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THE ACTION OF TOMATO AND *Aspergillus foetidus* PECTINESTERASES ON OLIGOMERIC SUBSTRATES ESTERIFIED WITH DIAZOMETHANE

OSKAR MARKOVIČ, EVA MACHOVÁ, AND ALEXANDER SLEZÁRIK
Institute of Chemistry, Slovak Academy of Sciences, 842 38 Bratislava (Czechoslovakia)
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ABSTRACT

The action of tomato and *A. foetidus* pectinesterases on oligo(D-galactosiduronic acids) partially esterified with diazomethane was studied. The pentamer was found to be the shortest substrate at which de-esterification catalyzed by tomato pectinesterase occurred, whereas *A. foetidus* pectinesterase was able to cause attack on the dimer. With increasing degree of polymerization of the esterified oligomers, the reaction rates increased for both enzymes. Differences were, however, found in the relative activities towards the oligomeric substrates, as well as in the extent of their de-esterification. From these results, it is apparent that the two enzymes studied differ in both the size and the character of the active site.

INTRODUCTION

Pectinesterase (pectin pectyl-hydrolase, EC 3.1.1.11) catalyzes the de-esterification of methyl D-galactopyranosyluronate residues of pectin.

Pectinesterases produced by *Aspergillus* sp. (*A. niger*[1], *A. japonicus*[2], and *A. foetidus*[3]) differ from those of higher plants (tomato[4,5], orange[6], and alfalfa[7]) in several properties: the pH optima of *Aspergillus* sp. pectinesterases lie in the acid region (3.5–4.8), whereas those of higher plants are in the alkaline range (7.0–8.5). The isoelectric points of *Aspergillus* sp. pectinesterases are acidic (3.7–3.9), but those of higher plants are basic (7.5–11.0). The activity of the higher-plant pectinesterases is significantly stimulated by Na^+ and Ca^{2+} ions, but *Aspergillus* sp. pectinesterases are only slightly influenced by these ions[8]. Pectinesterases of *A. niger*[1,9], *A. japonicus*[2], and *A. foetidus*[10] catalyze random de-esterification of methyl esters of pectin, whereas the de-esterification by tomato, alfalfa, and orange pectinesterases proceeds linearly along the D-galacturonan chain, giving rise to blocks of free carboxyl groups[9–11]. Differences have also been reported as to the effect of pectic acid on the activity of pectinesterases of various origins: tomato[4], orange[6,12], and alfalfa[7] pectinesterases are competitively inhibited by pectic acid, but the enzyme of *A. niger* is activated in its presence[13].

The length of the substrate at which the action of pectinesterase takes place is one of the questions so far unanswered. Earlier study with orange pectinesterase

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showed that this enzyme does not hydrolyze the ester groups of the mono- and dimethyl ester of di(D-galactosiduronic acid), the dimethyl ester of di(D-galactosiduronic acid) methyl glycoside, and the trimethyl ester of tri(D-galactosiduronic acid) methyl glycoside, but readily attacks the polymethyl ester of D-galacturonans having a degree of polymerization (d.p.) of 10 and above [14].

The availability of oligo(D-galactosiduronates) having defined degrees of esterification has permitted us to investigate and compare the action of tomato and *A. foetidus* pectinesterases on these substrates.

EXPERIMENTAL

Substrates. — Oligo(D-galactosiduronic acids) of d.p. 2–8 were prepared according to Rexová-Benková[15], D-galacturonan of d.p. 10 ±2 according to McCready and Seegmiller[14], and pectic acid of $[\eta]^a = 336$ mL/g was prepared by de-esterification of citrus pectin with tomato pectinesterase[10]. D-Galactopyranuronic acid was a product of Fluka (Switzerland). All of these substances were esterified with diazomethane to a predetermined degree of esterification (d.e.) by using a method described previously[17].

Citrus pectin of d.e. 65% was a product of Københavns Pektinfabrik (Denmark).

Enzymes. — Tomato pectinesterase (one of five multiple forms) was prepared and characterized according to procedures previously described[18,19].

