Esterase Activity Shown by Subtilisin, a Proteolytic Enzyme from *Bacillus subtilis*

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A proteolytic enzyme preparation from *Bacillus subtilis* catalyses the transformation of ovalbumin to plakalbumin. The preparation of crystals containing this enzyme is described by Güntelberg and Ottesen [1]. Water extracts of the crude enzyme as well as the crystalline preparation, shows hydrolysing effect toward methyl esters of the lower fatty acids, as demonstrated for methyl butyrate by Güntelberg and Ottesen [2], and as will be shown, there is reason to believe that the active component is identical with the protease.

The proteolytic effect is described by Linderstrøm-Lang and Ottesen [3,4], Ottesen and Villee [5] and Ottesen and Wollenberger [6].

The investigation here deals with the esterase activity. The determinations are made by automatic titrations in a "pH-stat". Experiments were carried out in 100 ml beakers containing 50 ml of phosphate buffer (0.01 *M* sec. phosphate, 0.0005 *M* prim. phosphate and 0.1 *M* KCl) adjusted to the desired pH value with *N* NaOH. 0.0922 ml ester is used and the experiments are made at 20° C. As a measure of the activity we use the amount of ester hydrolysed in ten minutes, expressed as scale readings at the burette, containing 0.825 *N* NaOH. (100 scale readings equal 0.0653 ml). Standardizing of the proteolytic activity is made in the same way, only substituting the ester with 250 mg of casein ("Casein nach Hammarsten"), and in stead of ten minutes hydrolysis the slope of the curve at the origin is used.

To investigate whether the esterase and the protease were identical or not, the following experiments were carried out.

6 g of the crude enzyme was extracted with 60 ml 0.15 *M* $CaCl_2$ and centrifuged. The supernatant fluid (I) was made 50 % with respect to acetone and again centrifuged. The precipitate was discarded and to the new supernatant, acetone was added until the content had increased to 75 %. The precipitate was separated by centrifugation and dissolved in 17 ml of distilled water (II). An equal amount of 24 % Na_2SO_4 was added. The resulting precipitate was dissolved in a small amount of water and then dialyzed against distilled water (III). Similarly the supernatant was dialyzed (IV) in order to remove the bulk of the sulphate.

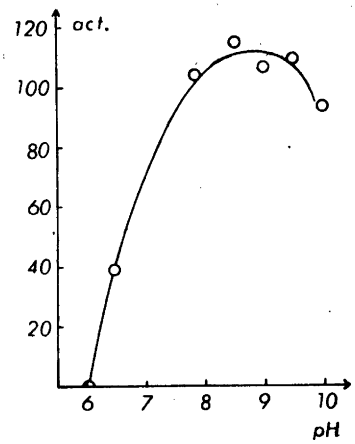

Fig. 1. Activity as function of pH.

The fractions I—IV were then standardized using casein and methyl propionate respectively as substrate. In the determination of the esterase activity of a certain fraction the amount of enzyme was always 25 times the amount used in the determination of the protease activity of the same fraction. The values of the activities of the different fractions are evidently not mutually comparable as the specific activities of the fractions are different and unknown.

		I	II	III	IV
Casein activity	(A)	138	162	20	80
ester "	(B)	113	137	15	60
F = A/B		1.22	1.18	1.33	1.33

This is taken as an evidence for the identity of the esterase and the protease, because it is estimated that the variations in F do not significantly exceed the random errors.

The esterase activity plotted against pH, using methyl propionate as sub trate and a water extract of the crude enzyme as source of enzyme was investigated (Fig. 1). The optimal pH value is approximately 8.5, and the enzyme hereby shows

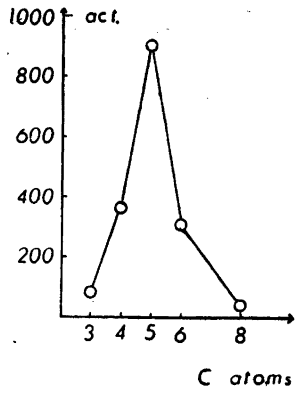

Fig. 2. Relationship between activity and chainlength (number of C-atoms) in the acid radical.

resemblance to esterases isolated from other materials as investigated by Purr [7] and others; they all find an optimum at pH 8.0–8.5.

The activity toward a few different methyl esters of the lower fatty acids was also investigated (Fig. 2).

The distict maximum found at methyl valerate is also shown by a liver esterase described by Schulte [8].

Investigations dealing with the kinetics of the ester hydrolysis are in progress in this laboratory.

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A Preliminary Note on the Electrophoretic Purification of Alkaline Phosphatase

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The application of electrophoresis in separation of enzymes has as yet been limited, due in particular to the laboriousness and the low yield of preparative experiments in the Tiselius apparatus. The development of a method (Carlson 1954 [1]) for electrophoresis in starch medium, where extraction of the proteins out of the medium is avoided, seemed to offer new possibilities for the use of electrophoresis in isolation of enzymes.

The electrophoresis is carried out in a vertical starch column and after the electrophoresis the separated components are forced out of the column by buffer flow and collected in fractions of suitable volume. The fractions are then available for analysis of *e. g.* protein concentration and enzyme activity.

The phosphatase preparation * used in this study was the 40 %–60 % acetone concentration fraction of a beef-kidney homogenate [2].

150 mg of this preparation was extracted with ammonium buffer of pH 9.0 and ionic strength 0.1 and the insoluble material removed by centrifugation. This extract was then electrophoretically separated in the same buffer during a period of 18 hours.

The protein concentration of the fractions was determined according to the modified biuret reaction of Goa [3]. The phosphatase activity determinations were carried out at pH 9.0 in 0.02 *M* Na-β-glycerophosphate with addition of $MgSO_4$ and phenylalanine to a final concentration of 0.01 *M* each.

The result is given in Fig. 1. As is evident, the phosphatase activity is localized to one of the slowest moving protein components holding 32 % of the protein in the preparation. Theoretically this would give a threefold enrichment of the enzyme. The

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