IMMOBILIZATION OF PECTIN ESTERASE FROM TOMATOES AND *Aspergillus foetidus* ON VARIOUS SUPPORTS

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The pectin esterases from tomatoes and *Aspergillus foetidus* were immobilized by covalent attachment to CNBr-activated Sepharose 4B and by adsorption to polyethylene terephthalate and the properties of the immobilized enzymes were compared. The relative activity of tomato pectin esterase after the immobilization to both supports was almost 7%, whereas the activity of *A. foetidus* pectin esterase covalently immobilized on CNBr-activated Sepharose 4B was close to 11·5% and a value of almost 23% was measured with the enzyme immobilized by adsorption to polyethylene terephthalate. The pH-optima of both pectin esterases were unchanged after their immobilization, their temperature stability and temperature optimum of activity, however, significantly increased. The differences in the action of free and immobilized pectin esterases were also observed when the final esterification degree of the substrate was compared: the immobilized enzymes, unlike the free pectin esterases, did not act on pectin showing a higher esterification degree. An increase in $K_{m.app}$ which was 5-fold for the tomato pectin esterase and 4—7-fold for the *A. foetidus* pectin esterase was observed after the immobilization.

The immobilization of both pectin esterases on Enzacryl AA which had been activated by diazotization resulted in complete loss of activity; this indicates the role of the residues of tyrosine (and histidine, resp.) in the catalytic action of these enzymes, which has been observed in earlier experiments.

The degradation of pectins is a multienzyme process requiring the action of pectin esterase (EC 3.1.1.11) and polygalacturonase (EC 3.1.1.15); the factor determining the depolymerization rate is the deesterification of pectin. The favorable results which have been obtained with other immobilized enzymes stimulated efforts to use also pectic enzymes in immobilized form. Mixed preparations of pectic enzymes[1-3], of polygalacturonase[4-9], of pectin lyase[10,11], and of pectin esterase[12,13] have been immobilized on various supports.

Pectin esterase from tomatoes (a crude preparation) has been immobilized on porous glass *via* the benzoyl azide groups of the support[12]. The relative activity of the immobilized pectine sterase was below 4%, $K_{m.app}$ increased 5 times and an additional pH-optimum of the enzyme in the acidic region was observed. Likewise, when fungal pectine sterase from *Aspergillus* sp. (a product of ROHM, FRG) was immobilized on a polyisonitrile derivative of nylon *via* the C-terminal group of the enzyme[13]

a marked increase of $K_{m,app}$ was observed; the relative activity of the enzyme was close to 10% and a slight shift of the pH-optimum toward the acidic region was also observed.

This paper reports on the immobilization of pectin esterases from tomatoes and *A. foetidus* on CNBr-activated Sepharose 4B, on diazotized Enzacryl AA, and on polyethylene terephthalate (Sorsilen) and on the comparison of the properties of the immobilized preparations.

EXPERIMENTAL

Preparation of Enzymes

Tomato pectin esterase was prepared by the procedure described earlier[14] using extraction of ripe tomatoes, salting-out with ammonium sulphate and chromatography on DEAE-Sephadex A-50, Sephadex G-75, and CM-Sephadex C-50. The preparations used for immobilization were the product after chromatography on Sephadex G-75, showing a specific activity of 6·6 mol s^{-1} . kg^{-1} and the isolated form of pectin esterase after chromatography on CM-Sephadex C-50, showing a specific activity of 10·83 mol s^{-1} kg^{-1}.

The *A. foetidus* pectin esterase of specific activity 0·36 mol s^{-1} kg^{-1} was prepared from Pektofoetidin, a commercial preparation (USSR), by salting-out with ammonium sulphate and chromatography on Sephadex G-50, G-75, DEAE-Sephadex A-50, and SE-Sephadex C-50 (ref.[15,16]).

Assay of Enzyme Activity

The activity of pectin esterase was determined by a modification of the method of continuous titration[17] of enzymatically liberated carboxyl groups from pectin using 0·01 mol l^{-1} NaOH in an autotitrator set with pH-stat (Radiometer Copenhagen, Denmark). The substrate was 1% citrus pectin and the assay was carried out at pH 7·0 (with tomato pectin esterase) or at pH 4·6 (with *A. foetidus* pectin esterase) and at a temperature of 30°C.

