DEESTERIFICATION MODE OF PECTIN BY PECTIN ESTERASES OF *Aspergillus foetidus*, TOMATOES AND ALFALFA

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The effect of microbial pectin esterases (*Aspergillus foetidus*) and higher plants (tomatoes, alfalfa) on high-esterified pectin was investigated. Pectin derivatives of esterification degree E 42—66% were prepared by a partial deesterification catalyzed by the above-mentioned enzymes. The distribution pattern of free and esterified carboxyl groups in the linear pectin macromolecule was determined on the basis of activity coefficients of counterions $\gamma_{Ca^{2+}}$ as found in solutions of the proper calcium pectinates. The activity coefficients $\gamma_{Ca^{2+}}$ were compared with the corresponding $\gamma_{Ca^{2+}}$ values determined in pectins partially deesterified with alkali, with a random distribution pattern of free carboxyl groups. The plant pectin esterases under investigation lead to a block-wise arrangement of the free carboxyl groups in the pectin molecule, whereas *A. foetidus* pectin esterase cleaves randomly the ester groups in the same mode as do the alkali hydroxides. Significant differences were observed with the final products of enzyme deesterification of pectin even in the selectivity of $Ca^{2+} \rightarrow 2\ K^{+}$ cation exchange, as documented by the change of electrostatic free enthalpy of exchange of these cations. The different mechanism of action of plant and *A. foetidus* pectin esterases was thus unambiguously proved by a physico-chemical method.

The deesterification of pectin catalyzed by pectin esterases (E.C. 3.1.1.11) of oranges[1-4] and tomatoes[5,6] affords a block-wise arrangement of free (deesterified) carboxyl groups in the linear pectin macromolecule. Deesterification of pectin by alkali and acids proceeds, on the other hand randomly with a statistical distribution pattern of free and esterified carboxyl groups. This conclusion has been deduced from a different action of pectin esterase upon pectinates obtained by a partial deesterification with acids, alkali or pectin esterases, further upon the kinetics of alkaline deesterification, chromatography of products on DEAE-cellulose, dissociation of pectinic acids, as well as upon differences in the bond strength of calcium to carboxyl groups of these substances. It was presumed that the mode of deesterification of pectin with pectin esterases of higher plants (oranges, tomatoes) can be analogously valid with certain probability also for pectin esterases produced by microorganisms[6,7].

As Ishii and coworkers[8] have recently shown, preparations of a low-esterified pectin behaving as homogeneous substances when chromatographed on DEAE-cellulose can be obtained by pectin esterase produced by *Aspergillus japonicus*; these form considerably strong gels in a 20% saccharose solution with an addition

of calcium ions, in contrast to heterogeneous preparations obtained by the action of plant pectin esterases. These authors assume that *A. japonicus* pectin esterase causes a random cleavage of esterified carboxyl groups similarly, as alkali and acids. Baron and coworkers[9] obtained preparations homogeneous upon chromatography on DEAE-cellulose by pectin esterase of *A. niger*, as well; therefore, the same mechanism of action, the multi-chain attack of the enzyme, was suggested as in[8]. The action of endo-D-galacturonanase on preparations of pectin partially deesterified with sodium hydroxide, pectin esterase of oranges and pectin esterase of *A. niger* was investigated by Dongowski and Bock[10] who concluded from the different activity of endo-D-galacturonanase of *A. niger* on these preparations that the mode of action of pectin esterase of *A. niger* is close to the mechanism of action of alkali, *i.e.* it affords preparations with more or less statistical distribution of free carboxyl groups in the pectin molecule.

As has been shown in one of our preceding papers[6], the mechanism of action of pectin esterases can be characterized substantially better than by procedures hitherto applied by means of the activities of calcium counterions bound to free carboxyl groups of pectin partially deesterified by the enzyme (pectin esterase of tomatoes). This paper deals with the mode of action of pectin esterase of *A. foetidus* and pectin esterases of tomatoes and alfalfa on the high-esterified pectin employing this method.

EXPERIMENTAL

Enzymes used: Pectin esterase of microbial origin was prepared from the commercially available preparation Pektofoetidin of *A. foetidus* (USSR) by precipitation with ammonium sulfate, separation on Sephadex G-75 and G-50 columns and chromatography on DEAE-Sephadex A-50 (ref.[11]). The accompanying endo-D-galacturonanase was separated from pectin esterase by chromatography on a SE-Sephadex C-50 column. Pectin esterase thus obtained corresponded to a 150-fold purified product having 0·36 mol s^{-1} kg^{-1} specific activity and pH optimum at 4·6.