Aspergillus foetidus pectinesterase was prepared from the commercial product Pektofoetidin (USSR) by salting out with ammonium sulfate, gel filtration on Sephadex G-50 and G-75, and ion-exchange chromatography on DEAE-Sephadex A-50 (ref. 5), followed by chromatography on SE-Sephadex C-50 in 0.1M citric buffer, pH 3.9, with a concentration gradient of NaCl.

Enzyme assay. — The activity of pectinesterase was determined in a thermostatically controlled vessel by continuous titration with 0.01M NaOH under nitrogen at 30° and pH 7.0 (for tomato pectinesterase), or at pH 4.6 (for *A. foetidus pectinesterase*), by using a Radiometer pH-stat and autotitrator set (Copenhagen, Denmark). In a standard, experimental procedure, a 10-mL sample of substrate (concentration of methyl D-galactopyranosyluronate units ~0.03M) was incubated with 50 μL of tomato pectinesterase (total activity, 87 nmol of released carboxyl groups per s) or 300 μL of *A. foetidus* pectinesterase (total activity, 48.3 nmol.s^{-1}). In the case of tomato enzyme, the activity measurements were performed in the presence of 0.15M NaCl.

The initial reaction rates were computed by using the procedure of Booman and Niemann[20].

[a] Limit viscosity number

RESULTS

Tomato pectinesterase. — The partially esterified penta(D-galactosiduronate) was found to be the shortest substrate attacked by tomato pectinesterase; partially esterified oligomers of d.p. 2–4 did not serve as substrates. The initial rate of pentamer de-esterification was 4.48% of that of pectic acid partially esterified with diazomethane (taken as 100%). The reaction rates for higher-oligomeric substrates increased with their d.p. (see Table I).

The time course of de-esterification of individual oligomers (see Fig. 1) shows that the reaction stopped at a relatively low extent of ester hydrolyzed, particularly with the lower oligomers (see Table I). The de-esterification of these substrates continued after addition of a new portion of the enzyme. An example of this situation is demonstrated in Fig. 2. This result indicates that the first portion of enzyme was inhibited by reaction products, but that there was still sufficient esterified substrate available for subsequent reaction. On the other hand, the activity of the enzyme inhibited by partially de-esterified, oligomeric substrates could be initiated by addition of the natural substrate (citrus pectin). The reaction rate of reactivated pectinesterase was 20–25% lower than that for pectin in the absence of partially de-esterified oligomers (see Fig. 3 — exemplified by the octamer). This indicates that tomato pectinesterase is competitively inhibited by reaction products of oligomeric substrates. The competitive inhibition of orange pectinesterase by pectic acid and various oligo(D-galactosiduronic acids) was reported by Termote *et al.*[12].

A. foetidus *pectinesterase.* — The monomethyl ester of di(D-galactosiduronic

TABLE I

ACTION OF TOMATO PECTINESTERASE ON SUBSTRATES ESTERIFIED WITH DIAZOMETHANE

Substrate						
		Concentration		*Enzyme action*		
d.p.	*d.e. (%)*	*Methyl ester[a] (mM)*	*Oligomer (mM)*	v_0 *(10^2 μmol.s^{-1})*	*Relative activity (%)*	*Ester hydrolyzed (%)*
2	50.0	26.5	26.5	—	—	—
3	66.6	34.8	17.4	—	—	—
4	50.0	26.5	13.3	—	—	—
5	60.0	31.9	10.6	0.32	4.58	0.188
6	66.6	35.4	8.8	0.91	13.00	1.285
7	57.0	30.6	7.6	1.43	20.50	3.265
8	62.5	34.4	6.9	1.92	27.50	6.546
10 ±2	54.5	29.4	5.3	3.36	48.11	20.115
E.P.A.[b]	65.0	31.5		7.00	100.0	82.300
C.P.[c]	65.0	31.5		8.70	125.28	

[a]Methyl D-galactopyranosyluronate units. [b]Diazomethane-esterified pectic acid. [c]Citrus pectin.

