ESTERASE ACTIVITY OF THE BERRIES OF WITHANIA COAGULANS DUNAL

Part I.—Kinetic and Inhibition Studies of Esterase I

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The pulp of the berries of *Withania coagulans* contains an esterase enzyme (esterase 1), the optimum pH of which is 8.5. Its Michaelis and Menten constant is 8.5×10^{-5} M for *p*-nitrophenyl acetate. Inhibition experiments show that the esterase I most probably contains SH groups as an integral part of the active centre of the enzyme.

The berries of *Withania coagulans* Dunal, which is commonly found in Khyber and Kurram agencies and also in certain parts of the former provinces of Panjab and Sind, are used by the locals for making cheese. Quite a few papers[1–4] have been published on the vegetable rennet from this plant and it has been reported that Cheddar cheese, made by employing crude enzyme preparation, develops bitter taste during ripening. While improving the quality of the rennet to make it industrially useful it was observed by the present authors that the berries contained some esterase enzyme system which showed *p*-nitrophenyl esterase activity. This system appears to have at least two such enzymes—one of which can be extracted at pH 5.0 and the other at pH 10.0. During the present study they have been designated as esterase I and esterase II, respectively. In the present communication only the properties of esterase I have been described. Esterase II being a different enzyme system, has been studied separately and would be dealt with in a later communication. To the authors' knowledge no such study has so far been reported in the literature.

Materials and Methods

Extraction of Esterase I.—Air-dried berries of *Withania coagulans* Dunal were purchased from the local market. The outer husk was manually removed and the pulp was ground to a coarse powder. The degree and nature of peptization of the pulp was determined from the protein content and esterase activity of the supernatant obtained by soaking the pulp (5.0 g) in distilled water (50 ml) for ½ hr, then adjusting the pH from 2.0 to 11.0, allowing it to remain at room temperature for 24 hr and centrifuging. Preliminary experiments on the extraction of the pulp with distilled water at various pH values revealed that there were at least two esterases—one extracted at pH 5.0 (esterase I) and the other extracted at pH 10 (Fig. 1). For the succeeding experiments the esterase I was similarly extracted with 0.01M sodium acetate buffer pH 5.0 and dialysed against the same buffer for 24 hr. The enzymic activity of the extract remained practically unchanged even after dialysis.

Substrate.—*p*-Nitrophenyl acetate was synthesized from *p*-nitrophenol by the method of Spasov.[5] The purity of the substrate was established by its m.p., 77–78°C, and its ester carbonyl band at 1760 cm^{-1} (Nujol). The solution of *p*-nitrophenyl acetate (1μM) was prepared according to the method of Huggins and Lapides.[6]

Measurement of Esterase Activity.—The *p*-nitrophenyl esterase activity of esterase I was determined by the method of Huggins and Lapides.[6]

Determination of the Nitrogen Content.—The nitrogen content of fractions concerned was determined by the method of Lowry, Rosenbrough, Farr and Randall.[7]

Inhibitors.—Metal ions and other potential inhibitors used here were, as far as possible, of ANALAR quality either of Merck (Darmstadt) or B.D.H. (England).

Results

Effect of Varying Enzyme Concentration.—Enzyme concentrations of 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 ml were employed. The volume of the reaction mixture was kept constant (10 ml) by adding suitable amounts of distilled water. The experiments were carried exactly for 20 min at 37°C and pH 7.0. A zero order reaction was observed throughout this range of concentrations (Fig. 2).

Effect of Different Incubation Periods.—One ml aliquots were removed from the incubating reaction mixture at 10 min intervals up to 1 hr and the amounts of *p*-nitrophenol liberated were measured spectroscopically. As shown in Fig. 3, the data reveals that the reaction is essentially of zero order for the first 30 min. The results beyond 40 min indicate a shift to first order reaction.

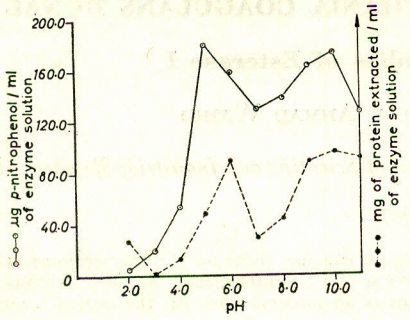

Fig. 1.—Esterase activity of the extract from berries of *w. coagulans* at different pH.

