Procedures for the Isolation of Crystalline Bovine Pancreatic Carboxypeptidase A. II. Isolation of Carboxypeptidase A_α from Procarboxypeptidase A*

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A procedure is described for the preparation of crystalline carboxypeptidase A_α from acetone powders of bovine pancreatic tissue. The method involves the isolation of the zymogen by chromatography, activation with trypsin, and dialysis of the activation mixture. One or two recrystallizations suffice to yield a product of maximum specific activity. The procedure is considerably simpler than those previously described and offers the advantage of as much as a 12-fold increase in yield.

Two procedures have been described for the isolation of crystalline carboxypeptidase A from bovine pancreas. In one of these (Anson, 1937; Putnam and Neurath, 1946) the fresh tissue was allowed to autolyze for 1 or 2 days to produce an exudate containing partly activated procarboxypeptidase A.[1] Activation was completed by raising the temperature of the exudate to 37° for 1 hour, and carboxypeptidase A was isolated from this mixture. In the second procedure (Allan *et al.*, 1964), a mixture of proteins containing procarboxypeptidase A was extracted from an acetone powder of the glands, and the activation was initiated by the addition of trypsin to the extract. The procedures following the activation step were similar in the two methods, each involving ammonium sulfate fractionation, isoelectric precipitation, and fractional crystallization.

Keller *et al.* (1956) have reported the isolation of crystalline carboxypeptidase A on a small scale after the activation by trypsin of a highly purified preparation of procarboxypeptidase A. The use of the purified zymogen allowed the activation to be carried out under well-defined and reproducible conditions. The isolation of the enzyme from the activation mixture was again similar to that of Anson (1937) and of Putnam and Neurath (1946).

The present report describes a simple and efficient method for the preparation of carboxypeptidase A from the partially purified zymogen; the procedure is rapid and reproducible and provides the enzyme in high yield and purity. The method originated from two observations in these laboratories. First, partially purified procarboxypeptidase A can be obtained in good yield by a single chromatographic separation on DEAE-cellulose[2] (Keller *et al.*, 1958; Yamasaki *et al.*, 1963). Second, carboxypeptidase can be induced to crystallize in nearly pure form simply by dialysis of a concentrated solution of the zymogen which has been incubated with trypsin.

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[1] Procarboxypeptidase A is the precursor of carboxypeptidase A. A new nomenclature for the various chemical species of carboxypeptidase A is given by Bargetzi *et al.* (1963). The chemical differences among the various preparations are described by Sampath Kumar *et al.* (1963a).

[2] Abbreviations used in this work: DEAE, diethylaminoethyl; HPLA, hippuryl-DL-phenyllactic acid.

MATERIALS AND METHODS

Acetone powders were prepared from freshly collected pancreatic tissue by the method described by Fischer and Stein (1954) and by Keller *et al.* (1956). The powders which were used had no detectable trypsin activity and a level of carboxypeptidase activity less than 10% of that obtained upon full activation with trypsin.

DEAE-cellulose was obtained from the Brown Co., Berlin, N.H., and was washed prior to chromatography as described by Wintersberger *et al.* (1962).

Trypsin, twice crystallized and containing 50% $MgSO_4$, was obtained from Worthington Biochemical Corp.

Carboxypeptidase activity was determined with HPLA as described by Bargetzi *et al.* (1963) using substrate prepared in this laboratory by Mr. W. O. McClure.

Procarboxypeptidase concentrations in 0.5-ml aliquots of the DEAE-cellulose effluent were estimated by their enzyme activity towards HPLA after incubation with 25 μl of 0.8% trypsin-$MgSO_4$ at 37° for 2 hours.

Protein concentration was calculated from the absorbancy at 278 mμ using an absorbancy index of 18.8 for a 1% solution of carboxypeptidase in a light path of 1 cm (Bargetzi *et al.*, 1963). The location of protein in the effluent from chromatograms was monitored by the absorbancy of aliquots at 278 mμ.

Sedimentation analyses were carried out in a Spinco Model E ultracentrifuge.

Isolation Procedure

Extraction.—Acetone powder of bovine pancreas (150 g) was stirred for 4 hours with 1600 ml of distilled water at 4°. All operations were carried out at 4° unless otherwise stated. Two drops of octanol were added to suppress foaming. The suspension was centrifuged for 45 minutes at 20,000 rpm in a Spinco Model L ultracentrifuge, No. 21 rotor.

