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CHROMATOGRAPHIC SEPARATION OF ORANGE PECTINESTERASE ISOENZYMES ON PECTATES WITH DIFFERENT DEGREES OF CROSS-LINKING

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SUMMARY

A selected sodium pectate preparation, suspended in an alkaline mixture of ethanol and water, was cross-linked with epichlorohydrin. By varying the amount of epichlorohydrin in the reaction mixture, preparations were obtained with different degrees of cross-linking. The preparations were used as chromatographic media for the separation and purification of two orange pectinesterase isoenzymes. The binding of the isoenzymes was found to depend on the degrees of cross-linking of the pectate. On pectate with an intermediate degree of cross-linking (0.46) both isoenzymes were retained and subsequently eluted, with a sodium chloride gradient, as separated peaks with a 7- to 8-fold increase in specific activity. Both pectinesterase isoenzymes saponified 93% esterified pectin with a high degree of cross-linking. It is thought that the binding of these enzymes to cross-linked pectate involves biospecific affinity.

INTRODUCTION

Pectinesterase (pectin pectylhydrolase, E.C. 3.1.1.11) occurs in many microorganisms and in a wide variety of plants[1,2]. The enzyme de-esterifies pectin, producing pectate and methanol. Some of the plant enzymes have a great impact on fruit and vegetable technology, because of their potential effects on the quality of the finished products[3]. Multiple forms of pectinesterase have been demonstrated, *e.g.*, in banana[4,5] and tomato[6–8]. Recently we purified two pectinesterase isoenzymes from the orange fruit[9]. These isoenzymes, I and II, have a molecular weight of 36,200 and isoelectric points of 10.0 and $\geqslant$11.0, respectively. Pectate is a competitive inhibitor for both enzymes, but pectinesterase II is much more strongly inhibited than pectinesterase I.

An important step in the purification procedure was the separation of the two isoenzymes on a column of pectate, cross-linked with epichlorohydrin. Pectinesterase I did not bind to this column, but pectinesterase II was retained and could be eluted with a sodium chloride gradient. We found it of interest to explore the effect of the degree of cross-linking of pectate on the separation and purification of

pectinesterase isoenzymes. For this purpose, pectates with different degrees of cross-linking were made from a selected pectate preparation, using different amounts of epichlorohydrin. The pectate was cross-linked as a suspension in a mixture of ethanol, water, sodium hydroxide and epichlorohydrin, a modification of the method of Tibenský and Kuniak[10].

EXPERIMENTAL

Materials

The pectins, pectates and alginates were obtained from commercial suppliers listed in Table I. Epichlorohydrin "zur Synthese" was obtained from E. Merck (Darmstadt, G.F.R.). Pectinesterase was extracted from Navel oranges (*Citrus sinensis* L.) as described by Versteeg *et al.*[9]. After ammonium sulphate precipitation the preparation had a specific activity of 34.1 units of pectinesterase per milligram of protein. Ultrazym 100, a pectinase derived from *Aspergillus niger*, was a gift from Ciba-Geigy (Basle, Switzerland). The pectic enzymes present in this preparation are polygalacturonase, pectinesterase and pectin lyase.

Preparation of pectates

Alkaline de-esterification of pectins was carried out at 4° by suspending 100 g of pectin in 500 ml of 95% ethanol–water (3:1). The mixture was stirred and, at intervals of 1 h, four equal portions of a total volume of 500 ml of 2 *M* sodium hydroxide in ethanol–water were added. Three hours after the last portion had been added, the mixture was filtered over a G3 sintered-glass filter. The pectate was re-suspended in 1 l of 0.5 *M* sodium hydroxide in ethanol–water. After stirring the mixture overnight at 4°, it was washed with ethanol–water until neutral, then with 95% ethanol and dried in air.

Enzymatic saponification of pectin was carried out at room temperature by dissolving 20 g of pectin in 1 l of 0.01 *M* sodium chloride solution. Orange pectinesterase (800 units) was added and the pH was maintained at 8.5 by continuous titration with 1 *M* sodium hydroxide solution. After 24 h the pectate was precipitated at pH 2.5 by adding 1 *M* hydrochloric acid. The pectate gel was homogenized with a mixer (Ultra-Turrax, Janke & Kunkel, Staufen, G.F.R.), washed with ethanol–water until free of chloride ions, then with 95% ethanol, and dried in air.