Purified citrus pectin (polyuronide content 88·2%, degree of esterification (d.e.) 65%, limit viscosity number $[\eta] = 350$ ml g^{-1}), was prepared from a commercial product of Genu Pektin Rapid Set (Københavns Pektinfabrik, Denmark) by ethanolic precipitation (in 60 and 90% ethanol[18]). The low molecular weight components and the neutral saccharides were separated from unpurified pectin by precipitation with 60% ethanol acidified by HCl (0·6 mol l^{-1} HCl in 60% ethanol).

The specific activity is expressed in mol of ester groups hydrolyzed in 1 kg of protein in 1 s. The relative activity is the ratio of the activity of the immobilized enzyme to the activity of an identical quantity of the free enzyme, expressed in per cent. The kinetic constants of free and immobilized pectin esterases were calculated from the initial reaction rates at various substrate concentrations and were evaluated by the polynome program[19,20].

The enzyme activity of tomato pectin esterase as a function of pH was examined over the pH-range 3·5—8·5 in 0·1 mol l^{-1} acetate and phosphate buffers; pectin esterase from *A. foetidus* was examined at pH 3·7—5·6 in 0·1 mol l^{-1} acetate buffer. The temperature stability was determined from the activity values (at the pH-optimum and 30°C) after 20 min incubation of the enzymes at various temperatures.

Immobilization of Pectin Esterases

Three different supports were used for the immobilization of pectin esterase from tomatoes and *A. foetidus*: *1)* CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) was treated according to the producer's instructions: 10 g of the support was washed with 2 000 ml of 0·001 mol l^{-1} HCl on a glass filter (S-3) and subsequently suspended in 100 ml of 0·1 mol l^{-1} sodium carbonate buffer, pH 8·1, in 0·5 mol l^{-1} NaCl. This gel was used for the immobilization of pectin esterases in carbonate buffer at pH 8·1. The enzyme (5—20 mg), dissolved in 6 ml of the buffer, was stirred with 1 g of the support (corresponding to 3·5 ml of the swollen gel) for 16 h at 4°C. The gel with the immobilized enzyme was washed with the proper buffers (0·1 mol. l^{-1} phosphate was used for tomato pectin esterase and 0·1 mol l^{-1} acetate for the *A. foetidus* pectin esterase). The protein content of the filtrate was assayed by absorbance measurement at 280 nm and the enzyme activity of the three washing solutions was determined.

The remaining active groups of the support were blocked by treatment of the gel with an excess of 1 mol l^{-1} ethanolamine for 2 h. The residual product was washed in turns with 0·1 mol l^{-1} acetate buffer at pH 4·6 and with 0·1 mol l^{-1} borate buffer at pH 8·5; both buffers were made in 1 mol l^{-1} NaCl. The gel with the immobilized *A. foetidus* pectin esterase was washed several times with 0·1 mol l^{-1} acetate buffer, pH 4·6, and was stored in the same solution at 4°C. Immobilized tomato pectin esterase was treated with 0·1 mol l^{-1} phosphate buffer, pH 7·5, under identical conditions. *2)* Polyethylene terephthalate (Sorsilen), a spherical bead support of pore size 2—3 cm^3 g^{-1} and specific surface 80—100 m^2 g^{-1}, was prepared in the Department of Polymers, Prague Institute of Chemical Technology, according to Kubánek and coworkers[21,22]. The support, freed of nonsedimenting fines by repeated decantation in water, was subsequently dehydrated in acetone and dried at room temperature. Before immobilization of the enzyme 1 g of the support was suspended in 20 ml of 0·1 mol l^{-1} acetate buffer, pH 4·5 and 7—12 mg of *A. foetidus* pectin esterase was added. Tomato pectin esterase was immobilized on 1 g of the support suspended in 20 ml of phosphate buffer, pH 7·5. The quantity of immobilized enzyme was assayed by determination of enzymatic activity of supernatants after washing of the immobilized product. The immobilized enzymes were stored at 4°C. *3)* Enzacryl AA (Koch-Light, Ltd., England) was a polyacrylamide based support whose aromatic amino groups were activated by diazotization according to the manufacturer's instructions: 100 mg of Enzacryl AA was weighed in a polyethylene centrifugation tube and mixed by a magnetic stirrer with 10 ml of 2 mol. l^{-1} HCl at 0°C. A 4% solution of $NaNO_2$ (4 ml) at 0°C was added and the stirring was continued for 15 min. The diazo-Enzacryl AA was washed 4 times with 0·2 mol l^{-1} phosphate buffer, pH 7·5. The supernatant was decanted off and 1 mg of tomato pectin esterase or 3 mg of *A. foetidus* pectin esterase dissolved in 1 ml of 0·2 mol l^{-1} phosphate buffer, pH 7·5 was added. The stirring of the suspension was continued for 48 h at 4°C. After immobilization of the enzyme the suspension was treated with 3 ml of 0·01% phenol in 10% sodium acetate (4°C); after 15 min of stirring the conjugate was washed with 0·1 mol l^{-1} phosphate buffer (3-times) and the enzymatic activity of the washings was determined. Tomato pectin esterase was washed with 0·1 mol l^{-1} phosphate buffer, pH 7·5, and stored in the same solution; 0·1 mol l^{-1} sodium acetate, pH 4·5, was used for *A. foetidus* pectin esterase.

