Mode of pectin deesterification by *Trichoderma reesei* pectinesterase

O. Markovič and R. Kohn

Institute of Chemistry, Slovak Academy of Sciences, CS-842 38 Bratislava (Czechoslovakia), 25 October 1983

Summary. *Trichoderma reesei* pectinesterase (isoelectric point 8.3–9.5; pH-optimum 7.6) catalyzes deesterification of pectin by giving rise to blocks of free carboxyl groups in the macromolecule; a mode similar to that observed with higher plant pectinesterases which also have I_p and pH-optima in the alkaline region.

Pectinesterases of higher plants (tomato[1–3], orange[4–7] and alfalfa[3]) catalyze deesterification of pectin linearly along the chain of the molecule, giving rise to blocks of free carboxyl groups. On the other hand, deesterification of pectin by alkali and acids results in a statistical distribution of free and esterified carboxyl groups[4,5]. Ishi et al.[8] were the first to show that *Aspergillus japonicus* pectinesterase causes a random cleavage of esterified carboxyl groups. Baron et al.[9] as well as Dongowski and Bock[6] obtained the same results with *A. niger* and Kohn et al.[3,7] with *A. foetidus* and *A. niger* pectinesterases, respectively.

The present study reports the mode of deesterification of pectin catalyzed by *Trichoderma reesei* pectinesterase, which has its pH-optimum and isoelectric point in the alkaline region.

Material and methods. Pectinesterase was prepared from culture filtrate of the fungus *Trichoderma reesei* QM 9414 cultivated on pectin as a carbon source, after ammonium sulfate precipitation and chromatography on Sephadex G-25 and G-100 columns[10]. This preparation included 5 multiple forms of pectinesterase with isoelectric points in the range 8.3–9.5 and pH-optimum 7.6.

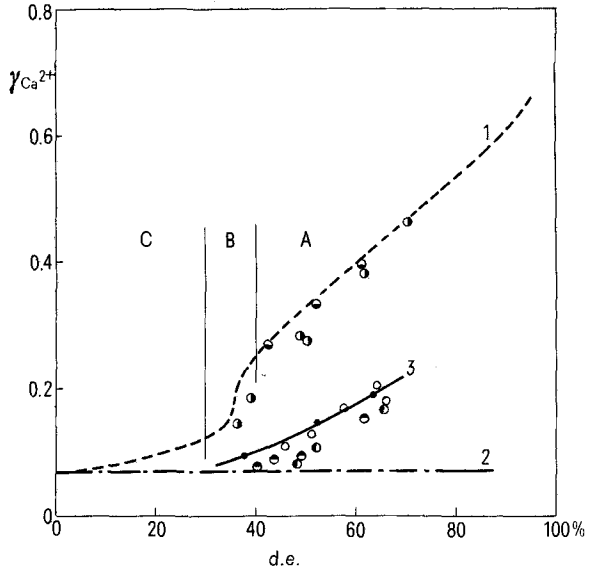
Preparation and characterization of pectin samples deesterified by pectinesterase. Purified citrus pectin (Genu Pectin, Medium Rapid Set, Københavns Pektinfabrik, Denmark) was esterified to 94.7% with methanolic 1 M H_2SO_4 at 3 °C. This pectin contained 88.2% of highly esterified D-galacturonan and exhibited a limit viscosity number $[\eta] = 147$ ml g^{-1}. The preparation was deesterified by *Trichoderma reesei* pectinesterase at pH 7.3 under continuous titration with 0.1 M NaOH at 25 °C using a Radiometer pH-stat and autotitrator set (Denmark). The 1% pectin solution (250 ml) was treated with 1 ml enzyme (total activity 1.2×10^{-3} mol sec^{-1}) until the required degree of esterification (d.e.) was obtained. The enzyme was inactivated by 10 min heating on a boiling water bath and after cooling the pectin was precipitated with acidified ethanol (final concentration 0.33 M HCl in 60% ethanol) and washed several times with 60% ethanol to remove chloride ions.