When enzymatic saponification was followed by alkaline saponification, the pH was increased to 10.0 and maintained at this value for 24 h, by automatic titration.

Cross-linking of polyuronides

Coarse preparations of sodium pectate, pectic acid or sodium alginate were ground in an analytical mill with a 0.7-mm screen (Culatti, Zürich, Switzerland). Amounts of 2–10 g were weighed into 100-ml glass-stoppered erlenmeyer flasks and epichlorohydrin, or a mixture of epichlorohydrin and ethanol, was added. The slurry was homogenized by stirring with a glass rod. Sodium hydroxide solution (5–10 *M*) was added while stirring. Different proportions of polyuronide, epichlorohydrin and sodium hydroxide, with or without ethanol, were used. The reaction mixtures were incubated at 40° on a rotary shaker (200 rpm) for 4 h. The reaction was stopped by neutralization with 1 *M* acetic acid. The reaction mixture was filtered on a medium-

fine (G3) sintered-glass filter, washed with 95% ethanol–water (3:1), then with 95% ethanol and dried in air.

The methyl ester derivative of cross-linked pectate was prepared by treatment of cross-linked pectate with a mixture of concentrated sulphuric acid and absolute methanol, according to Heri *et al.*[11].

Analysis of polyuronides

The anhydrogalacturonic acid content of pectates was determined in an AutoAnalyzer using the carbazole method[12]. Their degree of esterification was determined by the titrimetric method[13]. The molecular weights of pectates and alginates were calculated from intrinsic viscosity values determined with Ubbelohde glass capillary viscosimeters[14].

Analysis of cross-linked products

The solubility was tested by boiling 100 mg of product in 50 ml of 0.1 *M* sodium phosphate buffer (pH 7.0). The degree of swelling of cross-linked polyuronides expressed as millilitres of bed volume per gram of dry cross-linked product, was determined by suspending 0.5 g of product in excess of 0.1 *M* sodium acetate buffer (pH 4.2) and by reading the bed volume after swelling for 24 h in a 10-ml measuring cylinder[15]. The biodegradability of cross-linked pectates was arbitrarily measured as the decrease in bed volume of the product, suspended in 0.1 *M* sodium acetate buffer (pH 4.2), after exhaustive digestion (24 h) with 100 mg of Ultrazym 100 per gram of dry product.

The amount of epichlorohydrin, that had not reacted with pectate was determined in the filtrate after filtering and washing the cross-linked product with ethanol–water on a G3 sintered-glass filter. Following the method of Hamerstrand *et al.*[16], the epichlorohydrin was converted into glycerol with hot alkali. The glycerol was subjected to periodate oxidation to yield formaldehyde, which was determined colorimetrically using acetylacetone[17].

Epichlorohydrin, which had reacted with pectate, could either be bound as monoether side-chains or as diether cross-links. The side-chains, yielding formaldehyde upon alkaline hydrolysis and periodate oxidation[15], were also determined by the methods of Hamerstrand *et al.*[16] and Nash[17]. Corrections (10–20% of the values) had to be made for apparent formaldehyde formation from the pectate itself. The number of cross-links was determined by difference (amount of epichlorohydrin used in the reaction minus epichlorohydrin in filtrate and monoether side-chains). Both the number of cross-links [DS(CL)] and the number of side-chains [DS(SC)] were expressed per anhydrogalacturonic acid unit.

Chromatography and analysis of fractions

Chromatography of orange pectinesterase was carried out on small columns of cross-linked pectate. After loading and washing, elution was effected with a sodium chloride gradient. The concentration of sodium chloride in the eluted fractions was measured with a conductivity meter (Type PR 9500; Philips, Eindhoven, The Netherlands). Protein concentrations were determined by using Folin reagent[18] with bovine serum albumin (A-4378; Sigma, St. Louis, Mo., U.S.A.) as a standard. Pectinesterase activity was assayed according to Versteeg *et al.*[9], by automatic titration of

TABLE I

PROPERTIES OF PECTATES AND ALGINATES USED

Some preparations were obtained commercially, others were prepared by different methods of saponification of commercial pectins.