Pectin esterase of tomato (*Lycopersicum esculentum*, var. *Immuna*) was obtained after extraction and fractional salting-out with ammonium sulfate, by chromatography on DEAE-Sephadex A-50, Sephadex G-75 and CM-Sephadex C-50 (ref.[12]). The final product was one of the five multiforms of pectin esterase of mean relative molecular mass ($\bar{M}_r$) 27 500, specific activity 6·3 mol . s^{-1} kg^{-1} and pH optimum 7·5–8·5.

Pectin esterase of alfalfa (*Medicago sativa*, var. *Nitranka*) was isolated from leaves and stems after homogenization, extraction with a 0·4M-NaCl solution at pH 7·5, salting-out with ammonium sulfate, dialysis, chromatography on DEAE-Sephadex A-50 and Sephadex G-75. After desalting on Sephadex G-25 and freeze-drying the product had specific activity 0·6 mol s^{-1} kg^{-1} and pH optimum 7·5–8·5 at a 0·02M-NaCl concentration (ref.[13]).

Preparation of Samples of Pectin Partially Deesterified by Pectin Esterase

The highly esterified citrus pectin (Genu Pectin, Medium Rapid Set, Københavns Pektinfabrikl Denmark) was first purified by washing with an acidified 60% ethanol (5 ml of concentrated HC, in 100 ml of 60% ethanol), neutral ethanol 60% and 96%, respectively and methanol. Pectin was

esterified with a methanolic 1M-H_2SO_4 at 3°C for 3 weeks; the preparation was then washed with 60% and 96% ethanol, ether, and dried under diminished pressure and temperature not exceeding 60°C. The high-esterified pectin had the esterification degree of carboxyl groups with methanol $E = 94{\cdot}7\%$ and contained 88·2% of polyuronide (a high-esterified D-galacturonan), and 0·44% sulfate ash; the limit viscosity number $[\eta] = 147$ ml g^{-1}.

The high-esterified pectin was partially deesterified by pectin esterases using autotitrator TTT 11 (Radiometer Copenhagen, Denmark) at pH 4·6 (pectin esterase *A. foetidus*) or pH 7·5 (pectin esterases of tomato and alfalfa) by a continuous titration with 0·1M-NaOH at 25°C. The 1% pectin solution (250 ml) was treated with the enzyme (1 ml) of total activity $0{\cdot}75 \cdot 10^{-3}$ mol s^{-1} (pectin esterase *A. foetidus*), $1{\cdot}1 \cdot 10^{-3}$ mol s^{-1} (pectin esterase of tomato) and $1{\cdot}2 \cdot 10^{-3}$ mol s^{-1} (pectin esterase of alfalfa). The enzyme was deactivated by a 10-min heating on a steam bath after reaching the required degree of esterification. Pectin was then precipitated after cooling with an acidified ethanol (the final concentration 0·33M-HCl in 60% ethanol). The chloride ions were removed from the coagulate by washing with a 60% ethanol, 96% ethanol and ether. The product obtained in this way (H^+ form) was dried and characterized by the esterification degree (E) and limit viscosity number $[\eta]$.

Analytical Methods

The content of free carboxyl groups of pectin was determined alkalimetrically by a potentiometric titration with a 0·05M-KOH (carbonate free), the overall content of carboxyl groups by a method of precipitation of insoluble copper pectates[14,15]. The amount of copper bound to pectin carboxyl groups was determined chelatometrically by titration with Complexon IV (0·01M-solution), the point of equivalence being estimated by a spectrophotometric indication employing an interference filter IF 600 nm (Zeiss, Jena). The chloride content was determined argentometrically with 0·01M-$AgNO_3$ using silver electrode. The limit viscosity number $[\eta]$ was measured in potassium pectinate solutions (pH ~ 7) employing the Ubbelohde viscometer, in 0·15M-NaCl—0·005M-sodium oxalate at 25·0 ± 0·1°C. The mean molecular mass $\overline{M}_r$ was calculated from the $[\eta]$ values according to Owens and coworkers[16].

