Purification of Alkaline Phosphatase by Tryptic Digestion

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After fractional precipitation of kidney, intestinal and faecal phosphatase with acetone in the cold, further attempts at purification by either repeated acetone or ammonium sulphate fractionation were ineffective. Treatment with aluminium hydroxide was likewise of little use, and there was considerable loss of enzymic activity. Digestion with trypsin, at this stage, proved to be of great advantage in eliminating inactive proteins which are otherwise difficult to remove.

In most of the current procedures for the preparation of phosphatase, autolysis is used as an indispensable stage in the liberation of the enzyme from the tissues. Ehrenward (1933) discovered that the activity of alkaline phosphatase is not affected by trypsin. Kutscher & Pany (1938) found later that acid phosphatase is resistant to papain. Schmidt & Thannhauser (1943) used artificial proteolysis in the form of tryptic digestion for bringing the calf intestinal phosphatase into solution. Fischer & Greep (1948) have recently reported on an 'activation' of the alkaline phosphatase by trypsin. Thus the tryptic digestion of crude phosphatase preparations is well known, but its effect on highly concentrated purified enzyme preparations has not been well investigated. For this reason, it seemed desirable to conduct such a study to find out the best conditions for purification with minimum loss of enzyme activity.

EXPERIMENTAL

Enzyme solutions from ox kidney and dog intestinal mucosa treated by fractionation with acetone in the cold, and dog faecal phosphatase prepared by fractional precipitation with (NH₄)₂SO₄ and charcoal decolorization, have been used. A very potent trypsin powder (U.S.P.X., Armour Laboratories, Chicago) has been used throughout. The enzyme solutions were adjusted to pH 8 by Na₂CO₃ solution and kept at 37° with various concentrations of trypsin powder. Activities expressed in King phenol units in relation to protein N by micro-Kjeldahl (see Abul-Fadl, King, Roche & Thoi, 1949) have been determined in the presence of Mg⁺⁺ on samples withdrawn from the incubated enzyme solutions at regular intervals.

After tryptic digestion there is always a considerable amount of non-protein N, which is best eliminated by prolonged dialysis.

RESULTS AND DISCUSSION

Table 1 shows the effect of tryptic digestion on faecal and intestinal phosphatases. An initial increase of the enzyme activity in relation to protein nitrogen

during the first few hours of incubation is followed by a gradual fall. It is, therefore, inadvisable, with 0.1-0.2 % trypsin, to incubate for longer than

Table 1. *Effect of trypsin digestion on partially purified alkaline phosphatase*

Enzyme solution	Tryp- sin con- cen- tra- tion (%)	Time of incuba- tion at 37° (hr.)	Protein N (mg./ml.)	Phosphatase activity	
				(Units/ml.)	(Units/mg. protein N)
Intestinal	0.1	0	0.20	300	1500
		2	0.18	300	1720
		4	0.16	255	1569
		6	0.16	255	1569
		8	0.16	240	1480
Intestinal	0.2	0	0.17	315	1852
		1.5	0.14	293	2192
		3	0.13	243	1868
		4.5	0.12	225	1800
		6	0.12	200	1660
Faecal	0.5	0	0.40	240	600
		2	0.28	240	857
		4	0.24	200	833
		6	0.24	185	770
		24	0.24	144	600
		30	0.24	144	600*
		48	0.24	126	525*
		60	0.24	102	425

* More trypsin added.

Fig. 1. Effect of tryptic digestion on kidney phosphatase. 0.1 % trypsin, 37°, pH 8.0.

2-3 hr. at 37°. This depends on the degree of contamination of the enzyme with inactive protein. With kidney preparations (Fig. 1), however, a longer

incubation is required (4–5 hr.) for the maximum activity relative to protein nitrogen to be attained, since these are more contaminated by inactive protein than either faecal or intestinal preparations. It is interesting to note that trypsin does not appear to act on the protein part of the enzyme, since the protein nitrogen of the incubated enzyme solution remains constant after a certain time, and is not affected by any further incubation, even if more trypsin is added. The decline in enzyme activity appears to be due to inactivation of the enzyme, when incubated in alkaline buffer, rather than to direct trypsin action on the enzyme itself. This inactivation was pointed out by Fischer & Greep (1948) to be much more extensive in the absence of trypsin than when it is present. Thus trypsin, in addition to

digesting contaminating proteins, seems to protect the enzyme to a certain extent against inactivation during incubation in alkaline buffer. Excess of trypsin, however, does not seem to increase the degree of protection of the enzyme; on the contrary, it seems to decrease it, or to cause inhibition of the phosphatase.

SUMMARY

1. Further purification of the alkaline phosphatase, beyond that attainable by fractional precipitation and salting-out procedures, was obtained by incubation with 0.1–0.2 % trypsin at 37° for a period of not more than 4 hr.

2. Addition of more trypsin, or incubation for longer periods, resulted in loss of enzyme activity.

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Reactivation of Alkaline Phosphatases after Dialysis

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Albers, Bayer, Bohnenkamp & Muller (1938) ascribed the inactivation of the kidney alkaline phosphatase through prolonged dialysis to a separation of the phosphatase into an inactive protein apoenzyme and a dialyzable coenzyme or prosthetic group. These workers showed that this coenzyme cannot be magnesium. It appeared to be a labile organic substance, which could be obtained by incubating the holoferment at 100° for a few minutes in a neutral medium. This treatment destroyed the apoenzyme while not affecting the coenzyme. By mixing the latter with an enzyme preparation partially inactivated by dialysis or previous incubation in an acid medium, active phosphatase solutions were obtained. On combining the dialyzable liquid with the inactivated protein solution of dialyzed kidney preparations, with the addition of magnesium, Albers (1939) claimed to have obtained an almost 100 % reactivation. von Euler & Hahn (1947) described a partial reactivation of intestinal phosphatase, inactivated by dialysis at pH 4.5, by the addition of boiled enzyme solution. Cloetens (1941*a*, *b*, 1942), after repeating Albers's experiments, came to the conclusion that the activating action of a heated enzyme solution (Kochsaft), or the dialysate

liquid, was not due to the presence of a cophosphatase, but more probably to a zinc or cobalt ion, which is present in very small amount and which forms a dissociable complex. After studying the influence of different metallic ions, as well as substances which form weakly dissociable combinations with metals, Cloetens concluded that alkaline phosphatase is composed of a slightly active or completely inactive part, EG_2^1 (i.e. protein + metal-combining groups), and the two metals magnesium and zinc. This postulate was supported by the work of Hove, Elvehjem & Hart (1940), who used zinc salts in combination with amino-acids and with protein split products (from autolyzed mucosal tissue) to activate dialyzed intestinal phosphatase. Thoai, Roche & Roger (1947) reported the inactivation of intestinal phosphatase by prolonged dialysis at 37°, and its complete reactivation by incubation with amino-acids in alkaline medium and determination of the enzyme activity in presence of certain inorganic ions. Comparing these findings with that of Albers, it would appear possible that the dialyzable prosthetic group may be a combination of a metal other than magnesium (possibly zinc) with a compound containing amino-acid groups.