RESULTS AND DISCUSSION

The immobilization of pectin esterase from tomatoes and *A. foetidus* by covalent attachment to CNBr-activated Sepharose yielded rapidly sedimenting gels which could readily be removed from the substrate at the end of the reaction.

The highest relative activity (7·3%) was obtained with immobilized tomato pectin esterase when 8 mg of the isolated form of the enzyme was immobilized on 1 g of the support. The preparation purified by chromatography on Sephadex G-75 yielded after immobilization (10 mg of enzyme per 1 g of support) a product whose relative activity was 4·5%. Weibel and coworkers[12] were able to obtain a covalentlty immobilized tomato pectin esterase showing a relative activity of 3·95%. Pectin esterase from *A. foetidus* immobilized on CNBr-activated Sepharose 4B (15 mg of enzyme per 1 g of support) yielded a product of relative activity 11·5%, a value very close to that obtained with *A. niger* pectin esterase immobilized on nylon[13].

The products obtained by adsorption of tomato pectin esterase to polyethylene terephthalate (Sorsilen) showed approximately the same relative activity as the products immobilized covalently (Table I). Preparations with highest relative activity (7·5%) were obtained when 10 mg of the isolated form of pectin esterase (after chromatography on CM-Sephadex C-50) was immobilized on 1 g of the support. The immobilization of pectin esterase after Sephadex G-75 yielded products with relative activity of 3·5%. Pectin esterase from *A. foetidus* immobilized by adsorption to Sorsilen (10 mg per 1 g support) showed a relative activity of up to 23%, *i.e.* double the activity of the preparation obtained by covalent bonding (Table II).

All preparations of immobilized pectin esterases showed increased temperature optima (Table I, II) and increased temperature stability when compared with the free enzymes (Fig. 1 and 2). The value of $K_{m.app}$ increased 5 – 6 times with immobilized tomato pectin esterase and 4 – 7 times with the enzyme from *A. foetidus* (Table I, II).

TABLE I

Comparison of characteristics of free and immobilized tomato pectin esterase

Values	Free enzyme	Pectin esterase immobilized on	
		CNBr-Sepharose 4B	polyethylene terephthalate
Relative activity (%)	100	7·3	7·5
K_m, $K_{m.app}$ (mol l^{-1} . 10^{-3})	4·1	21·6	20·4
pH-optimum	7·5	7·5	7·5
Temperature optimum (°C)	50	62	61
d.e.[c] of final product (%)	11·3[a]	23·2[a]	24·1[a]
	1·3[b]	10·2[b]	10·0[b]

[a] In presence of 0·15 mol l^{-1} NaCl in autotitrator; [b] in presence of 0·3 mol l^{-1} potassium phosphate and of 0·3 mol l^{-1} KCl; [c] degree of esterification.

TABLE II

Comparison of characteristics of free and immobilized *A. foetidus* pectin esterase

Values	Free enzyme	*A. foetidus* pectin esterase immobilized on	
		CNBr-Sepharose 4B	polyethylene terephthalate
Relative activity (%)	100	11·5	23·1
K_m, $K_{m,app}$ mol l^{-1} . 10^{-3}	11·3	75·3	40·7
pH-optimum	4·8	4·6	4·6
Temperature optimum (°C)	40	50	52
d.e.[c] of final product (%)	25·0[a]	31·2[a]	47·0[a]
	15·2[b]	23·1[b]	32·0[b]

[a] At pH 4·6 in the autotitrator; [b] in 0·2 mol l^{-1} acetate buffer, pH 4·6; [c] degree of esterification.

