MODIFICATION OF TYROSINE RESIDUES OF TOMATO PECTIN ESTERASE

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Selective acetylation of tyrosine residues in tomato pectin esterase with an 80 molar excess of N-acetylimidazole reduced the activity of the enzyme to 50%. Deacetylation with hydroxylamine restored the original activity of the enzyme. The difference in absorptivities at 278 nm showed that 2 tyrosine residues of the enzyme had been acetylated. Spectrophotometric pH-titration of native pectin esterase revealed that 2 of the 12 tyrosine residues in the enzyme molecule were ionized at pH 9·3–9·5, and the remaining 10 residues at pH > 10·5. Acetylation of the pectin esterase with acetahydride resulted in an irreversible inhibition of the enzyme.

Nitration of the enzyme with tetranitromethane also suggested a role of the tyrosine residues in the catalytic function of the enzyme. However, 10 min after the start of the nitration precipitation set in.

Tomato pectin esterase (EC 3.1.1.11) is sensitive to inhibition by iodine, and the extent of this inhibition increases with purity of the enzyme. On the basis of these studies it was assumed that iodination affected tyrosine or histidine residues. Any role of SH-groups in the catalytic function of the enzyme was however, excluded[1].

The present paper describes the results of modification of the tyrosine residues of tomato pectin esterase, effected by acetylation with acetanhydride or N-acetylimidazole, and by nitration with tetranitromethane. Spectrophotometric pH-titration of the native enzyme is also described.

EXPERIMENTAL

Preparation of the enzyme. One of the five multiple forms of the pectin esterase, present in tomatoes in the greatest quantity[2], was prepared in our laboratory by a procedure previously described[3], involving extraction, precipitation with ammonium sulphate, column chromatography on DEAE-Sephadex A-50, Sephadex G-75 and CM-Sephadex C-50. The molecular weight of the employed form of pectin esterase, containing 12 tyrosine residues, is close to 27 000 (refs[2,4]).

Determination of activity of the enzyme. The activity was determined, using purified citrus pectin (Genu Pectin, Københavns Pektin-fabrik, Denmark) as substrate, by continuous titration in an autotitrating equipment PHM 29/TTT 1 (Radiometer, Copenhagen, Denmark) under nitrogen in a thermostaticaly controlled vessel at 30°C and pH 7·0.

Acetylation of pectin esterase. Acetylation with acetanhydride[5] was conducted in a 1M acetate buffer of pH 5·8, the concentration of acetanhydride being $6 \cdot 10^{-2} - 1 \cdot 10^{-1}$ mol l^{-1} per $3 \cdot 10^{-6}$ mol l^{-1} pectin esterase.

Acetylation with N-acetylimidazole[6] (Fluka, Switzerland), recrystallized from benzene and kept *in vacuo* over P_2O_5, was carried out at 25 °C in Tris-0·01M-HCl buffer of pH 7·5. The concentration used was $0{\cdot}2 \cdot 10^{-4} - 1{\cdot}6 \cdot 10^{-4}$ mol l^{-1} N-acetylimidazole per $2 \cdot 10^{-6}$ mol l^{-1} pectin esterase, which is 10 to 80 mol of the agent per mol of the enzyme.

Deacetylation was effected with hydroxylamine hydrochloride *puriss*, A. R. (Fluka, Switzerland) in a final concentration of 0·7 mol l^{-1} at pH 7·5 and 25 °C for 60 min. The excess of the agent was removed by dialysis through membranes (Kalle, Aktienges., Wiesbaden-Biebrich, F.R.G.) against a 500-fold volume of 0·05M-Tris-HCl buffer pH 7·5, exchanged three times, for 12 h at 4°C.

Nitration of pectin esterase. Nitration[7] was effected with redistilled tetranitromethane (Fluka, Switzerland), 0·85 mol l^{-1} in absolute ethanol. The agent was added to the enzyme dissolved in 0·1M-Tris-HCl, pH 8·0. Ten to 50-fold molar excess of the agent was employed. The concentration of ethanol did not exceed 5% and did not affect the activity of the pectin esterase. After 60 min the reaction mixture was passed through a column of Sephadex G-25 Fine (1 × 10 cm) equilibrated with 0·05M ammonium hydrogen carbonate (to remove the nitrated enzyme from the unreacted agent).

Spectrophotometric measurements were recorded with a spectrophotometer Unicam SP 1800. The number of acetylated tyrosine residues was calculated from the relation $\Delta\varepsilon_{278} \times M/1\,160 \times$ $\times\, c$, where $\Delta\varepsilon_{278}$ is the difference between the absorbances of the acetylated and the deacetylated samples, M the molecular weight of the enzyme, c the concentration of the enzyme (mg/ml) and 1 160 is the molar absorptivity of O-acetyltyrosine[6].

Spectrometric titration of the native pectin esterase was conducted in buffers, Tris-HCl, glycine — NaOH, lysine — NaOH and in NaOH (with ionic strength brought to 1·1—1·14 μ with KCl) of pH 8—13, the enzyme concentration being 1 mg/ml. The change in absorptivity at 295 mm associated with ionization of one tyrosine residue was calculated from the relation $2\,540M^{-1} \times$ $\times$ cm^{-1} (ref.[8]), where M is the molecular mass of the enzyme.