Product	*Supplier*	*Method of saponification*	*Mol. wt.*	*Anhydrouronic acid content (%)*	*Degree of esterification (%)*
Polygalacturonic acid	ICN Pharmaceuticals (Cleveland, Ohio, U.S.A.)	–	18,400	88.0	<1.0
Citrus pectin NF	Sunkist Growers (Ontario, Calif., U.S.A.)	Alkali	26,200	71.0	4.0
Apple pectin*	Obipectin (Bischofszell, Switzerland)	Alkali	31,400	70.7	0.8
Citrus pectin NF	Sunkist Growers	Pectinesterase	53,000	71.0	7.0
Citrus pectin B	Copenhagen Pectin Factory (Copenhagen, Denmark)	Pectinesterase and alkali	64,500	78.9	5.2
Alginic acid**	Kelco (Chicago, Ill., U.S.A.)	–	12,000	90	0
Sodium alginate***	Kelco	–	114,000	70	0

* Green Ribbon.
** Kelacid.
*** Kelco Gel HV.

carboxyl groups liberated from green ribbon pectin. In this assay, one unit of pectinesterase liberates one micromole of carboxyl groups per minute.

RESULTS

The pectates and alginates used for cross-linking, listed in Table I, cover a wide range of molecular weights. The preparations were cross-linked by two different methods (Table II). In one method, similar to that of Tibenský and Kuniak[10], the preparations were mixed with epichlorohydrin and sodium hydroxide solution to give a dry, crumbly mixture. In the other method, ethanol was added, giving a suspension that could be kept in motion on a rotary shaker. This could not be achieved by adding more water, as the polyuronides would then swell and eventually dissolve. Pectates and the alginate of low molecular weight did not yield insoluble products. Preparations of higher molecular weight gave insoluble products with varying bed volumes. The cross-linked products were degraded to some extent by pectinase. There was not much difference in the results obtained with the two methods. However, the addition of ethanol to the reaction mixture ensured a more homogeneous mixture of reactants and prevented clotting. The preparation with the smallest bed volume and best resistance against enzymatic attack was obtained from pectate (molecular weight 31,400) prepared by heterogeneous alkaline saponification of a commercial pectin.

Cross-linking of sodium pectate under varying reaction conditions

In order to obtain preparations with different degrees of cross-linking, variations were made in the experimental conditions. Sodium pectate (molecular weight 31,400) was used in cross-linking experiments in alcoholic suspension as described in Table II. Variations of the molar ratio of sodium hydroxide to pectate (as monomers) from 0.6 to 3.5 showed little effect on bed volume or biodegradability of the cross-linked product. Also, the time of the reaction (1–24 h at 40°) and the temperature (30–50°, 6 h) were not very critical. However, the ratio of epichlorohydrin to pectate affected the cross-linking considerably (Table III). With increasing molar ratio of epichlorohydrin to pectate (monomers) the bed volume and the biodegradability decreased, whereas the number of diether cross-links and monoether side-chains increased. Apparently, the most efficient way to obtain preparations with varying degrees of cross-linking is by selecting the molar ratio of epichlorohydrin to pectate in the reaction mixture. As part of the sodium hydroxide is consumed by epichlorohydrin, which hydrolyses to glycerol[19], care should be taken that excess of sodium hydroxide is present throughout the reaction period in reaction mixtures with relatively large amounts of epichlorohydrin.

Cross-linking experiments were carried out with some other modifications. Pectate could be cross-linked in alkaline acetone–water mixtures, but experiments with pectate suspended in pyridine, as an organic base, were not successful. Also, cross-linking reactions with water-dissolved pectate did not yield water-insoluble products. No beneficial effect was noticed when sodium borohydride[19] was added to reaction mixtures as described in Table II.

TABLE II

CROSS-LINKING OF PECTATES AND ALGINATES OF VARYING MOLECULAR WEIGHTS IN THE PRESENCE OR ABSENCE OF ETHANOL

Two-gram portions of polyuronide were mixed with 0.75 ml of epichlorohydrin or with 0.75 ml of epichlorohydrin in 6 ml of 95% ethanol. Then 2.0 ml of sodium hydroxide solution (concentration 5 *M* for sodium uronides; 10 *M* for free uronic acids) were added. The reaction mixtures were incubated on a rotary shaker at 40° for 4 h. The molar ratio of uronide (as monomers), epichlorohydrin and sodium hydroxide was *ca.* 1:1:1.

