CARBOXYPEPTIDASE

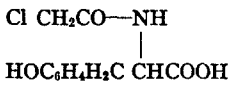
I. The Preparation of Crystalline Carboxypeptidase*

By M. L. ANSON

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Accepted for publication, September 15, 1936)

Carboxypeptidase (CP) splits the amide linkages of certain amino acid compounds such as chloracetyl-tyrosine, tyrosyl-tyrosine, and leucyl-glycyl-tyrosine. In each case an amino acid is liberated which in the intact compound has a free carboxyl group. Since chloracetyl-tyrosine,



is attacked, it is clear that the substrate of carboxypeptidase need not have a free amino group nor need it, despite the name of the enzyme, be a peptide. It has been assumed, but not proven experimentally, that chloracetyl-tyrosine is attacked by only a single enzyme and that the same enzyme which attacks chloracetyl-tyrosine also attacks the other supposed substrates of carboxypeptidase. It may be, however, that what has hitherto been called carboxypeptidase is in reality a mixture of enzymes. The previous work on carboxypeptidase has been reviewed by Waldschmidt-Leitz (1931) and Bergmann (1934).

The present paper (Part I) describes the preparation from autolyzed beef pancreas of a crystalline, water-insoluble protein which attacks chloracetyl-tyrosine and peptic digests of proteins. This crystalline carboxypeptidase is active even in the presence of formaldehyde which abolishes the free amino groups of both enzyme and substrate. Car-

* A brief account of the preparation of crystalline carboxypeptidase has been published in *Science* (Anson (1935)).

boxypeptidase is the only proteolytic enzyme of those which have been crystallized which attacks simple substrates of known structure. The two following papers describe (Part II) the extraction from fresh pancreas of the inactive precursor of carboxypeptidase, pro-carboxypeptidase, its partial purification, and its activation by trypsin; and (Part III) the estimation of carboxypeptidase and of its inactive precursor.

Two sources of material have been used for the preparation of crystalline carboxypeptidase, the turbid fluid which exudes when sliced frozen bovine pancreas is thawed overnight at 5°C.,[1] which is not always available in large quantities from commercial sources; and ordinary commercial frozen pancreas, which can be obtained from any of the large meat packers. The turbid fluid is by far the more convenient starting material. 1 liter of this fluid yields roughly a gram of crystals.

The turbidity of the fluid which exudes when frozen pancreas is thawed is due to a small amount of dark, slimy material which at the pH of the fluid cannot readily be removed by filtration. If the fluid is acidified (green to brom cresol green) and warmed, the dark, slimy material clots and can then readily be filtered off. The filtrate on dilution with water yields a precipitate which contains most of the carboxypeptidase and most of the proteinase of the original fluid. This precipitate is only partially soluble in barium hydroxide in slightly alkaline solution (pink to phenolphthalein). When the soluble part, which contains all the carboxypeptidase, is neutralized (orange to phenol red) a protein crystallizes out which attacks chloracetyl-tyrosine (Fig. 1). On repeated recrystallization this protein becomes free of proteinase but retains all its original carboxypeptidase activity.

When crystalline carboxypeptidase is prepared directly from commercial frozen pancreas the early stages of the preparation are modified. An acidified sodium chloride extract is filtered, the filtrate is 0.6 saturated with ammonium sulfate, and the precipitate formed is dialyzed against water. The fraction which remains precipitated after dialysis is treated with barium hydroxide.

[1] This same material was originally used for the isolation of trypsin (Northrop and Kunitz (1932–33)).

The specific activity of crystalline carboxypeptidase is

$$0.081\ [\text{CP.u.}]^{\text{CT}}_{\text{mg. N}} \quad \text{and} \quad 0.103[\text{CP.u.}]^{\text{PDE}}_{\text{mg. N}}.^{2}$$