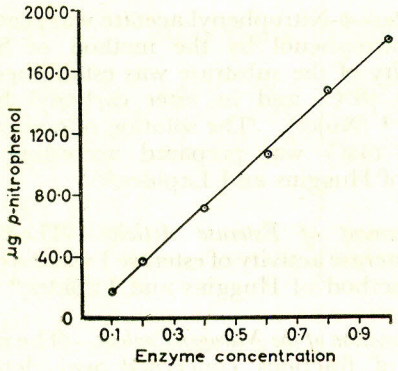

Fig. 2.—Effect of varying enzyme concentration.

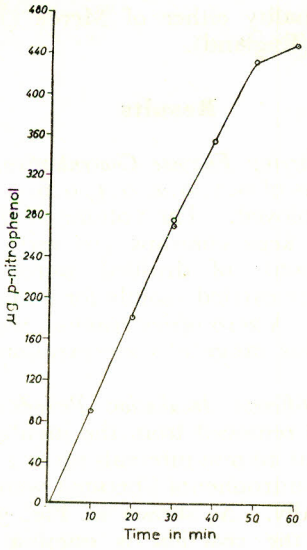

Fig. 3.—Effect of varying incubation timings.

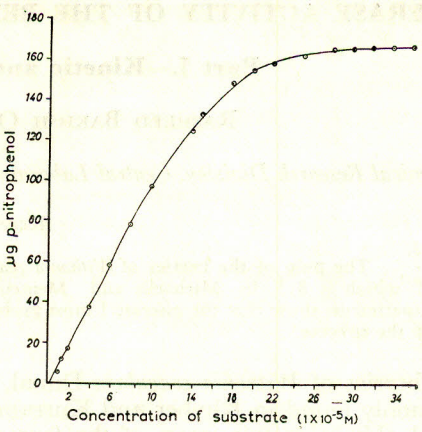

Fig. 4.—The effect of substrate concentration on rate of enzymic hydrolysis of *p*-nitrophenyl acetate.

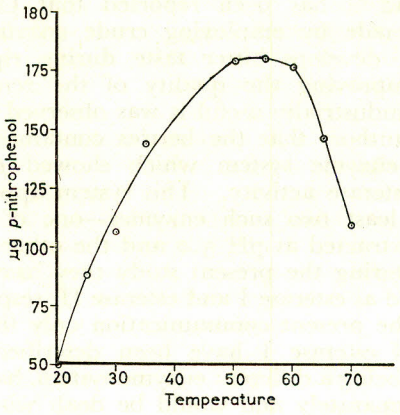

Fig. 5.—Effect of temperature on *p*-nitrophenyl esterase activity of esterase I.

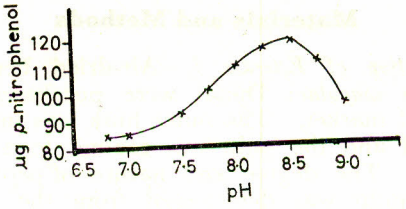

Fig. 6.—The effect of pH on the rate of hydrolysis of *p*-nitrophenol acetate .

Substrate Concentration and Velocity of Enzyme Action.—Various concentrations of *p*-nitrophenyl acetate, ranging from 0.1×10^{-4} to 3.4×10^{-4}M final concentration were used. The results shown in Fig. 4 reveal that with substrate concentration up to about 1.25×10^{-4}M the reaction was of zero order, i.e. linear response to increased substrate concentration, but beyond this value a first order

reaction resulted. The value of Michaelis constant Km was found to be 8.5×10^{-5}M.[6]

p-Nitrophenyl acetate underwent hydrolysis in 0.01 M phosphate buffer even at pH 7 at 25°C. Therefore, inconveniently large amounts of colour were produced non-enzymically when sufficiently high substrate concentrations was used to keep the enzyme saturated. For reducing the colour blank much lower concentrations of substrate were used than were required to saturate the enzyme, and experimental conditions were therefore determined under which a linear relationship resulted between enzyme concentration and the *p*-nitrophenol liberated.

Effect of Temperature on p-Nitrophenyl Esterase Activity.—The optimum temperature of *p*-nitrophenyl esterase activity of esterase I was found to be 55°C; at 37°C the enzyme activity is 82% of the optimum (Fig. 5). The temperature coefficient was also determined, A_{10}=velocity at $T°+10$/ velocity at $T°$. Between 27–37°C, Q_{10} was 1.15 for *p*-nitrophenyl acetate.