Chromatography on DEAE-Cellulose.—Washed DEAE-cellulose was equilibrated with 0.005 M potassium phosphate buffer at *p*H 8.0 and thoroughly degassed by stirring under a vacuum produced with a water aspirator. A column 4.4 cm in diameter was packed to a height of 50 cm by air pressure of 5 psi. The column was washed with 2 liters of the same buffer and the clear extract of the acetone powder was pumped directly onto the column at a flow rate of about 170 ml/hour. After the column had been washed overnight with the starting buffer a linear phosphate gradient from 0.005 M to 0.33 M was applied using 4 liters of each buffer. Fractions (20 ml) were collected, the protein peaks were located by absorbancy measurements at 278 mμ, and procarboxypeptidase A was located by activity measurements (Fig. 1). Procarboxypeptidase B, which after activation also possesses

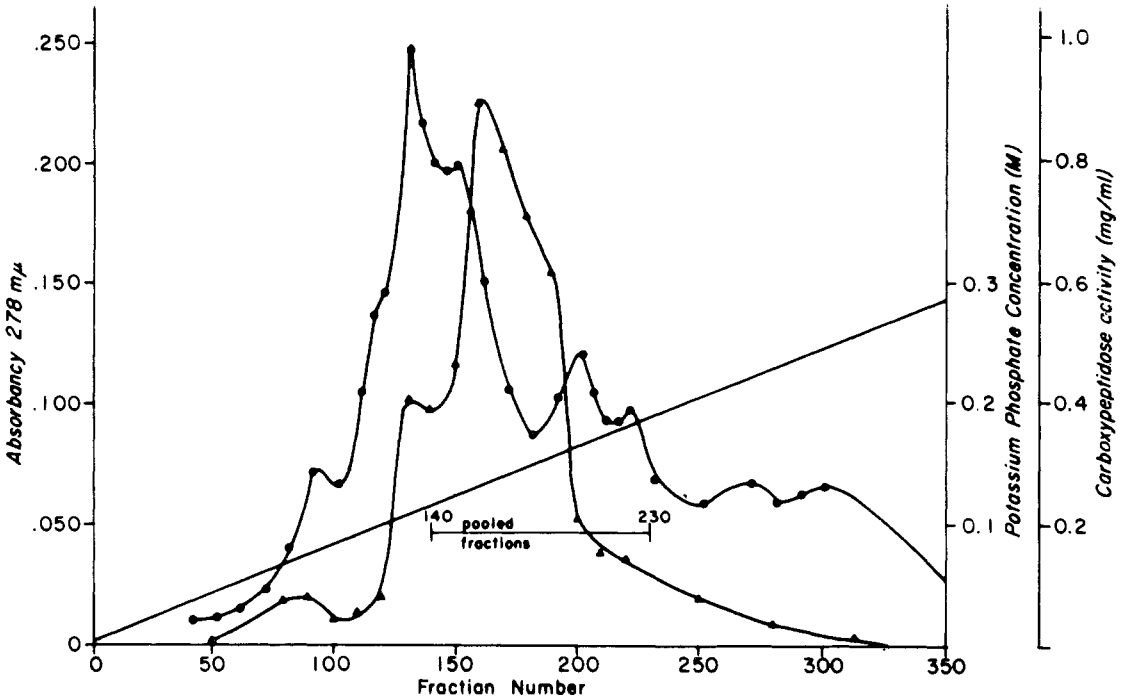

FIG. 1.—Separation of a crude fraction of procarboxypeptidase A on DEAE-cellulose. Conditions of chromatography are described in the text. Circles represent absorbancy at 278 mμ; triangles represent carboxypeptidase activity assayed against HPLA after activation with trypsin as described in the text. Enzymatic activity is recorded in terms of carboxypeptidase concentration.

activity toward HPLA (Wintersberger *et al.*, 1962) is eluted at much lower salt concentrations and hence does not appear in the region of the chromatogram represented in Figure 1.

