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MULTIPLE FORMS OF PECTINESTERASE IN LIMES AND ORANGES

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Key Word Index—*Citrus aurantifolia*; *Citrus sinensis*; Rutaceae; lime; orange; pectinesterase; multiple forms.

Abstract—Two forms of pectinesterase, apparently similar in MW but differing in charge and response to cation concentration, have been demonstrated in West Indian limes and Washington Navel oranges, and these enzymes have been purified at least 100-fold. In the orange one pectinesterase is located almost exclusively in the peel while the other is located within the segment covers and juice sacs. The location of the two pectinesterases in lime has not been determined.

INTRODUCTION

Apart from the demonstration [1] of pectinesterase activity in various component parts of the lime, no detailed studies have been carried out on the natural pectolytic enzymes of this fruit. Purification of a buffered extract of the whole lime fruit has now revealed the presence of two pectinesterases of similar MW. Whilst pectinesterase is distributed widely in higher plants, multiple forms have only been reported for tomato [2] and banana [3]. In view of the results with lime it was of interest to re-examine the orange since previous studies [4] were restricted to the enzyme extracted from the peel.

This paper describes the purification and some of the properties of two forms of pectinesterase which have been isolated from both the orange and lime fruits.

RESULTS AND DISCUSSION

Purification of orange and lime pectinesterases

The pectinesterases present in buffered extracts of orange and lime fruits were purified by similar procedures which are summarized in Tables 1 and 2. On Sephadex G-75 chromatography the pectinesterase activity eluted as one fraction with the same elution volume in both cases. Further purification of the G-75 fractions on DEAE-Sephadex A-50 resolved each pectinesterase into two active fractions, designated in order of elution LPE I, LPE II, OPE I and OPE II for the lime and orange enzymes respectively.

The failure to resolve the pectinesterases from either fruit on Sephadex G-75 and their identical elution volumes suggest similar MWs for all 4 enzymes. Since both fruit extracts were separated into two fractions on DEAE-Sephadex A-50, the two molecular forms from each fruit would appear to have different charges. The elution profiles of the lime and orange pectinesterases on DEAE-Sephadex A-50 were identical and suggested a structural similarity between OPE I and LPE I and also OPE II and LPE II, but this was not borne out by the results of cation activation and extent of de-esterification of various pectins.

Effect of substrate concentration on activity of pectinesterases

The initial rates of de-esterification of a pectin (degree of esterification, DE 69%) were measured for various pectin concentrations at pH 7. From the double reciprocal plot of initial velocity against substrate concentration the K_m values for the lime pectinesterases I and II were 0.015 and 0.054% w/v, and for the orange pectinesterases I and II 0.025 and 0.021% w/v.

Effect of pH *and* NaCl *concentration*

The effect of pH and NaCl concentration on the activity of the pectinesterases isolated in these studies agreed with previous findings [4, 5] for orange and tomato pectinesterases that activation by cations occurs and the pH optimum shifts to a lower pH value. Table 3 gives the

Table 1. Purification of pectinesterase from lime

Fraction	Volume (ml)	Total protein (mg)	Total units	Units/mg protein	Yields %
1. Crude extract from 5 kg fruit	5000	—	30 000	—	100
2. Dialyzed 0–70% $(NH_4)_2SO_4$	540	—	27 500	—	92
3. 40–65% $(NH_4)_2SO_4$	34	9690	24 100	2.5	81
4. Sephadex G-75 0–75% $(NH_4)_2SO_4$	11.5	403	17 800	44.3	60
5. DEAE-Sephadex A-50*					
LPE I	2	8.3	1200	144	32
LPE II	5	23.3	7600	328	

* Eluates concd by ultrafiltration.

Table 2. Purification of pectinesterase from orange

Fraction	Volume (ml)	Total protein (mg)	Total units	Units/mg protein	Yields %
1. Crude extract from 1 kg fruit	1400	—	23 400	—	100
2. Dialyzed 0–70% $(NH_4)_2SO_4$	100	—	22 000	—	94
3. 40–65% $(NH_4)_2SO_4$	30	6120	18 400	3	79
4. Sephadex G-75/0–75% $(NH_4)_2SO_4$	8.1	131	13 800	105	59
5. DEAE-Sephadex A-50*					
OPE I	5	12.6	2800	222	52
OPE II	5	21.2	9400	444	

* Eluates concd by ultrafiltration.

Table 3. Minimum NaCl concentrations for optimum activity at various pH values

	Minimum NaCl concn (M)			
	Orange		Lime	
pH	PE I	PE II	PE I	PE II
6	0.20	0.08	0.20	0.10
7	0.15	0.04	0.20	0.075
8	0.10	0.02	0.20	0.05
9	0.075	0.01	0.10	0.025

minimum NaCl concentration necessary for optimum activity at different pH levels.