Preparation	*Mol. wt.*	*Cross-linked without ethanol*			*Cross-linked with ethanol*		
		Yield (%)	*Bed volume (ml/g)*	*Bio-degradation (%)*	*Yield (%)*	*Bed volume (ml/g)*	*Bio-degradation (%)*
Pectates	18,400	Soluble*			Soluble		
	26,200	Soluble			Soluble		
	31,400	107	3.0	7	99	4.2	13
	53,000	108	8.8	28	105	7.9	17
	64,000	105	9.0	24	102	8.7	21
Alginates	12,000	Soluble			Soluble		
	114,000	108	4.9		99	6.6	

* Almost completely soluble in boiling 0.1 *M* sodium phosphate (pH 7.0).

TABLE III

CROSS-LINKING OF SODIUM PECTATE WITH VARIOUS AMOUNTS OF EPICHLOROHYDRIN

Five-gram portions of sodium pectate (mol.wt. 31,400) were mixed with 0.50–3.10 ml of epichlorohydrin in 15 ml of 95% ethanol. Then 5.0 ml of 5 *M* sodium hydroxide solution was added. The reaction mixtures were incubated on a rotary shaker at 40° for 4 h. The molar ratio of pectate (as monomers) to sodium hydroxide was 1:1.

*ECH:AGU ratio**	*Yield (%)*	*Bed volume (ml/g)*	*Biodegradation (%)*	*DS(CL)***	*DS(SC)****
0.37	52§	15.4	74	0.18	0.029
0.56	96	6.0	35	0.36	0.030
0.82	97	5.0	20	0.46	0.041
2.34	101	3.6	11	0.57	0.063

* ECH:AGU ratio = molar ratio of epichlorohydrin to anhydrogalacturonide monomers.
** DS(CL) = number of glyceryl diether cross-links per monomer.
*** DS(SC) = number of glyceryl monoether side-chains per monomer.
§ Partly soluble in 0.1 *M* sodium phosphate buffer (pH 7.0).

Chromatographic separation of orange pectinesterase isoenzymes on cross-linked pectates

In a previous paper[9] it was shown that crude orange pectinesterase contains two isoenzymes, which can be separated by chromatography on cross-linked pectate. We have now extended this study by chromatographing crude orange pectinesterase on columns containing pectates with degrees of cross-linking of 0.57, 0.46 and 0.36 (Fig. 1). Because of the instability of pectinesterase I, chromatography of the enzyme had to be carried out at relatively high salt concentrations, or at pH values of 6 and