The specific activity of twice crystallized carboxypeptidase is not

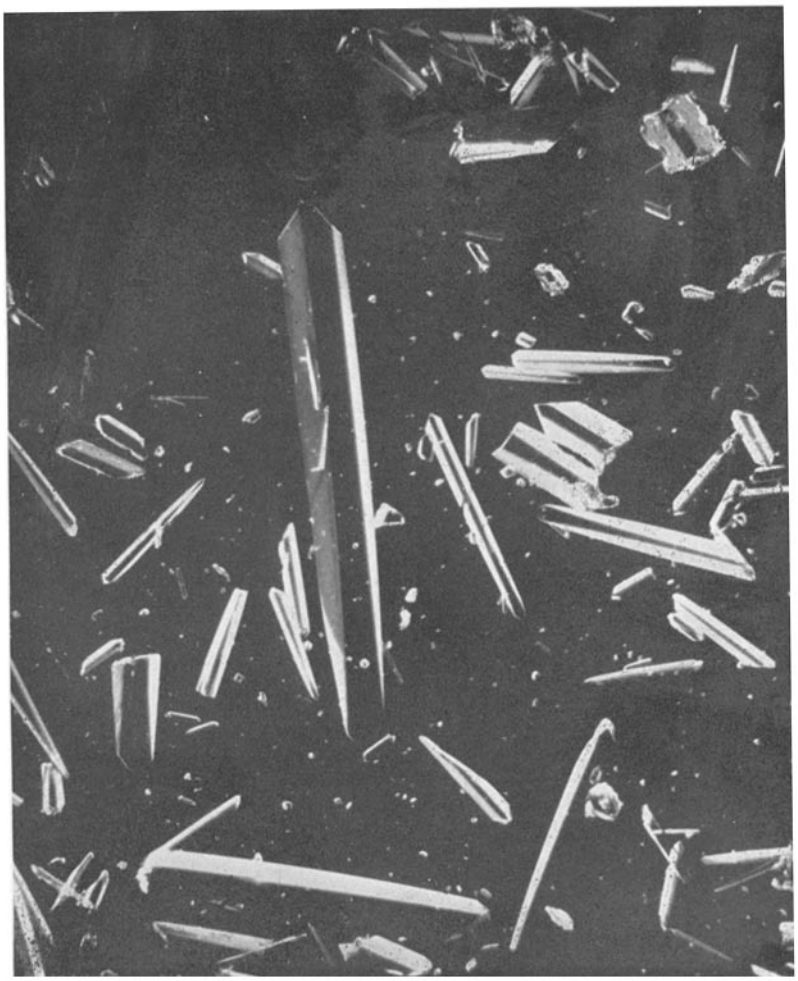


FIG. 1. Carboxypeptidase crystals × 85

changed by repeated or fractional recrystallization. If crystalline carboxypeptidase is partially denatured and precipitated, the surviving

[2] Part III (*J. Gen. Physiol.*, in press) will describe the estimation of carboxypeptidase and define the carboxypeptidase unit (CP.u.). Two substrates are used for the estimation of carboxypeptidase, chloracetyl-tyrosine (CT) and a formolized peptic digest of edestin (PDE).

soluble protein has the same specific activity as the original crystalline material.

Recrystallized carboxypeptidase gives no test for proteinase, dipeptidase, amino polypeptidase, and amylase.

The elementary analysis of carboxypeptidase is: C, 52.6 per cent; N, 14.4 per cent; H, 7.2 per cent; S, 0.47 per cent; P, 0.00 per cent; ash, 0.68 per cent.

An amount of carboxypeptidase containing 0.20 mg. N gives the same color with the phenol reagent as 0.15 mg. tyrosine.

Preparation from Pancreatic Fluid

The Starting Material.—The pancreatic fluid which exudes when frozen pancreas is thawed consists mainly of proteins and protein split products and is highly variable in composition. As a result the absolute amount of crystalline carboxypeptidase that is obtained finally, and the yields of carboxypeptidase at each step, are also highly variable. If the directions are followed, however, a large crop of crystals is obtained without fail. It is not necessary to measure the carboxypeptidase content of the original fluid which is usually around 0.07 $[\text{CP.u.}]^{\text{PDE}}_{\text{ml.}}$.

The First Globulin Precipitate.—5 N acetic acid is added with stirring to the crude fluid until the solution is definitely green to brom cresol green. The acidified solution in 4 liter Erlenmeyer flasks is left in a 37°C. bath for 2 hours and then filtered. The filtrate contains all the carboxypeptidase. Ten volumes of tap water are added to each volume of filtrate in large glass vessels. A precipitate forms which settles. At the end of the day the supernatant fluids are siphoned off and rejected and the precipitates are collected in a single vessel. The next morning the supernatant is again siphoned off and rejected and the precipitate is filtered on Schleicher and Schüll folded filter paper No. 588, 50 cm. diameter. The smaller and also thinner paper of the same number is less satisfactory. Finally the precipitate is evenly suspended in enough water to give a volume roughly one-fifth of the original volume of pancreatic fluid. A sample is dissolved with a little di-potassium phosphate and sodium chloride and its activity is measured. From 35 to 90 per cent of the original activity is recovered in the first globulin precipitate.