Determination of Calcium Ion Activities in Calcium Pectinate Solutions

Solutions of pectinic acids (4—5 mmol (COOH) l^{-1}) were centrifuged at 20 000 *g* for 20 min and neutralized (pH ~ 7·2) by potentiometric titration with a saturated calcium hydroxide solution (0·021M-$Ca(OH)_2$). The calcium ion activities were determined in calcium pectinate solutions (3·00 mmol $(COOCa_{0.5})$ l^{-1}) by a metallochromic indicator (tetramethylmurexide) method as already described[17,18] at room temperature. Tabulated values of the single-ion activity coefficients $\gamma_{Ca^{2+}}$ calculated by the Debye and Hückel theory of strong electrolytes[19,20] were employed for determination of $a_{Ca^{2+}}$ activities in calibration $CaCl_2$ solutions.

Determination of Electrostatic Free Enthalpy of $Ca^{2+} \rightarrow 2\,K^+$ Cation Exchange in the Studied Pectin Samples

A solution of pectinic acid (3·00 mmol (COOH) l^{-1}) was titrated potentiometrically with potassium and calcium hydroxides at 25·0 ± 0·01°C. The functional relationship pH = f(DN), where DN is the neutralization degree of pectinic acid with hydroxide, was expressed. The electrostatic free enthalpy of exchange of these cations $\Delta(G_{el}/N)_K^{Ca}$ value refers to one mol of free carboxyl group.

The measurement was carried out by a compensation spectrophotometer Uvispec (Hilger), a Research pH-meter PHM 64 (Radiometer), glass electrode G202 B (Radiometer), redistilled water (carbonate free), and analytical grade chemicals.

RESULTS AND DISCUSSION

Characteristic of Partially Enzyme-deesterified Pectin Samples

Samples of pectin partially deesterified by pectin esterases were obtained from the starting citrus pectin of a high esterification degree E 94·7% and the polyuronide content 88·2%. The samples were deesterified to an esterification degree E 40 – 65%, *i.e.* to the region well enabling the study of the mechanism of action by the technique chosen. It was of importance to get macromolecules of pectin without a greater degradation due to the action of endo-D-galacturonanase which can be present in the enzyme preparations as a contaminant. Therefore, we determined the limit viscosity number $[\eta]$ of the partially deesterified pectin samples as a characteristic of the relative molecular mass of pectin. The esterification degree of pectin samples was constant throughout the experiments, this being an evidence for a total inactivation of enzymes after a partial deesterification of the pectin samples tested.

Calculation of the mean relative molecular mass of pectin ($\bar{M}_r$) on the basis of the limit viscosity number $[\eta]$ has so far not been unambiguously cleared. Therefore, the $\bar{M}_r$ values listed in Table I were obtained by the most conventionally used Owens and coworkers equation[16]. The comparison of $[\eta]$ or $\bar{M}_r$ values of the starting sample of pectin with those of partially deesterified ones shows that no virtual change of the relative molecular mass took place by the action of alfalfa pectin esterase; the scattering of values is in the experimental error range. Application of the remaining two pectin esterases was associated with the relative molecular mass decrease by 9 to 29%.

The method of estimation of pectin esterase action pattern is based upon interpretation of activity coefficients $\gamma_{Ca^{2+}}$ determined in solutions of the corresponding calcium pectinates. Samples of pectin for $a_{Ca^{2+}}$ measurements must have a sufficiently high relative molecular mass, when $\gamma_{Ca^{2+}}$ is no more the function of the macromolecule chain length but the function of its linear charge density only. Our preceding paper[22] showed that this condition is fulfilled in the range of $[\eta]$ values corresponding to systems investigated (Table I). (The activity coefficient $\gamma_{Ca^{2+}}$ in calcium polyuronate solutions is independent on the relative molecular mass at a polymerization degree DP greater than 30, ref.[23,24]). Small changes in molecular mass of the investigated pectin samples do not influence, from this point of view, the determination of activities $a_{Ca^{2+}}$ in solutions of the corresponding calcium pectinates. Samples of pectin prepared in the H^+ form did not contain any accompanying low-molecular electrolytes.