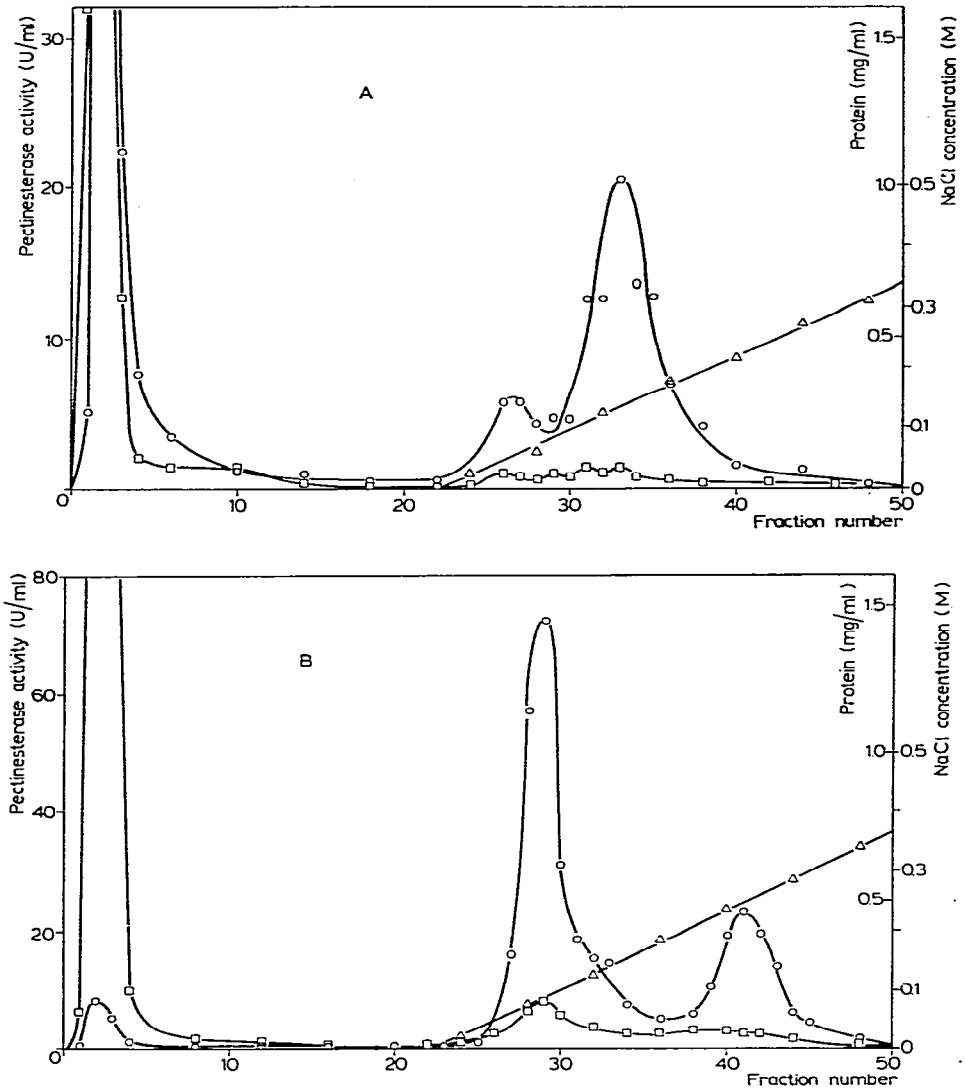

Fig. 1.

(Continued on p. 158)

higher. Fig. 1A confirms our previous results. Separation of the two pectinesterase isoenzymes was achieved on pectate with a degree of cross-linking of 0.57, but pectinesterase I was not bound and passed through the column together with a large amount of non-esterase proteins. A large peak of pectinesterase II eluted with a sodium chloride gradient (fractions 30–42, Fig. 1A). This peak was preceded by a small peak of pectinesterase activity which, according to its pH–activity profile[9], was additional pectinesterase I.

On a pectate column with a lower degree of cross-linking (0.46), both isoenzymes were bound and eluted in the salt gradient as well separated peaks. On a third column of pectate with a degree of cross-linking of only 0.37, both isoenzymes