RESULTS AND DISCUSSION

In the exposure to $6 \cdot 10^{-2}$ mol l^{-1} acetanhydride, the activity of pectin esterase ($3 \cdot 10^{-6}$ mol l^{-1}) decreased by 50% in 120 min. At an acetanhydride concentration of 10^{-1} mol l^{-1} the activity of the enzyme was reduced to 20% (Fig. 1). Partial deacetylation with hydroxylamine was achieved so long as the concentration of acetanhydride was under $8 \cdot 10^{-2}$ mol l^{-1}; at higher concentration the inhibition was irreversible. The acetylation with acetanhydride is assumed to involve amino groups and to bring about partial denaturation of the protein[6].

N-acetylimidazole proved to be a milder and more selective acetylation agent than acetanhydride in the reaction with pectin esterase as well as other enzymes[6]. To reduce the enzyme activity to 50% the sufficient concentration of N-acetylimidazole was $1{\cdot}6 \cdot 10^{-4}$ mol l^{-1} (for $2 \cdot 10^{-6}$ mol l^{-1} pectin esterase). With this agent in a concentration range of $0{\cdot}2 \cdot 10^{-4}$ to $1{\cdot}6 \cdot 10^{-4}$ mol l^{-1}, practically complete reactivation of the enzyme was attained (Fig. 2). The deacetylation was followed optically

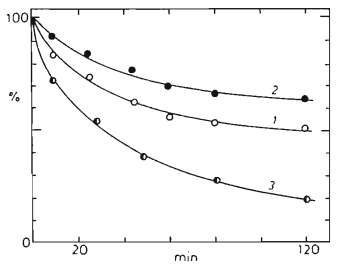

FIG. 1

The action of acetanhydride on pectin esterase. The enzyme, $3 . 10^{-6}$ mol l^{-1}, was in 1M acetate buffer, pH 5·8; *1* acetanhydride, $6 . 10^{-2}$ mol l^{-1}; 2 acetahydride, $6 . 10^{-2}$ mol l^{-1}, and deacetylation with 0·7M hydroxylamine, pH 7·5; *3* acetanhydride, 10^{-1} mol l^{-1}. The axis of abscissas gives the relative enzymic activity in %

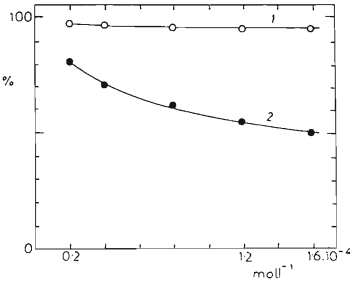

FIG. 2

Activity of pectin esterase in relation to concentration of N-acetylimidazole. The enzyme, $2 . 10^{-6}$ mol l^{-1}, was in 0·01M Tris, pH 7·5. *1* 60 min acetylation; *2* deacetylation with hydroxylamine. The axis of ordinates gives the concentration of N-acetylimidazole, the axis of abscissas the relative enzymic activity in %

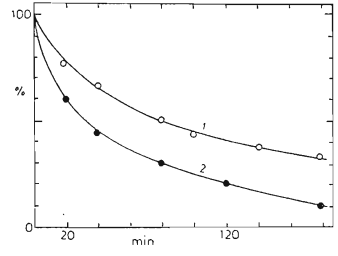

FIG. 3

The action of tetranitromethane on pectin esterase. *1* A ten-fold molar excess of the agent; 2 a 50-fold molar excess of the agent (in 0·1M Tris, pH 8·0). The axis of abscissas gives the relative enzymic activity in %

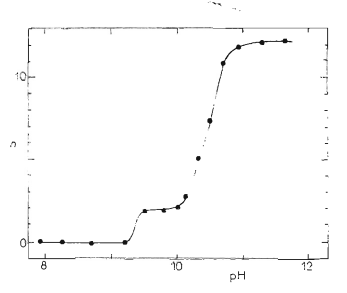

FIG. 4

Spectrophotometric pH titration of tyrosine residues in native pectin esterase at 295 nm; n denotes the number of ionized tyrosine residues

by the change of absorbance at 278 nm. The difference in absorptivity showed that 2 tyrosine residues were acetylated if an 80-fold molar excess of N-acetylimidazole was used. Under these conditions the enzyme activity decreased to 50%, but the action of hydroxylamine reactivated the enzyme fully.

The action of tetranitromethane on pectin esterase also indicates a role of the tyrosine residues in the catalytic function of the enzyme A 10-fold molar excess reduced the enzymic activity to 55% in 60 min and to 30% in 180 min. A 50-fold molar excess reduced it to 20% in 180 min (Fig. 3). The action of tetranitromethane on the enzyme was accompanied by visible precipitation in the course of 10–20 min. This circumstance prevented us from determining the degree of the nitration. The same phenomenon was observed in nitration of other proteins with tetranitromethane. The precipitation of collagen and polymerization of γ-globulins were described[9]. The precipitation accompanying the nitration of trypsinogen and trypsin was ascribed to the formation of cross-links[10]. Also in dialysis of nitrated L-maleate oxidoreductase from pigeon liver the formation of an insoluble material was probably due to cross-linking[11].

The reactivity of tyrosine residues in relation to conformation of the protein was followed by spectrophotometric pH titration. The course of the titration curve (Fig. 4) showed that out of the 12 tyrosine residues in a molecule of tomato pectin esterase[12], two were ionized at pH 9·3 to 9·5 and represented the accessible tyrosine residues, whereas the remaining 10 masked residues[6] were ionized at pH 10·5. This results is consistent with the previous results obtained in iodination of tomato pectin esterase, where one molecule of iodine was involved in the interaction with the active site[1]. It is also in full accordance with the modification of tyrosine residues by N-acetylimidazole, in which two tyrosine residues of the pectin esterase seemed to be acetylated.

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