Mode of Action of Pectin Esterases

The bond strength of calcium ions to carboxyl groups of pectinates is given by the linear charge density of the macromolecule, *i.e.* by the mean distance of two neighbouring carboxyl groups. This principle can well be used to judge the distribution pattern of free carboxyl groups in the pectin molecule. Calcium ions are bound to carboxyl groups of pectin of an esterification degree E greater than 40% and a random distribution of free carboxyl groups in the molecule by an intramolecular electrostatic bond. In the region, where E is smaller than 40%, an abrupt change of the electrostatic bond in a stronger intermolecular chelate binding occurs. A chelate binding of calcium ions takes analogously place at a block-wise arrangement of uronic acid units with free carboxyl groups. This method has already been applied to characterize the mode of action of tomato pectin esterase in our previous communication[6]. The stability constants (K) of calcium pectinates, prepared from pectin samples partially deesterified either with alkali hydroxide or with enzyme investigated were compared. (The alkaline deesterification of pectin leads to a random, and at lower esterification degrees to a more or less regular distribution pattern of free carboxyl groups in the pectin molecule[3] due to electrostatic repulsions of the negatively charged carboxylate and hydroxyl groups). Interpretation of results let us conclude that the action of tomato pectin esterase results in formation of segments with a block-wise arrangement of free carboxyl groups in the pectin molecule. These alternate with segmetns containing fully esterified uronic acid units[6].

Interaction of cations with polyanions can best be considered on the basis of counterion activity coefficients. Fig. 1, curve *1* shows the relationship of $\gamma_{Ca^{2+}}$ in calcium pectinate solutions on the esterification degree of pectin E with a random distribu-

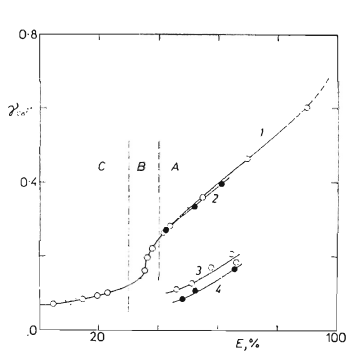

FIG. 1

Calcium ion binding to pectin partially deesterified by pectin esterases of plant and microbial origin. $\gamma_{Ca^{2+}}$ activity coefficient of calcium ions in calcium pectinate solutions of 3·00 mmol $(COOCa_{0.5})\,l^{-1}$ concentration; E esterification degree of pectin carboxyl groups; *A* region of electrostatic intramolecular binding of calcium ions, *C* region of intermolecular chelate binding, *B* transient region (regions *A* to *C* hold for pectin having a random distribution pattern of free carboxyl groups in the molecule). The mode of deesterification: *1* by alkali, *2*, *3*, *4* by pectin esterases of *A. foetidus*, tomato, and alfalfa, respectively.

tion pattern of free carboxyl groups, as already reported[25] (the samples of pectin were prepared by an alkaline deesterification of the starting pectin of esterification degree E 95%). Region A characterizes the intramolecular electrostatic binding of Ca^{2+} ions, region C the firmer intermolecular chelate binding, and B a transient region. The activity coefficient $\gamma_{Ca^{2+}}$ decreases with the decreasing esterification degree of pectin, *i.e.* with the shorter distance of neighbouring free carboxyl groups and reaches anomalously low values of $\gamma_{Ca^{2+}} = 0{\cdot}07 - 0{\cdot}10$.

The mode of action of pectin esterases of various origin is considered according to the single-ion activity coefficients $\gamma_{Ca^{2+}}$ determined in partially deesterified pectin samples with an esterification degree E 42 to 66%, *i.e.* in the range of values corresponding to an electrostatic binding of calcium ions to pectin with a random distribution pattern of free carboxyl groups in the molecule. The $\gamma_{Ca^{2+}}$ values (Table I, Fig. 1) refer to calcium pectinate solutions containing 3·00 mmol $(COOCa_{0{\cdot}5})\,l^{-1}$.

TABLE I

Binding of calcium ions to pectin partially deesterified by pectin esterases of plant and microbial origin; 3·00 mmol $(COOCa_{0{\cdot}5})\,l^{-1}$

No	E %	$[\eta]$ ml g^{-1}	$\bar{M}_r$	$\gamma_{Ca^{2+}}$	$\Delta(G_{el}/N)_K^{Ca}$ J $site^{-1}$
			Starting pectin		
1	94·7	147	31 000		
			Pectin partially deesterified by tomato pectin esterase		
2	66·1	121	27 000	0·183	
3	64·4	109	25 000	0·207 ± 0·004	2 080 ± 90
4	57·7	121	27 000	0·171	
5	51·2	118	26 000	0·129 ± 0·001	2 040 ± 30
6	46·0	126	28 000	0·112 ± 0·005	2 230 ± 150
			Pectin partially deesterified by alfalfa pectin esterase		
7	65·6	147	31 000	0·166 ± 0·002	1 860 ± 15
8	52·2	144	30 500	0·108 ± 0·001	2 275 ± 95
9	48·2	159	33 000	0·083 ± 0·000	2 430 ± 100
			Pectin partially deesterified by *A. foetidus* pectin esterase		
10	61·2	94	22 500	0·395 ± 0·003	1 070 ± 5
11	52·0	92	22 000	0·335 ± 0·018	1 265 ± 5
12	42·4	92	22 000	0·273 ± 0·008	1 250 ± 115