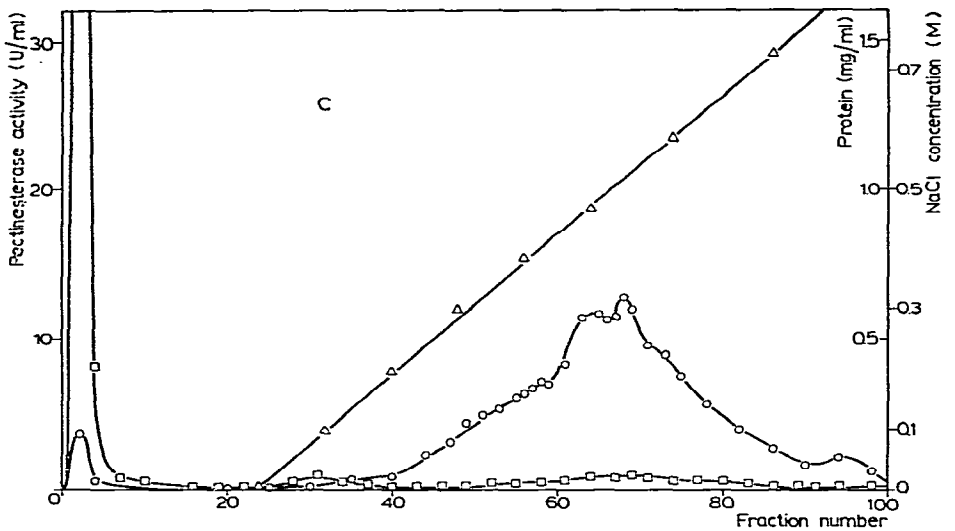

Fig. 1. Chromatography of orange pectinesterase on pectates with different degrees of cross-linking. Crude orange pectinesterase (33 ml, 360 units/ml) was dialysed against two portions of 1 l of 0.02 *M* sodium succinate buffer (pH 6.0). Portions of 11 ml were then applied to (A) a column (8 × 1.7 cm) of 5 g of pectate with a degree of cross-linking of 0.57, (B) a column (11 × 1.7 cm) of 5 g of pectate with a degree of cross-linking of 0.46 and (C) a column (11.2 × 1.85 cm) of 5 g of pectate with a degree of cross-linking of 0.36. Elution was carried out with 0.02 *M* sodium succinate buffer (pH 6.0) up to fraction 20 and then a gradient of sodium chloride in the same buffer. The flow-rate was 20 ml/h and fractions of 11.2 ml were collected. ○, Pectinesterase activity; □, protein; △ sodium chloride concentration.

were bound even more tightly, but did not elute as separate peaks (Fig. 1C). A small amount of pectinesterase (*ca.* 4%) did not bind to the columns (Fig. 1B and C). Upon re-chromatography, this enzyme material was retained on the column mentioned in Fig. 1B and eluted as pectinesterase I.

Obviously, the pectate with a degree of cross-linking of 0.46 was superior to the other two preparations. On a column of this material a separation of the isoenzymes and a purification of both enzymes by a factor of 7–8 was achieved (Table IV). The results with this column were also superior to separations achieved with cation exchangers. At pH 6 the binding of pectinesterase I to carboxymethylagarose was very weak, and elution of the enzyme had already started during washing of the column. On cross-linked alginate, used under the same conditions as described in Fig. 1, most of the pectinesterase was bound and eluted with a salt gradient, but no separation of the isoenzymes took place.

The nature of the binding of pectinesterase to cross-linked pectate was studied by determining enzymatic activity on the methyl ester of the matrix (Fig. 2). It appeared that this product was saponified by both enzymes, albeit much more slowly than the soluble substrate. Apparently, enzyme–substrate complex formation which leads to catalysis is possible, in spite of the cross-links and side-chains. Pectate is a competitive inhibitor of orange pectinesterase isoenzymes. Compared with pectinesterase I, pectinesterase II is much more strongly inhibited[9,20]. Hence it is likely that the binding of these enzymes to cross-linked pectate involves biospecific affinity, in addition to ion-exchange phenomena.

TABLE IV

FRACTIONATION OF ORANGE PECTINESTERASE ON PECTATES WITH DIFFERENT DEGREES OF CROSS-LINKING

Sample	*Volume (ml)*	*Activity (units)*	*Protein (mg)*	*Specific activity (units/mg)*	*Purification factor*	*Yield (%)*
Dialysed enzyme	11	3960	116	34.1	1	100
Pectate with degree of cross-linking of 0.57						
Pectinesterase I (fractions 2 to 7)	67	2508	99	25.3	0.74	63
Pectinesterase II (fractions 30–42)	146	1424	5.4	264	7.7	36
Pectate with degree of cross-linking of 0.46						
Pectinesterase I (fractions 25–36)	134	2837	10.5	270	7.9	72
Pectinesterase II (fractions 37–52)	179	1357	5.7	238	7.0	34
Pectate with degree of cross-linking of 0.36						
Pectinesterase I and II (fractions 40–100)	683	3620	14.5	250	7.3	91