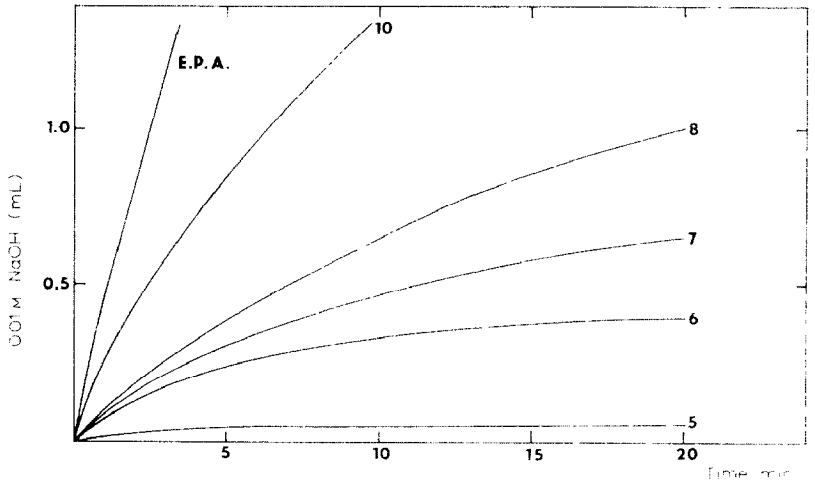

Fig. 1. Time course of de-esterification of oligomeric substrates by tomato pectinesterase. [Curves 5–8: partially esterified oligo(D-galactosiduronic acids) of d.p. 5–8. Curve 10: partially esterified D-galacturonan, d.p. 10 ± 2. Curve E.P.A.: 65% esterified pectic acid. In all cases, 10-ml samples of substrate (d.e. and concentration of methyl ester as in Table I) were incubated with 50 μl of enzyme (total activity 87 nmol s^{-1}).]

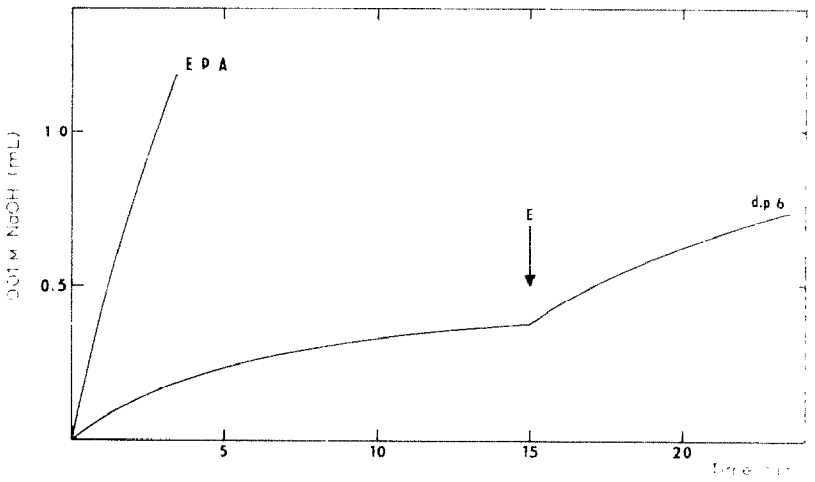

Fig. 2. Time course of de-esterification of hexamer with addition of a further portion of tomato pectinesterase. [Curve d.p. 6: 10 mL of 66.6% esterified hexamer (35mM methyl ester) with 50 μl of pectinesterase (87 nmol s^{-1}). ↓ E: addition of 100 μl of pectinesterase (174 nmol s^{-1}). Curve E.P.A.: as in Fig. 1.]

acid) was found to be the shortest substrate for this fungal pectinesterase (see Fig. 4, and Table II). The methyl ester of D-galactopyranuronic acid was not de-esterified, either alone or in an equimolar mixture with the free acid.