Barium Hydroxide Extraction.—The suspension of the first globulin precipitate is diluted to have roughly 0.25 $[\text{CP.u.}]^{\text{PDE}}_{\text{ml.}}$. 0.2 M barium hydroxide is added with vigorous stirring until the solution is definitely pink to phenolphthalein and the suspension is immediately filtered with Standard Super-Cel[3] on a Buchner

[3] Standard Super-Cel is the trade name of a particular Celite filter aid of medium porosity. Hyflo Super-Cel is a coarser grade, Filter-Cel a finer. Celite filter aids are made from diatomaceous earth (kieselguhr) by Johns-Manville, 22 East 40th Street, New York City. The names of the European sales agents and booklets describing the Celite filter aids may be obtained from the manufacturer.

funnel or centrifuged. Barium hydroxide dissolves only a part of the globulin precipitate whereas sodium hydroxide at the same pH dissolves it completely. If not enough barium hydroxide has been added the supernatant solution obtained by centrifugation is turbid and does not contain all the carboxypeptidase. If too much barium hydroxide is added the carboxypeptidase activity is slowly destroyed. As a precaution, when a large batch of material is being worked up, the barium hydroxide extraction is first carried out on a small scale, the amount of barium hydroxide added is recorded, and the activity of the solution is measured after removal of the undissolved protein. The solution should be clear and its activity should be the same as that of a complete solution of the globulin precipitate obtained by adding di-potassium phosphate and sodium chloride instead of barium hydroxide.

Crystallization.—Immediately after centrifugation, 1 N acetic acid is added to the barium hydroxide solution with vigorous stirring until the solution becomes just turbid (orange to phenol red). Crystals appear slowly if the solution is simply allowed to stand. In practice the solution is heavily seeded, allowed to stand the rest of the day at room temperature with occasional stirring, and finally left in the refrigerator overnight. The next morning the supernatant solution is siphoned off and rejected and the crystals which have settled to the bottom of the vessel are centrifuged. The supernatant solution is rejected, the crystals are again suspended in water, and again centrifuged. 40 to 80 per cent of the carboxypeptidase of the barium hydroxide extract is obtained in crystalline form.

At the pH used for crystallization carboxypeptidase is stable, its crystalline form is insoluble, and its amorphous form is soluble. At a more acid pH (green to brom cresol green) both the crystalline and amorphous forms are insoluble and so, since precipitation of the amorphous form is rapid compared with crystallization, carboxypeptidase is immediately and completely precipitated in the amorphous form. If the amorphous precipitate is dissolved at the acid pH with a solution of sodium chloride, the carboxypeptidase slowly coagulates and is inactivated. It is clear that carboxypeptidase is insoluble over a wide range of pH and that the proper pH for crystallization is far on the alkaline side of the pH of minimum solubility.

Recrystallization.—To a suspension of crystals having about 1.0 $[\text{CP.u.}]^{\text{PDE}}_{\text{ml.}}$ (the exact concentration is not important) 0.1 N sodium hydroxide is added slowly with vigorous stirring until almost all the material is dissolved. The undissolved material is removed immediately by centrifugation and 1 N acetic acid is immediately added to the supernatant solution until the first turbidity appears. The solution is then seeded and allowed to stand several hours and the crystals centrifuged. The total activity of the recrystallized protein is 80 to 100 per cent of the activity of the protein used for recrystallization. The crystals are stored in the cold with toluol as a preservative and so far as I know are stable indefinitely in aqueous suspension. If pure carboxypeptidase is dried, more or less of the protein is converted into an insoluble, inactive form, even if the protein is dried while frozen or if the water is removed with sodium sulfate. Impure carboxypeptidase, however, can be dried with sodium sulfate without any loss of solubility or activity.

This protective action of impurities has been noted with pepsin and many other proteins.

It is important when adding sodium hydroxide not to make the solution too alkaline. The crystals sometimes dissolve very slowly and the process of solution should not be hurried by adding sodium hydroxide in excess. After the carboxypeptidase has been once recrystallized it usually dissolves completely when the solution is definitely alkaline to phenolphthalein. This pH is entirely safe. It is not important that the crystals be completely dissolved. Undissolved crystals can be saved and added to the supply of once crystallized material.

Changes in Specific Activity.—In general the variable specific activities ($[CP.u.]_{mg.\ N}$) of the various fractions were not measured since they need not be known for preparative purposes. Typical values are given in Table I.