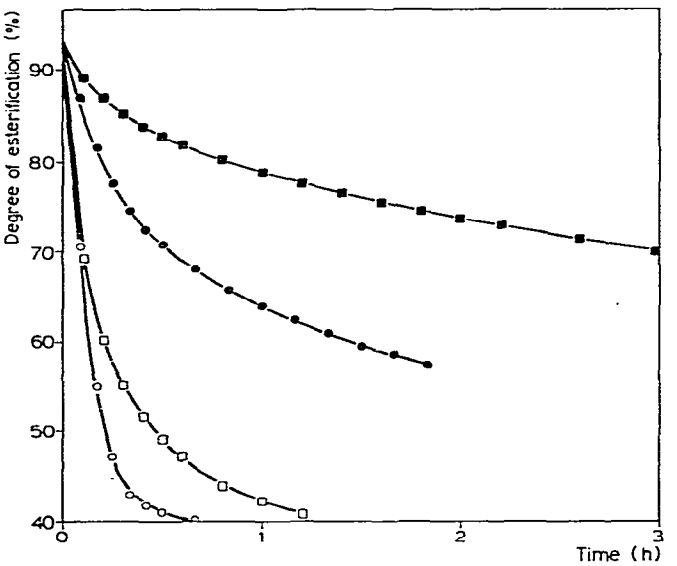

Fig. 2. Activity of pectinesterase isoenzymes on pectin and on cross-linked pectin. Reaction mixtures (20 ml) contained 50 mg of substrate, 2 mmole of sodium chloride and 10 units of pectinesterase. Activity was measured at 30° by continuous titration with 0.02 *M* sodium hydroxide solution. ○, Pectinesterase I on 93% esterified pectin; ●, pectinesterase I on 93% esterified cross-linked pectin with a degree of cross-linking of 0.57; □, pectinesterase II on pectin; ■, pectinesterase II on cross-linked pectin.

DISCUSSION

Cross-linking of pectate with epichlorohydrin

Under the alkaline conditions necessary for the cross-linking of polysaccharides with epichlorohydrin, pectins are extremely rapidly degraded by a *trans*-elimination reaction[21], which is why pectins have to be saponified prior to cross-linking. Saponification can be carried out under alkaline conditions, but at low temperatures (2–4°), where the *trans*-elimination reaction is less important.

In the method for cross-linking of pectate described by Tibenský and Kuniak[10], polyuronide is mixed with a small volume of sodium hydroxide solution and epichlorohydrin. In this system it is difficult to prevent clotting of the pectate, and also epichlorohydrin is poorly water-soluble. By using ethanol or acetone in the reaction mixture, a suspension of only slightly swollen polyuronide granules is obtained and, at the same time, the solubility of epichlorohydrin is increased. In cross-linking of polysaccharides with epichlorohydrin a number of side-reactions occur[19,22]. Addition of ethanol to the reaction mixture creates the possibility of glyceryl mono- and diether formation with ethanol. However, in model experiments, carried out under the same conditions as the cross-linking reactions, we found that this reaction does not take place.

Cross-linking of low-molecular-weight polyuronides was not successful (Table II). The same is true for dextrans, and it can be concluded that, although the reaction is known to have occurred, an increased amount of polysaccharide, is not incorporated in the insoluble part of the network[19]. Some high-molecular-weight pectates gave cross-linked products with very high bed volumes. This property may be determined by the physical state (the low bulk density) of the dry pectate powder. Similarly, the concentration of dextran in gels is known to determine the swelling properties of the cross-linked product[19,22]. Pectates with different degrees of cross-linking are most conveniently obtained by varying the amount of epichlorohydrin in the reaction mixture (Table III). The results shown in this table are in agreement with data obtained by Kohn *et al.*[23].

Chromatography of orange pectinesterase on cross-linked pectates

Rexová-Benková and Tibenský[24] showed that fungal *endo*-polygalacturonase could be selectively purified by affinity chromatography on cross-linked pectate. Preparations with an intermediate degree of cross-linking, which at the same time provided a reasonable binding capacity and were not enzymatically degraded, performed well[23]. In a subsequent study on tomato enzymes, Rexová-Benková *et al.*[8] showed that *endo*-polygalacturonases could be separated from pectinesterases. Starch gel electropherograms indicated five molecular forms of pectinesterase, but they were unable to separate them on cross-linked pectate.

Crude enzyme extracts from orange fruits contain only trace amounts of polygalacturonase[9,25], and there is no risk of enzymatic attack on the cross-linked pectate. For reasons of enzyme stability, separation of orange pectinesterase isoenzymes was carried out at pH 6.0. However, at lower pH values pectate is a more powerful inhibitor of orange pectinesterase[20], and binding of pectinesterases to cross-linked pectate would be enhanced. Elution could be effected with a salt gradient, which was anticipated from the decreased degree of inhibition of orange pectinesterase

at higher ionic strengths[20]. Only oligogalacturonates with a degree of polymerization of 8 or higher show pectinesterase inhibition[20]. Evidently, orange pectinesterase binds to longer sequences of galacturonide units. As both isoenzymes saponified pectin with a high degree of cross-linking (Fig. 2), carbon atoms 2 and 3 are of only limited importance for substrate binding and catalysis. They do, however, affect the binding, as pectinesterase I, which is less strongly inhibited by pectate, was only retained on pectate with a degree of cross-linking of 0.46 or lower.

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