The rates of de-esterification of the oligomers tested increased with their d.p. (see Table II); however, the relative activities, as well as the extent of ester hy-

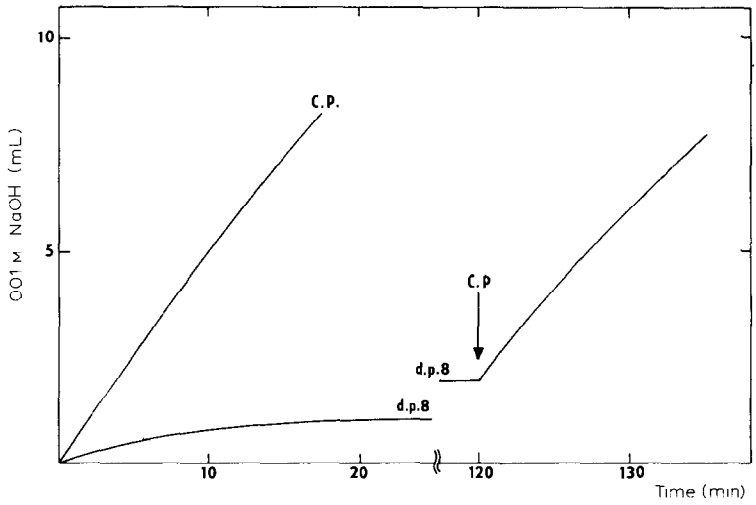

Fig. 3. Time course of de-esterification of octamer, and reactivation of tomato pectinesterase with the natural substrate. [Curve d.p. 8: 5 mL of 62.5% esterified octamer (36mM methyl ester) titrated to a volume of 7 mL. ↓ C.P.: addition of 7 mL of citrus pectin (66mM methyl ester). Curve C.P.: 14 mL of citrus pectin (33mM methyl ester) with 50 μL of enzyme (87 nmol.s^{-1}).]

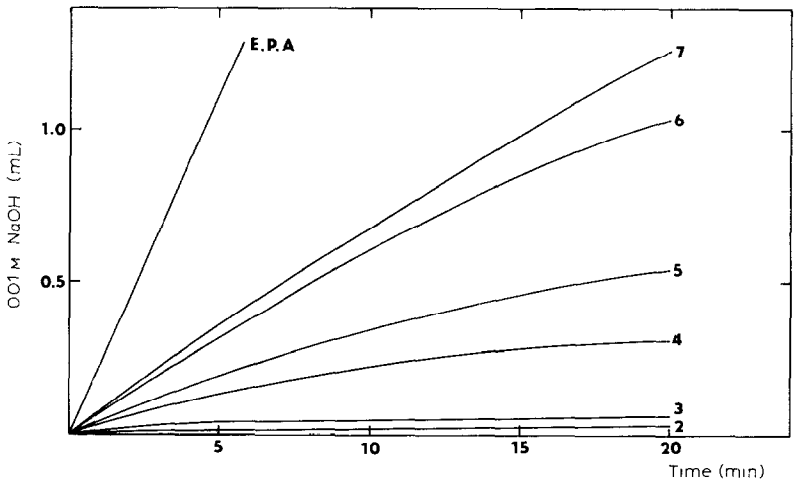

Fig 4. Time course of de-esterification of oligomeric substrates by *A foetidus* pectinesterase [Curves 2–7· partially esterified oligomers of d.p. 2–7. Curve E.P A.: 65% esterified pectic acid In all cases, 10-mL samples of substrate (d.e. and concentration of methyl ester as in Table II) were incubated with 300 μL of enzyme (total activity, 48.3 nmol.s^{-1})]

drolyzed for corresponding oligomers, were several times higher than those obtained with tomato pectinesterase (see Tables I and II).

Addition of citrus pectin to the reaction mixture, at the time when the de-esterification of oligomers by *A. foetidus* pectinesterase ceased, caused the reaction

TABLE II

ACTION OF *Aspergillus foetidus* PECTINESTERASE ON DIAZOMETHANE-ESTERIFIED SUBSTRATES