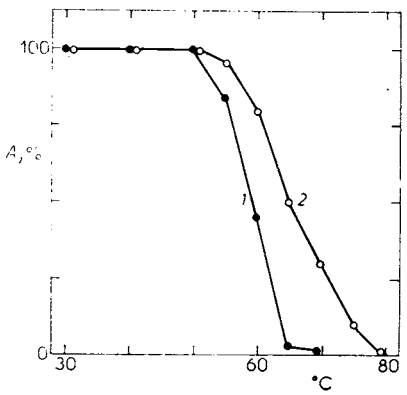

FIG. 1

Temperature stability of tomato pectin esterase. 20 min incubation at pH 7·0; determination of activity at pH 7·0 and 30°C. *1* Free enzyme, *2* enzyme covalently immobilized on CNBr-activated Sepharose 4B; *A* (%): residual activity

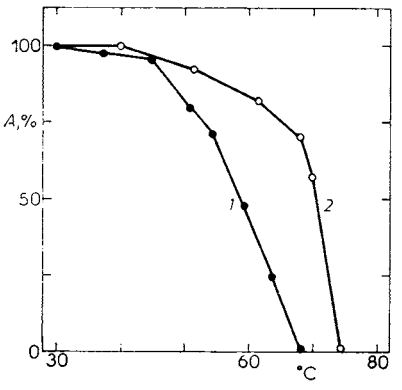

FIG. 2

Temperature stability of *A. foetidus* pectin esterase. 20 min incubation at pH 4·6; determination of activity at pH 4·6 and 30°C. *1* Free enzyme, *2* enzyme immobilized by adsorption to Sorsilen; *A* (%): residual activity

The differences between pectin esterase from tomatoes and *A. foetidus* became apparent when the relative activities of the individual immobilized preparations were compared: the tomato enzyme showed a maximal relative activity of about 7% after immobilization on both types of supports, whereas the enzyme from *A. foetidus* gave products of relative activity of 11% after covalent immobilization and of even 23% after immobilization by adsorption. We observed differences in the degree of esterification (*d.e.*) of the resulting products: free tomato pectin esterase deesterified pectin under appropriate conditions of the reaction[23] to a *d.e.* below 2%, whereas the immobilized form of the enzyme gave products of *d.e.* 10% (Table I). Covalently immobilized pectin esterase from *A. foetidus* gave final products of *d.e.* 35%, the enzyme adsorbed to Sorsilen yielded products of *d.e.* 47%, whereas free pectin esterase from *A. foetidus* deesterified pectin to a *d.e.* of 23% (Table II).

The observed differences reflect the different properties of tomato and *A. foetidus* pectin esterase: the enzymes differ in pH-optima and isoelectric points (tomato pectin esterase – alkaline pH-region, *A. foetidus* pectin esterase – acidic pH-region) in the mode of deesterification of highly esterified pectin[24] as well as in the action on partially esterified oligomers of D-galactopyranuronic acid[25].

The low relative activity of immobilized pectin esterase from tomatoes as well as the lower affinity of this enzyme for the substrate (especially observed with the crude product, *i.e.* 3–5%) can be explained by postulating that pectin, a high molecular weight substrate whose size does not change after the deesterification, has a limited access to the immobilized enzyme because of sterical and diffusion effects. Fungal pectin esterase from *A. foetidus* showed higher relative activities after immobilization and likewise yielded final products with a higher *d.e.*; this seems to indicate differences in the localization of the binding sites of pectin esterases in the complex of the immobilized enzyme.

The immobilization of pectin esterase from tomatoes and *A. foetidus* to diazotized Enzacryl AA, *i.e.* immobilization by preferential binding *via* the tyrosine hydroxyl, yielded preparations with completely attached enzymes yet inactive. This result indicates the role of tyrosine residues in the active site of tomato pectin esterase, a role which has been observed in experiments with the inhibition of the enzyme by iodine[26] and is in accordance with the earlier observed role of "free" tyrosine residues in the catalytic effect of tomato pectin esterase[27]. Pectin esterase from *A. foetidus* was inhibited by the presence of iodine[15]; hence, the complete loss of the activity of the enzyme immobilized on the diazotized support permits us to assume that tyrosine and histidine residues play a role also in the catalytic action of this enzyme.

The results of this study show that fungal pectin esterase from *Aspergillus foetidus* (or pectin esterase produced by other *Aspergilli*) immobilized on Sorsilen is suited for use in enzyme reactors. The pH-optimum of this enzyme lies in the range of pH-optima of polygalacturonases and its relative activity is 3 times higher than the

activity of immobilized tomato pectin esterase whose pH-optimum lies in the alkaline region.

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