The content of free carboxyl groups of pectin was determined by potentiometric titration with 0.05 M KOH, and the overall content of carboxyl groups by precipitation of insoluble copper pectates[11,12]. The amount of copper bound to pectin carboxyl groups was determined chelatometrically. The limit viscosity number $[\eta]$ of pectin solutions adjusted to pH ~ 7 with KOH and containing 0.15 mol NaCl – 0.005 mol sodium oxalate in 1 l was measured using an Ubbelohde viscometer at 25.0 ± 0.1 °C.

Determination of calcium ion activities in Ca-pectinate solutions. Solutions of pectinic acids (4–5 mmol COOH l^{-1}) were centrifuged at 20,000 × g for 20 min and the supernatants neutralized with a saturated calcium hydroxide solution (0.021 M $Ca(OH)_2$) to pH ~ 7.2. Single-ion activity coefficients $\gamma_{Ca^{2+}}$ of calcium ions bound to carboxyl groups of pectin were determined by the metallochromic indicator (tetramethylmurexide) method[13].

Results and discussion. Samples of highly esterified citrus pectin (d.e. 94.7%) were deesterified with *Trichoderma reesei* pectinesterase to the esterification degree 38–63%, which is suitable for the study of the mode of deesterification by the method chosen (table). As evident from the values of the limit viscosity number $[\eta]$ of the substrate its deesterification was practically not accompanied by its depolymerization.

The elucidation of the pectinesterase action pattern is based upon determination of activity coefficient $\gamma_{Ca^{2+}}$ in solutions of the corresponding calcium pectinates. The activity coefficient $\gamma_{Ca^{2+}}$ is a complex function of the linear charge density of the macromolecule, i.e. of the mean distance of two neighboring free carboxyl groups. This principle can well be applied determining the distribution pattern of free carboxyl groups in the molecule[3]. Calcium ions are bound to pectin with d.e. > 40%, having a random distribution of free carboxyl groups, by intramolecular electrostatic bonds[14] (fig., curve 1, region A). With decreasing d.e. the $\gamma_{Ca^{2+}}$ values continuously decrease. At d.e. < 40% the electrostatic bond changes suddenly into a stronger chelate bond (region C; region B – transient region). The molecules with long segments of block-wise arrangement of free carboxyl groups exhibit the same anomalously low $\gamma_{Ca^{2+}}$ value



Calcium ion binding to pectin partially deesterified by pectinesterases 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; d.e., degree of esterification of pectin carboxyl groups; A, region of intramolecular electrostatic binding of calcium ions; C, region of intermolecular chelate binding; B, transient region. Distribution of free carboxyl groups: 1, random (by alkali); 2, block-wise (long chains). Mode of deesterification: ●, *Trichoderma reesei* (curve 3); ◒ *Aspergillus foetidus*[3]; ◑, *Aspergillus niger*[7]; ○, tomato[3]; ◓, orange[7]; ◐, alfalfa[3].

Binding of Ca^{2+} ions to pectin partially deesterified by pectinesterase of *Trichoderma reesei*

Sample No.	d.e. (%)	η (ml g^{-1})	$\gamma_{Ca^{2+}}$
1[a]	94.7	147	–
2	63.4	129	0.191 ± 0.003
3	52.1	115	0.143 ± 0.009
4	37.7	140	0.094 ± 0.019

[a] Starting sample; ($COOCa_{0.5}$) = 3.00 mmol l^{-1}.

as found for calcium pectate[14] (fig. 1, straight line 2). The $\gamma_{Ca^{2+}}$ values determined in solutions of calcium pectinates prepared by the action of pectinesterases of different origins are compared with those corresponding to pectinates with a random distribution of free carboxyl groups achieved by alkaline deesterification[14] (curve 1) and with the $\gamma_{Ca^{2+}}$ value assigned to block-wise arrangement (straight line 2).