Effect of pH on p-Nitrophenyl Esterase Activity.—0.01M barbitone hydrochloric buffer solutions of pH 6.8–9.0 were prepared. The experimental system consisted of 1 ml buffer and 0.5 ml enzyme solution, while in control tubes heat-denatured enzyme solution was used in place of the active enzyme. After a preincubation period of 5 min, 2 ml substrate was added at 1-min intervals to both sets, and after exactly 20 min, 6.5 ml 0.01M phosphate buffer at pH 7.0 was added to bring the system to neutral pH and reaction tubes were read at 400mμ in colorimeter. All the pH values of the reaction mixtures were determined potentiometrically at the end of the reaction and the final pH of these solutions differed from 6.91 to 7.15; the free *p*-nitrophenol was read from calibration curve made at pH 7.0. The plot of the activity of *p*-nitrophenyl esterase against pH showed that the optimum activity was at pH 8.5 (Fig. 6). When the system was buffered at pH 7.0 as in the present case, the enzyme activity was about 70% of its maximum. The instability of the substrate at pH 8.5 precluded its use in routine analysis at the optimum pH for enzyme.

Inhibition.—The effect of metal ions and some other potential inhibitors on the *p*-nitrophenyl esterase activity of the enzyme was studied by the following method:

Enzyme solution (1 ml) and inhibitor solution (1 ml)were mixed and the mixture was incubated for ½ hr at 37°C. The 0.01M phosphate buffer, pH 7.0 (7 ml) and 1 ml *p*-nitrophenyl acetate solution (1μM) were added. Controls were also run, one without inhibitor and the other as control in which 1 ml of enzyme was substituted by 1 ml of heat-inactivated enzyme. The final concentration of inhibitor in each case is given in Table 1. The esterase activity was determined in the usual way after 40-min incubation using Spino/Beckman spectrophotometer.

Table 1 shows that the enzyme is completely inhibited by certain metal ions like Al^{+++}, Zn^{++} and Cu^{++}, and also to a great extent by Hg^{++}. The activity of the enzyme is also lost by di-iodate and fluoride ions. Again esterase I is totally inhibited by cystine and oxidised glutathione, and practically so by *p*-chloromercuribenzoate, EDTA and Tris.

Discussion

The present study shows that the berries of *W. coagulans* contain an esterase enzyme extractable at pH 5.0 designated here as esterase I. Its kinetic study revealed that the Michaelis and Menten constant of the enzyme for *p*-nitrophenyl acetate is 8.5×10^{-5}M while its optimum pH and temperature are 8.5 and 55°C, respectively. Since the substrate is not very stable under these optimum conditions, all the experiments were made at 37°C and at pH 7.0. It is clear from Fig. 5 that the enzymatic activity begins to decrease sharply at 60°C.

TABLE 1.—INFLUENCE OF METAL IONS AND OTHER POTENTIAL INHIBITORS ON THE ESTERASE ACITVITY OF ESTERASE 1.

Inhibitor	Final concentration of inhibitor (M)	% inhibition of Enzyme activity
Aluminium chloride	1×10^{-3}	100
Calcium chloride	1×10^{-3}	42
Cobalt sulphate	1×10^{-3}	62
Cadmium sulphate ..	1×10^{-3}	72
Copper sulphate	1×10^{-3}	96
Magnesium sulphate ..	1×10^{-3}	48
Manganese sulphate ..	1×10^{-3}	36
Mercuric chloride ..	1×10^{-4}	75
Sodium chloride	1×10^{-3}	6
Sodium fluoride	1×10^{-3}	96
Sodium hydrogen diiodate ..	2×10^{-3}	98
Zinc sulphate	1×10^{-3}	98
Chloroacetate	1×10^{-3}	45
p-Chloromeruribenzoate ..	1×10^{-4}	95
Cystine	1×10^{-3}	100
EDTA	1×10^{-3}	90
Glutathione (oxidised) ..	1×10^{-3}	100
Pyridine	1×10^{-3}	9
Tris	1×10^{-3}	90

It is interesting to note (Table 1) that *p*-chloromercuribenzoate inhibits the *p*-nitiophenyl esterase activity of the enzyme. This shows that esterase I most probably contains essential thiol groups and these groups constitute an integral part of the active centre of the enzyme.[8] The fact that chloroacetate reduces the activity of the enzyme to half also supports this idea. This view is further strengthened by the fact that the esterase activity is totally stopped by cystine and oxidised glutathione. Al^{+++}, Zn^{++}, Cu^{++} and Hg^{++} are inactivating ions for the enzyme. The activity is also mainly lost by di-iodate, fluoride, EDTA and Tris.

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