The activity coefficients $\gamma_{Ca^{2+}}$ of calcium pectinates, prepared by pectin esterase of *A. foetidus* (Fig. 1, curve 2), are virtually identical with those of pectin samples obtained *via* alkaline deesterification (curve *1*). Pectin esterase of *A. foetidus* causes therefore a random splitting of esterified carboxyl groups in the pectin molecule. On the other hand, the activity coefficients $\gamma_{Ca^{2+}}$ determined with pectin samples partially deesterified by plant pectin esterases (curve *3* pectin esterase of tomato, curve *4* pectin esterase of alfalfa) are much lower, and, at an esterification degree $E \leqq 50\%$, they are very close to $\gamma_{Ca^{2+}}$ values corresponding to a chelate binding of calcium ions to a fully deesterified pectin. The little higher $\gamma_{Ca^{2+}}$ values of pectin samples partially deesterified by plant pectin esterases to a higher esterification degree $E \sim 65\%$ can be rationalized by a formation of shorter segments in molecules with a block-wise arrangement of free carboxyl groups, where $\gamma_{Ca^{2+}}$ is already the function of the segment length. These anomalously low $\gamma_{Ca^{2+}}$ values repeatedly document, in line with the preceding findings, that plant pectin esterases lead to a block-wise arrangement of free carboxyl groups in the macromolecule chain.

The distribution pattern of free carboxyl groups in the pectin molecule markedly influences also the selectivity of cation exchange in this natural polyelectrolyte. Therefore, the change of electrostatic free enthalpy $\Delta(G_{el}/N)_K^{Ca}$ of the exchange of ions $Ca^{2+} \rightarrow 2\,K^+$ (Table I, Fig. 2) was estimated. The selectivity of exchange of these cations with pectin samples prepared by a partial deesterification with a plant pectin esterase (curve 2 pectin esterase of tomato, curve *3* pectin esterase of alfalfa) is substantially higher (higher $\Delta(G_{el}/N)_K^{Ca}$ values) than that of pectin samples obtained by a deesterification by pectin esterase of *A. foetidus* (curve *1*). These results also indi-

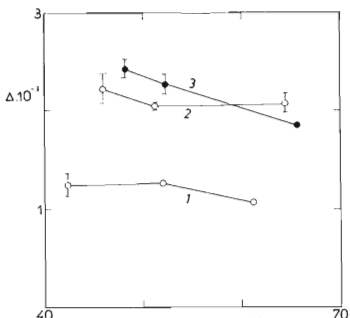

FIG. 2

Electrostatic free enthalpy $(\Delta(G_{el}/N)_K^{Ca})$ of $Ca^{2+} \rightarrow 2\,K^+$ cation exchange for pectin partially deesterified by pectin esterases of plant and microbial origin. *E* esterification degree of pectin; pectin esterases: *1* of *A. foetidus*, 2 of tomato, *3* of alfalfa; Δ means $\Delta(G_{el}/N)_K^{Ca}$, J site^{-1}.

cate the block-wise, or random distribution pattern of free carboxyl groups during the action of plant or *A. foetidus* pectin esterase.

Considering the elderly papers and our contribution to the effect of pectin esterases of plant origin (pectin esterase of orange[1-4], tomato[5,6] and alfalfa), or microbial pectin esterases (*A. japonicus*[8], *A. niger*[9,10], *A. foetidus*) one can assume that the action of pectin esterases of plant origin with a pH optimum >7 results in a block-wise arrangement of free carboxyl groups in the pectin molecule, whilst pectin esterases of *Aspergillus* species, with the pH optimum in the acid region investigated so far, cleave esters more or less randomly. The different distribution pattern of free and esterified carboxyl groups in the pectin molecule influences significantly the physicochemical properties of these pectin preparations, *e.g.* the gel-forming properties of pectin, binding of cations to these substances and selectivity of their exchange.

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