Substrate		*Concentration*		*Enzyme action*		
d.p.	*d.e.* (%)	*Methyl ester*[a] (mM)	*Oligomer* (mM)	v_0 (10^2 μmol s^{-1})	*Relative activity* (%)	*Ester hydrolyzed* (%)
1	100.0	30	30	—	—	—
2	50.0	23.4	23.4	0.137	3.56	1.67
3	75.0	35.1	15.6	0.188	4.89	4.56
4	75.0	35.3	11.8	0.467	12.18	12.86
5	75.0	35.5	9.4	1.020	26.61	13.84
6	75.0	35.6	7.9	1.105	28.83	14.38
7	75.0	35.6	6.8	1.278	33.55	18.59
F.P.A.[b]	65.0	31.5		3.833	100.00	79.17
C.P.[c]	65.0	31.5		4.830	126.08	

[a,b,c] As in Table I

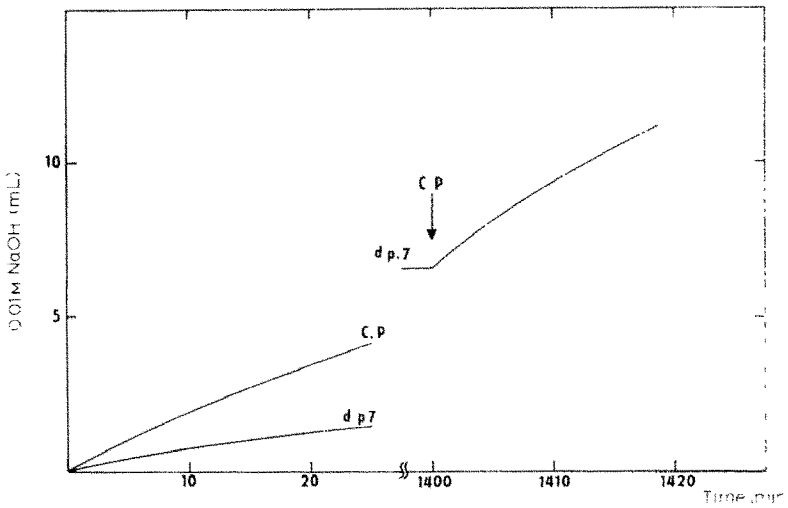

Fig. 5. Activating effect of partially de-esterified heptamer on *A. foetidus* pectinesterase [Curve d.p. 7: 10 mL of 75% esterified heptamer (35.6mM methyl ester) with 300 μL of enzyme (48.3 nmol s^{-1}) titrated to a volume of 17 mL. ↓ C.P.: addition of 8.5 mL of citrus pectin (66mM methyl ester). Curve C.P.: 25.5 mL of citrus pectin (22mM methyl ester) with 300 μL of enzyme (48.3 nmol s^{-1}).]

to resume at a rate 20–30% higher than that with citrus pectin alone (see Fig. 5 — demonstration with the heptamer). The activating effect of pectic acid had been observed for *A. niger* pectinesterase [13].

DISCUSSION

The rate of de-esterification of oligomeric substrates by the two pectinesterases studied depends on the d.p. of the oligomers at approximately the same concentration of methyl D-galactopyranosyluronate units, and is not determined by the molar concentration of the oligomers (see Tables I and II).

Based on the differences in the de-esterification of oligomeric substrates, it may be assumed that the size of the binding site of tomato pectinesterase is larger than that of *A. foetidus* enzyme. Considering the different mode of de-esterification of pectin[10] by tomato (in blocks) and by *A. foetidus* pectinesterase (random), the opposite effect of reaction products on the activity of the enzymes studied, and their different pH optima and isoelectric points, it may also be assumed that the active sites of these two enzymes have a different character.

It may be mentioned that the relative activities of tomato and *A. foetidus* pectinesterases were 25% lower with diazomethane-esterified pectic acid than the values obtained with natural citrus pectin (see Tables I and II). Solms and Deuel[21] reported that the activity of orange pectinesterase on citrus pectin dropped 25% after total esterification with diazomethane and subsequent alkaline de-esterification to the d.e. of natural pectin. However, it is not yet known how diazomethane influences the pectin molecule or the linear D-galacturonan chain.

Despite these observations, the action of tomato and *A. foetidus* pectinesterases was investigated by using the substrates prepared by esterification with diazomethane, as this method[17] provides, very readily and in quantitative yield, products having a predetermined d.e.

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