Fractions containing procarboxypeptidase A were pooled as indicated in Figure 1. Procarboxypeptidase A was concentrated by precipitation with 40% saturated ammonium sulfate, obtained by the gradual addition (over a period of 1 hour) of 283 g of ammonium sulfate per liter of effluent. The *p*H was maintained above 7.2 by occasional dropwise addition of 4 M sodium hydroxide. After 2 hours of stirring the suspension was centrifuged at 6000 × *g* for 60 minutes. The precipitated protein was suspended in water to a final slurry volume of *less than* 150 ml. Ammonium sulfate was removed by dialysis for 2 days against two 6-liter changes of distilled water.

Activation and Crystallization of the Enzyme.—A sparse, amorphous precipitate which usually remained after dialysis of the partially purified procarboxypeptidase was removed by centrifugation at 6000 × *g*. The clear, pale yellow supernatant was brought to 0.25 M potassium phosphate, *p*H 8.0, by the addition of one-third of its volume of 1 M buffer. Commercial trypsin (250 mg, containing 125 mg of $MgSO_4$) was added as well as a few crystals of thymol to suppress bacterial growth. Equivalent results were obtained with salt-free trypsin. The mixture was incubated at 37° for 16 hours. The progress of a typical activation is indicated in Figure 2. A small precipitate was removed by centrifugation and the activation mixture was dialyzed at 5° against 1 liter of 0.001 M potassium phosphate buffer, *p*H 8.0, for 24 hours. When dialysis was repeated against the same volume of the same buffer, carboxypeptidase crystallized in small needles.

Recrystallization.—The crystals were harvested by centrifugation, dissolved in 20 ml of 1 M NaCl, and dialyzed in succession for 24 hours each against the following salts present in 500 ml of 0.02 M sodium Veronal, *p*H 8.0: 0.5 M NaCl, 0.2 M NaCl, 0.15 M NaCl. The last dialysis usually induced crystallization; if not, dialysis was continued against 0.02 M sodium Veronal–0.10 M NaCl. Only two or three recrystallizations were generally necessary to yield material of maximum specific activity.

A typical protocol for the preparation of the enzyme is given in Table I.

Properties of Product

The enzyme prepared by the present method shows similar solubility characteristics in 1 M NaCl, *p*H 8.0, as the enzyme (A_δ) prepared by the method of Allan *et al.* (1964). Both preparations are considerably more soluble than the enzyme isolated by the procedure of Anson (1937).

The specific activity of the purified enzyme, its amino acid composition, and its amino-terminal and carboxyl-terminal residues, are described in other communications from this laboratory (Bargetzi *et al.*, 1963; J.-P. Bargetzi, E. O. P. Thompson, K. S. V. Sampath Kumar, K. A. Walsh, and H. Neurath, in preparation; Sampath Kumar *et al.*, 1963a). The specific activity of the enzyme toward HPLA is reported to be 0.208 μmole/min/μg of carboxypeptidase (Bargetzi *et al.*, 1963). This value may be compared with the value of 0.212 μmole/min/μg of carboxypeptidase reported for the enzyme prepared by the method of Allan *et al.* (1964).

Yield.—As shown in Table I, the present procedure yields approximately 3 g of pure enzyme from 1 kg of acetone powder. Even higher yields have often been obtained. Such yields are larger than those obtained by the procedure of Allan *et al.* (1964), which results in approximately 1 g of recrystallized enzyme per kg of acetone powder, in purity comparable to the product of the present procedure. The procedure of Anson (1937) yields only 0.5 g of partly pure first crystals from 5 kg of *wet* tissue (i.e., from the equivalent of 1 kg of acetone powder), and even this recovery is more variable than in either of the other preparations.

Sedimentation Analysis.—The sedimentation coefficient of carboxypeptidase A_α was measured at concen-

