. (1966) Biochemistry, 5, 4137—4145
5 Reeck, G.R. and Neurath, H. (1972) Biochemistry 11, 3947—3955
6 Kycia, J.H., Elzinga, M., Alonzo, N. and Hirs, C.H.W. (1968) Arch. Biochem. Biophys. 123, 336—342
7 Reeck, G.R., Walsh, K.A., Hermodson, M.A. and Neurath, H. (1971a) Proc. Natl. Acad. Sci. U.S. 68, 1226—1230
8 Reeck, G.R., Walsh, K.A. and Neurath, H. (1971b) Biochemistry 10, 4690—4698
9 Geokas, M.C., Largman, C., Brodrick, J.W., Raeburn, S. and Rinderknecht, H. (1975) Biochim. Biophys. Acta 391, 396—402
10 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
11 Walsh, K.A. (1970) Methods Enzymol. 19, 41—43
12 Walsh, K.A., and Wilcox, P.E. (1970) Methods Enzymol. 19, 31—40
13 Bieth, J., Spiess, B. and Wermuth, C.G. (1974) Biochem. Med. 11, 350—375
14 Largman, C., Brodrick, J.W. and Geokas, M.C. (1976) Biochemistry, 2491—2500
15 Studier, F.W. (1973) J. Mol. Biol. 79, 239—248
16 Studier, F.W. (1972) Science 176, 367—375
17 Weber, K. and Osborn, M.A. (1969) J. Biol. Chem. 244, 4406—4412
18 Davis, B. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
19 Reisfeld, R.A., Lewis, U.J. and Williams, D.E. (1962) Nature 195, 281—283
20 Crestfield, A.M., Moore, S. and Stein, W.H. (1963) J. Biol. Chem. 238, 622—627
21 Hirs, C.H.W. (1967) Methods Enzymol. 11, 197—203
22 Champion, A.B., Prager, E.M., Wachter, D. and Wilson, A.C. (1974) in Biochemical and Immunological Taxonomy of Animals (Wright, C.A., ed.), pp. 397—416, Academic Press, London
23 Prager, E.M. and Wilson, A.C. (1971a) J. Biol. Chem. 246, 5978—5989
24 Prager, E.M. and Wilson, A.C. (1971b) J. Biol. Chem. 246, 7010—7017