The activity coefficients $\gamma_{Ca^{2+}}$ of calcium pectinates, prepared by the action of *Trichoderma reesei* pectinesterase (table; fig., curve 3) are close to those of pectin samples partially deesterified by tomato[3], orange[7] and alfalfa[3] pectinesterases (fig.). The results demonstrate the formation of blocks of free carboxyl groups in the linear macromolecule of pectin. Somewhat higher $\gamma_{Ca^{2+}}$ values, corresponding to pectin samples deesterified only slightly (d.e. 55–65%) by these enzymes are caused by formation of shorter blocks of free carboxyl groups, where $\gamma_{Ca^{2+}}$ is already a function of the block length. On the other hand, $\gamma_{Ca^{2+}}$ values corresponding to pectin samples deesterified by *Aspergillus foetidus*[3] and *A. niger*[7] pectinesterases are very close to curve 1 and represent therefore a random distribution of free carboxyl groups in the pectin molecule.

Pectinesterases of tomato, orange, alfalfa and *Trichoderma reesei* are basic proteins with isoelectric points in the range 8.3–11.0 and pH-optima also in alkaline region. On the contrary, pectinesterases of *Aspergillus* species, catalyzing a random deesterification of pectin in the same way as alkali, have isoelectric points and pH-optima in the acidic region. From these findings it is possible to conclude that the mode of deesterification of pectin by pectinesterases is independent of their origin (fungal or higher plant), but is determined by the character of the enzyme proteins reflected e.g. in isoelectric points and pH-optima.

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0014-4754/84/080842-02$1.50 + 0.20/0

Peptide interactions with taste receptors: Overlap in taste receptor specificity

R.H. Cagan[1]

Veterans Administration Medical Center and Monell Chemical Senses Center and University of Pennsylvania, Philadelphia (Pennsylvania 19104, USA), 21 September 1983

Summary. Two hitherto unrelated areas of taste appear to provide an important insight into a class of taste receptor sites. A region of the sweet protein monellin is similar to a savory tasting beef octapeptide, and this peptide region could represent an overlap in the specificity of binding to a common peptide taste receptor site. Such an overlap in binding specificity explains the low but significant level of sweetness observed with savory tasting stimuli.

The biochemical basis of taste receptor specificity appears to depend upon recognition of stimuli in a weak binding interaction with receptors located in the cell plasma membrane[2]. An experimental model emerged from the discovery a decade ago[3,4] of the sweet-tasting protein monellin, which consists of 2 dissimilar polypeptide subunits of known sequence[5,6]. Native monellin tastes intensely sweet, being around 10^5 times more effective in eliciting a sweet taste (at threshold levels) than the common sugars[7]. The structural features of monellin required for sweetness are not elucidated in detail, but the evidence[8–12] demonstrates the importance of the tertiary and quaternary structures, and supports the hypothesis[7] that its conformation is critical in its ability to evoke a sweet sensation.

The specific regions of the protein that bind to taste receptor sites are not known. Partial methylation of the lysines of monellin does not markedly affect its taste, while extensive methylation leads to loss of sweetness[13]. The necessity for the sulfhydryl group of the single cysteine to remain in the reduced form for the protein to retain its sweetness was demonstrated[5,14], although the cysteine is buried in the interior and is relatively inaccessible to reaction. We demonstrated that ^{3}H-labeled methylated monellin[13] binds to taste tissue preparations from bovine and human circumvallate papillae[15].

A seemingly unrelated area is that of monosodium L-glutamate (MSG), the taste of which is called *umami* in Japanese[16,17], meaning 'delicious' or 'savory' taste[18]. Possible mechanisms of action of L-glutamate in eliciting this unique taste sensation were discussed in an earlier review[19], which stressed that the well known taste synergism of certain 5′-ribonucleotides in combination with MSG[17,20,21] could be involved in the mechanistic basis for the unique 'flavor enhancing' action of MSG. We demonstrated direct binding of L-[^{3}H]glutamate to bovine taste tissue preparations and enhancement of this binding by certain 5′-ribonucleotides[22]. Yamasaki and Maekawa[23,24] have isolated an octapeptide from beef gravy which elicits a 'delicious' or 'savory' taste. They established its amino acid sequence[23] and synthesized the octapeptide[24].