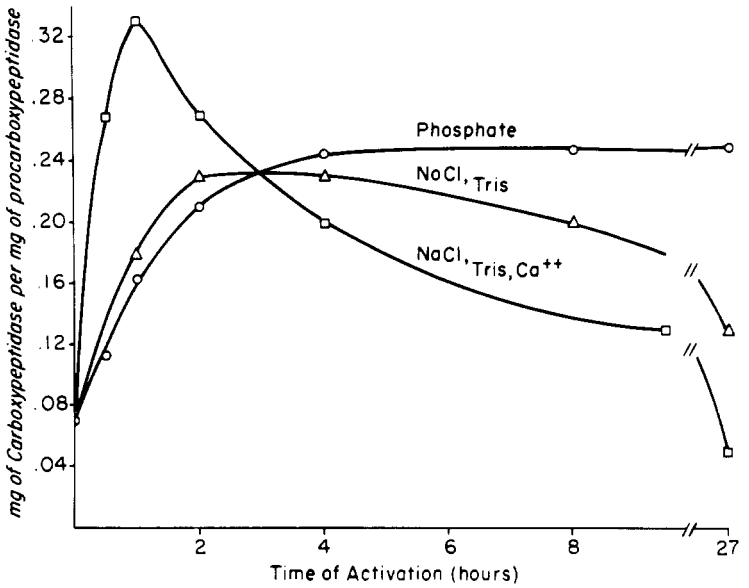

FIG. 2.—Activation of partially purified procarboxypeptidase with trypsin. Partially purified procarboxypeptidase, obtained in pooled fraction from a DEAE-cellulose column, was activated with trypsin at *p*H 8.0, 37.° (a) in 0.25 M potassium phosphate, (b) in 0.05 M Tris–0.5 M NaCl, and (c) in 0.05 M Tris–0.5 M NaCl–0.1 M $CaCl_2$. Carboxypeptidase was measured by its activity towards HPLA; procarboxypeptidase was estimated by absorbancy measurements.

trations ranging from 1.3 to 13 mg/ml in 1 M sodium chloride–0.01 M Tris-HCl, *p*H 8.0. All the sedimentation patterns were consistent with homogeneity of the preparation (Fig. 3). As in the case of the enzymes prepared by either the method of Allan *et al.* (1964) or of Anson (1937), the sedimentation coefficients increased with increasing concentration (Rupley and Neurath, 1960; Smith *et al.*, 1949). The value obtained by the least-squares extrapolation of $s_{20,w}$ was 3.2 Svedberg units, which is in agreement with that reported by Smith *et al.* (1949) for carboxypeptidase A_γ and by Rupley and Neurath (1960) for carboxypeptidase A_δ.

DISCUSSION

The present method of isolation of carboxypeptidase A_α is a much simpler operation than either of the two existing methods (Anson, 1937; Allan *et al.*, 1964). It avoids the variable degrees of autolysis and the ill-controlled activation of crude mixtures characteristic of the other procedures. The present procedure has been tested at least fifty times by six different individuals and results comparable with those in Table I have been consistently obtained. The yield of the purified enzyme is manyfold larger than that obtained from the previous methods of isolation, and the enzyme is invariably in a high state of purity even after the first crystallization. The specific activity of the twice-crystallized enzyme toward HPLA is approximately the same as that of the enzyme prepared by repeated crystallization by the procedure of Allan *et al.* (1964).

In the present method of purification, it is necessary that the acetone powder be essentially free of trypsin as assayed with benzoyl-L-arginine ethyl ester as sub-

TABLE I

TYPICAL PROTOCOL FOR THE PREPARATION OF CARBOXYPEPTIDASE A_α[a]

Stage of Preparation	Volume (ml)	Total Protein by Absorbancy at 278 mμ[d] (g)	Total Procarboxypeptidase (activity vs. HPLA) (g)	Total Carboxypeptidase (activity vs. HPLA) (g)	Purity in g of CP per 100 g Protein (%)	Recoveries of Carboxypeptidase (%)
Aqueous extract after centrifugation	1480	—	2.29	0.881[b]	—	100
Pooled fractions after chromatography	1805	11.2[c]	2.07	0.798[b]	7.2[d]	90
Ammonium sulfate precipitation, centrifuged (wet precipitate)	250[g]	1.5	1.40	0.540[b]	36.0[d]	61
Ammonium sulfate precipitation, supernatant	2085	9.7[c]	0.2	(0.075)[b]	(0.8)[d]	(8.5)
First crystals	25.2	0.405	—	0.364	90.2[f]	41.3
Second crystals, suspension in water	20.0	0.355[e]	—	0.362[cb]	102.0[f]	41.0

