CARBOXYPEPTIDASE

II. The Partial Purification of Pro-Carboxypeptidase

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Extracts of autolyzed pancreas contain carboxypeptidase which even in the presence of formaldehyde can digest chloracetyl-tyrosine and peptic digest of edestin. Part I[1] described the isolation of this carboxypeptidase in crystalline form. If fresh pancreas is extracted with cold salt solution, the extract does not attack a formalized peptic digest of edestin. On standing at 37°C., however, the extract slowly becomes active. The activation is enormously hastened by the addition of trypsin. Thus, fresh pancreas contains not active carboxypeptidase (CP) but an inactive precursor, pro-carboxypeptidase (PCP). The nature of this precursor is not known. It may be a protein which is different from carboxypeptidase. It may be carboxypeptidase combined with an inhibitor.

Pro-carboxypeptidase can be partially purified by fractionation with ammonium sulfate. Most of the pro-carboxypeptidase in the extract is precipitated by 0.35 saturated ammonium sulfate but not by 0.2 saturated ammonium sulfate. The protein can be freed of ammonium sulfate by precipitation by ferric chloride or by dialysis under carefully controlled conditions.

The pro-carboxypeptidase in the partially purified preparation, like the pro-carboxypeptidase in the crude pancreatic extract, is activated by trypsin. Partially purified pro-carboxypeptidase contains trypsinogen, which trypsin can convert into trypsin. The activation of impure pro-carboxypeptidase by trypsin, therefore, is partially due to the added trypsin and partially due to trypsin formed from the trypsinogen present. Until pro-carboxypeptidase is prepared free from trypsinogen, experiments on the kinetics of activation of pro-carboxypeptidase by trypsin are of dubious significance.

[1] *J. Gen. Physiol.*, 1936–37, **20**, 663.

Enterokinase can activate pro-carboxypeptidase. This activation may be due to the trypsin formed from trypsinogen by enterokinase. In the presence of sufficient trypsin inhibitor no activation takes place. From this result alone one cannot decide whether the inhibitor acts by eliminating activation by trypsin or by interfering with direct activation by enterokinase. The inhibitor does not affect the activity of activated carboxypeptidase.

Pro-carboxypeptidase is not activated by a small amount of chymo-trypsin. If a large amount of chymo-trypsin is used there is in time a partial activation which may be due to a slight impurity of trypsin.

In general the results agree with but do not prove the hypothesis that pro-carboxypeptidase, like chymo-trypsinogen (Kunitz and Northrop (1934–35)) is activated only by trypsin. More conclusive experiments are not possible with the impure pro-carboxypeptidase now available.

To estimate pro-carboxypeptidase, trypsin is added and the resulting carboxypeptidase is estimated. Part III[2] describes the activation and estimation of pro-carboxypeptidase and defines the pro-carboxypeptidase unit [PCP.u.].

Partial Purification of Pro-Carboxypeptidase.—Bovine pancreas is trimmed of fat, and rapidly frozen immediately after removal from the animal, and stored frozen. If it is impossible to freeze the pancreas immediately it should at least be promptly chilled in ice water. To each kilogram of ground frozen pancreas are added 3 liters of 5 per cent sodium chloride solution and 150 ml. of toluol (Eastman practical). The suspension is stirred and allowed to stand overnight at 5°C. In the morning the toluol and fat are skimmed off the top and the suspension is filtered through very fine gauze. 114 gm. of ammonium sulfate are added to each liter of solution which makes the solution 0.2 saturated with ammonium sulfate. 40 gm. of Filter-Cel and 20 gm. of Standard Super-Cel (Johns-Manville) are added to each liter of solution and the solution is filtered on a large Buchner funnel through a filter bed of Standard Super-Cel. To each liter of filtrate are added 89.4 gm. ammonium sulfate, which makes the solution 0.35 saturated with ammonium sulfate. The suspension is filtered on large folded papers in the cold, the precipitate is hardened on a Buchner funnel, and stored frozen. Half saturated ammonium sulfate precipitates only 10 per cent more pro-carboxypeptidase than 0.35 saturated ammonium sulfate.

The extraction of fresh pancreas is less efficient than that of autolyzed pan-

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creas. In a typical case, the sodium chloride extract contained 0.012 $[PCP.u.]^{PDE}_{ml.}$ and 3.5 mg. nitrogen per ml.

The specific activity of crystalline carboxypeptidase is 0.01 $[CP.u.]^{PDE}_{mg.\ N}$. The specific activity of the ammonium sulfate precipitate after activation is 10 per cent that of crystalline carboxypeptidase and 2.5 times that of the sodium chloride extract. Before activation, the carboxypeptidase activity of the ammonium sulfate precipitate is less than 3 per cent of its activity after activation.

The procedure outlined can be applied to unfrozen fresh pancreas. The specific activity of the product obtained is a quarter to a third less than that of the product obtained from frozen pancreas.

TABLE I

Activation Experiments

Activator	Time activation	Time digestion	Increase in formol titration
	min.	*min.*	*ml. 0.02 N sodium hydroxide*
Trypsin	5	10	0.6
Chymo-trypsin	5	30	0.0
	60	30	0.3
Enterokinase	5	10	0.45
Enterokinase plus inhibitor; inhibitor added before enterokinase	5	20	0.05
Enterokinase; inhibitor added after activation but before estimation	5	10	0.40

Pro-carboxypeptidase solution, final concentration in all cases: 1 gm. unhardened ammonium sulfate precipitate per 100 ml. 5 per cent sodium chloride.

Trypsin: 0.5×10^{-4} hemoglobin units (Anson and Mirsky (1933–34)) crystalline trypsin (Kunitz and Northrop (1935–36)) per ml.

Chymo-trypsin: 0.3×10^{-4} hemoglobin units crystalline chymo-trypsin (Kunitz and Northrop (1935–36)) per ml.

Enterokinase: 1 drop of concentrated solution (prepared by Dr. M. Kunitz) per 2 ml.

Inhibitor: 5 drops of concentrated solution of protein-free crude trypsin inhibitor (prepared from pancreas by Dr. Kunitz) per 2 ml.

Activation carried out for various times at 37°C. 0.5 ml. used for digestion of peptic digest of edestin (see Part III) for various times.

It is not possible to purify or crystallize pro-carboxypeptidase by the technique used to isolate carboxypeptidase. The ammonium sulfate precipitate is completely soluble in barium hydroxide.

Precipitation with Ferric Chloride.—Pro-carboxypeptidase is very easily denatured by acid. One can, however, precipitate 80 per cent of the partially

purified pro-carboxypeptidase with ferric chloride (which is acid) without any change in specific activity. To each gram of ammonium sulfate filter cake are added 10 ml. of a cold solution of 5 per cent sodium chloride containing enough ferric chloride to make the solution green to brom cresol green. The precipitate is filtered off in the cold. It is completely soluble at pH 8.0.

Dialysis.—1 gm. of ammonium sulfate filter cake is dissolved by the addition of 16 ml. of 0.1 M acid potassium phosphate and 4 ml. of 0.1 M di-potassium phosphate. The solution is dialyzed overnight at 10°C. in a shaking dialyzer (Kunitz and Simms (1927–28)) against a solution containing 2 per cent sodium chloride, 0.008 M acid potassium phosphate, and 0.002 M di-potassium phosphate. Under these conditions pro-carboxypeptidase can be dialyzed without destruction or activation.

Activation Experiments.—The experiments and their results are given in Table I.

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