Effect of degree of esterification of pectin

The effect of the degree of esterification of pectin on the rate of de-esterification was investigated using pectins of DE values 33, 69 and 90.7%. With all 4 pectinesterases the fastest initial rates of de-esterification were obtained with the pectin of lowest ester content. Increasing the degree of esterification decreased the initial rate of de-esterification. Table 4 gives the relative initial rates of de-esterification for a 0.2% solution of the various pectins at pH 7 with the initial rate of de-esterification of the 33% DE pectin normalized to 100. These results are in accord with previous findings with orange pectinesterase [6].

Both lime pectinesterases de-esterified all the pectin samples to *ca* 75% of the theoretical maximum. With the orange pectinesterases the extent of de-esterification decreased from *ca* 75% with the 33% DE pectin to *ca* 42% with the 90.7% DE pectin.

Location of pectinesterase within the fruit

Since no major differences in the activity were apparent between the pectinesterases of each fruit, the possibility of a difference in location within the fruit was considered. The small size of the lime fruit and the thinness of the peel (albedo and flavedo) made a detailed study of possible differences in distribution of the enzymes difficult. However, the orange was easily separated into flavedo and albedo, juice sacs and segment covers, and juice. The level of activity in the juice sacs and segment covers was *ca* 3 times that in the flavedo and albedo extract. No activity was present in the juice. Both active extracts were purified in the same way as the whole fruit extract but on DEAE-Sephadex A-50 each gave only one pectinesterase fraction. The elution volume of the pectinesterase fraction from the peel extract was identical to that of OPE I while the pectinesterase fraction from the juice sacs and membrane covers had the same elution volume as OPE II.

Table 4. Effect of degree of esterification of pectin on initial rate of de-esterification

% DE pectin	Relative initial rates			
	Lime		Orange	
	PE I	PE II	PE I	PE II
33	100	100	100	100
69	81.3	72.5	75	88
90 7	65.6	42 8	57	65

The significance of the multiple forms of pectinesterase in both the orange and lime fruits is not readily apparent from the *in vitro* studies described in this paper. In the fruit the pectinesterases are bound to the tissue so the activity of the pectinesterases *in vivo* may well be different from that observed in these studies. Furthermore, the complexity of pectin and the changes in its structure and distribution that occur during softening and ripening of fruits may require multiple forms of pectinesterase.

EXPERIMENTAL

West Indian limes *C. aurantifolia* were obtained by airfreight from L. Rose & Co., Dominica, Windward Islands. Washington Navel oranges *C. sinensis* were purchased locally. The fruits were sectioned to give the required component parts and then blended in 0.3 M Tris containing M NaCl at 4°. After 2 hr at 4° the homogenate was pressed through cheese cloth and the supernatant brought to 70% satn with solid $(NH_4)_2SO_4$. The pptd protein was collected by centrifugation and redissolved in 0.1 M Tris-HCl pH 7 containing 0.1 M NaCl (hereafter called Tris buffer), and dialyzed for 18 hr against the same buffer. The dialyzed soln was again fractionated with $(NH_4)_2SO_4$; the ppt. obtained between 40 and 65% satn being redissolved in the Tris buffer. Chromatography was first carried out on a 4.4 × 60 cm column of G-75 Sephadex equilibrated and eluted with the Tris buffer. Fractions (*ca* 5 ml) were collected and assayed for pectinesterase activity by addition of 0.1 ml of each fraction to 50 ml of 0.2% pectin in 0.1 M NaCl, pH adjusted to 7 at 25°. The pH was maintained by continuous titration with 0.01 N NaOH and results expressed in pectinesterase units (μeq ester hydrolyzed per min). The fractions containing pectinesterase activity were combined and brought to 70% satn with solid $(NH_4)_2SO_4$. After centrifugation the protein ppt. was redissolved in a minimum of Tris buffer. This fraction was further chromatographed on a 3.2 × 45 cm column of DEAE-Sephadex A-50 equilibrated

and eluted with the Tris buffer. The fractions containing pectinesterase activity were combined and concd to small vol. by ultrafiltration using a UM 10 membrane (Amicon Corporation). The pectin sample DE 33% was kindly supplied by H. P. Bulmer Ltd., Hereford. The pectin DE 69% was obtained from the Sigma Chemical Company. The sample DE 90.7% was prepared from the 69% DE pectin by the method of ref. [7]. Pectin solns (0.2%, 50 ml) containing NaCl adjusted to the required pH with 0.1 N NaOH were used for the assay of pectinesterase activities. Stepwise increments of NaCl were used to determine minimum concn necessary for maximum pectinesterase activity.

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