[a] These data apply to an extract of 150 g of pancreas acetone powder. [b] Values calculated by assuming that complete activation (by trypsin) yields 1 mole of carboxypeptidase per mole of procarboxypeptidase. [c] Approximately 50% of the absorbing material is dialyzable. [d] Arbitrarily, the value for carboxypeptidase A_α $E^{278m\mu}_{1\,cm}$ = 1.88 (mg × ml^{-1}) was assumed, and the molecular weight ratio of procarboxypeptidase A to carboxypeptidase A taken as 2.6. [e] One ml aliquot of the suspension was dissolved in a final concentration of 1 M NaCl and the measurements were made on this solution. [f] Specific activity. [g] The yield at this step could be substantially improved by keeping the volume of the slurried ammonium sulfate precipitate below 150 ml, as noted in the text.

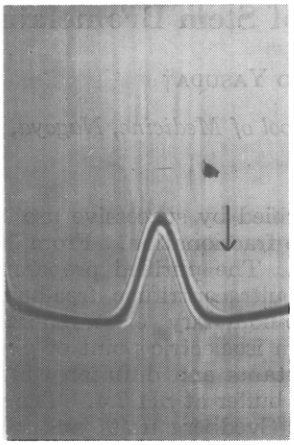

FIG. 3.—Sedimentation pattern of carboxypeptidase. The protein was dissolved to a concentration of 13 mg/ml in 1 M NaCl–0.01 M Tris chloride, *p*H 8.0, at 20.1°. Centrifugation was carried out at 59,780 rpm for 52 minutes in a synthetic-boundary cell. The initial position of the boundary is indicated by the arrow.

strate, so that the water extract will contain the unactivated zymogens.

The enzyme obtained by this procedure is probably derived primarily from the species of zymogen having a sedimentation coefficient of 6 S (procarboxypeptidase A-S6), since another species of zymogen (procarboxypeptidase A-S5), which appears on the DEAE-cellulose column as a shoulder on the leading edge of procarboxypeptidase A-S6 (Yamasaki *et al.*, 1963; Brown *et al.*, 1963), has been largely excluded from the pooled peak (Fig. 1).

The activation reaction is rather slow (see Yamasaki *et al.*, 1963), maximum carboxypeptidase activity being reached only after about 8 hours (Fig. 2). This behavior is in contrast to the more rapid activation occurring in whole pancreatic juice (P. J. Keller, unpublished data). Moreover, the enzyme cannot be crystallized by dialysis of the activation mixture after 8 hours of incubation, but it can if incubation is continued for an additional 8 hours. Since the immediate precursor of carboxypeptidase, subunit I, is not free in pancreatic extracts, but occurs in association with other subunit moieties of procarboxypeptidase A (Brown *et al.*, 1963), prolonged incubation may be required for digestion of the rest of the procarboxypeptidase A molecule (subunits II and III), and other proteins that may interfere with crystallization.

The data of Figure 2 demonstrate that the rate of activation is dependent on the nature of the buffer ions. Thus in a 0.01 M Tris chloride buffer, containing 1 M NaCl, the activity declined after having reached a maximum, in contrast to activation in a 0.25 M potassium phosphate buffer wherein the activity remained at the maximum level for up to 4 days, at 37°. When 0.1 M $CaCl_2$ was added to the Tris chloride buffer system the activation proceeded more rapidly and to a higher level of carboxypeptidase activity, but the activity rapidly declined after the maximum had been reached. These effects of calcium ions on the rate and course of tryptic activation remain unexplained at present.

The enzyme isolated by the present procedure is identical in sedimentation coefficient and specific activity with those prepared by the methods of Anson (1937) and Allan *et al.*, (1964). Other properties, relating to metal content, metal binding, and metal exchange (Vallee *et al.*, 1963), remain to be determined. As documented in preceding communications, the present enzyme (A_α) differs from those prepared by the other two methods in having an additional pentapeptide sequence at the N-terminus (Sampath Kumar *et al.*, 1963a). The peptide distribution (i.e., "fingerprints") and the amino acid sequences so far elucidated in this laboratory (Sampath Kumar *et al.*, 1963a, 1963b), are otherwise identical. Thus carboxypeptidases A_α and A_γ are *different* structurally though they seem to have practically identical specific activities; hence specific activity is not a good guide to a decision as to which is *the* enzyme of choice for further study.

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