Alternative Method of Preparation.—If the first globulin precipitate is dissolved with basic potassium phosphate and heated to 60°C. under

TABLE I

	Pancreatic fluid	Acetic acid filtrate	Dilution precipitate	Barium hydroxide extract	Once crystallized CP.	Twice crystallized CP.
$[CP.u.]^{PDE}_{mg.\ N}$	0.00277	0.00265	0.0128	0.0222	0.0814	0.103

suitable conditions much of the protein is digested and the remaining protein has a very high carboxypeptidase and a very low proteinase activity. But I have never succeeded in obtaining crystals or in obtaining a preparation of carboxypeptidase completely free of proteinase without a fractionation with barium hydroxide.

Preparation from Frozen Pancreas

Frozen beef pancreas is ground and stirred up with 3 times its weight of 2 per cent sodium chloride and 20 per cent of its weight of toluol (Eastman practical), and allowed to stand at room temperature. The next morning the fat and toluol are skimmed off and the suspension is filtered through gauze; 5 N acetic acid is added to the filtrate until it is green to brom cresol green. The acid solution is filtered on a Buchner funnel with the aid of Standard Super-Cel (Johns-Manville) and 390 gm. ammonium sulfate are added to each liter of filtrate. The precipitate formed is filtered off and dialyzed overnight against cold water in a shaking dialyzer (Kunitz and Simms (1927–28)). The dialyzed solution is centrifuged,

the supernatant is discarded, and the precipitate is then treated like the precipitate obtained from the fluid which exudes when frozen pancreas is thawed.

Recrystallization and Fractional Crystallization.—A sample of twice crystallized carboxypeptidase had a specific activity of 0.082[CP.u.]$^{CT}_{mg.\ N}$, of six times crystallized, 0.083[CP.u.]$^{CT}_{mg.\ N}$. A sample of twice crystallized carboxypeptidase had a specific activity of 0.095[CP.u.]$^{PDE}_{mg.\ N}$. It was recrystallized. The first 25 per cent which crystallized out had a specific activity of 0.104[CP.u.]$^{PDE}_{mg.\ N}$.

Fractional Heat Coagulation.—A test tube containing a solution of twice crystallized carboxypeptidase in half saturated sodium chloride solution containing 1.84 mg. nitrogen per ml. was placed in 52°C. water for 3 minutes and the suspension was then cooled and filtered. The filtrate contained 1.26 mg. nitrogen per ml. The specific activity of the carboxypeptidase in the filtrate was 0.104[CP.u.]$^{PDE}_{mg.\ N}$.

Fractional Denaturation by Acid.—To a solution of twice crystallized carboxypeptidase in 5 per cent sodium chloride containing 1.06 mg. nitrogen per ml. was added an equal volume of a solution containing 3 parts 0.2 M sodium acetate to 1 part 0.2 M acetic acid. The solution was kept 90 minutes at 37°C., cooled, and the denatured and precipitated protein filtered off. The filtrate contained 0.26 mg. nitrogen per ml. The specific activity of the carboxypeptidase in the filtrate was 0.092[CP.u.]$^{PDE}_{mg.\ N}$.

Other Enzymes.—Six times crystallized carboxypeptidase contains a trace of proteinase which can be detected by long digestion of hemoglobin (Anson and Mirsky (1933–34)) with a large amount of carboxypeptidase. This slight activity could be accounted for by an impurity of 1 part trypsin to 30,000 parts carboxypeptidase.

Thrice crystallized carboxypeptidase is free of dipeptidase and amino polypeptidase. Fruton[4] found that 1 mg. carboxypeptidase per ml. produced no detectable digestion of 0.05 M *dl*-leucyl-glycine or *dl*-leucyl-glycyl-glycine at pH 7.8 in 23 hours at 40°C.

Six times crystallized carboxypeptidase is free of amylase. A 1 per cent neutral solution of starch gave almost the original color with iodine after 24 hours at 37°C. despite the presence of 0.7 mg. per ml. of carboxypeptidase.

REFERENCES

Anson, M. L., 1935, *Science*, **81**, 467.
Anson, M. L., and Mirsky, A. E., 1933–34, *J. Gen. Physiol.*, **17**, 151.
Bergmann, M., 1934, *Science*, **79**, 439.
Kunitz, M., and Simms, H., 1927–28, *J. Gen. Physiol.*, **11**, 641.
Northrop, J. H., and Kunitz, M., 1932–33, *J. Gen. Physiol.*, **16**, 267.
Waldschmidt-Leitz, E., 1931, *Physiol. Rev.*, **11**, 358.

[4] Dr. Joseph Fruton, private communication.