PURIFICATION AND A PARTIAL CHARACTERIZATION OF EXO-D-GALACTURONANASE OF BANANA

Kvetoslava HEINRICHOVÁ

Institute of Chemistry,
Slovak Academy of Sciences, 842 38 Bratislava

Received November 18th, 1980

Exo-D-galacturonanase (E.C.3.21.67) was obtained from the aqueous extracts of banana peel by salting out with ammonium sulfate and separating the mixture of proteins on a DEAE Sephadex A-50 packed column followed by a gel filtration through Sephadex G-100 and Sephadex G-75. The purified enzyme was stable in acid medium up to 50°C. The pH optimum for the action of exo-D-galacturonanase depended upon the chain length of the degraded substrate and was found to be 4·4 for di- and tetragalacturonic acids, 4·6 for hexa- and heptagalacturonic acids, and 4·9 for polygalacturonic acid. The chain length of galacturonan affected action of the enzyme. Oligomer of polymerization degree $Dp = 6$ was the optimal substrate. The enzyme was classified as an exo-D-galacturonanase on the basis of its terminal mode of action, the ability to cleave digalacturonic acid and the decrease of viscosity in relation to the number of cleaved glycosidic bonds.

Although the changes of pectin-like substances during the growth, ripening and work-up of bananas were reported by various authors[1,2], attention to the isolation and characterization of pectolytic enzymes, exceptiog pectinesterase[3-5], has not been paid. Our preceding paper[6], dealing with the isolation and characterization of multiple forms of pectinesterase and endo-D-galacturonanase, pointed out also the presence of exo-D-glacturonanase in bananas. Presence of exo-D-galacturonanase in addition to endo-D-galacturonanase in bananas indicated the necessity to characterize the banana exo-D-galacturonanase, the further polygalacturonanase of higher plants, aiming to ascertain differences in features of polygalacturonanases with a terminal mode of action in relation with their exclusive presence in plants (as *e.g.* in carrots[7,8], cucumbers[9] and ripening apples[10]), or jointly with endo-D-galacturonanase (as *e.g.* with peaches[11], tomatoes[12] and bananas[6]). Knowledge of features and mechanism of action of both groups of these enzymes could throw more light on the function of exo-D-galacturonanases in higher plants.

EXPERIMENTAL

Material

Sodium pectate ($\overline{M}_r$ 27 000, content of polygalacturonan 75·5%) used as a substrate for activity determination of endo-D-galacturonanase was prepared from an apple pectin by a repeated alkaline deesterification with 0·1M-NaOH at pH 10 and 22°C. The deesterified product was precipitated upon adjusting the pH of the medium to pH 2·5 with sulfuric acid.

Oligogalacturonic acid (polymerization degree 10 ± 1) was obtained by an acid hydrolysis of pectic acid[13]. A product with a great scattering of the polymerization degree. which was separated by a multiple gel chromatography on Sephadex G-25 Fine and desalted with Sephadex G-10, was obtained. Criterion for the polymerization degree was the ratio of carboxyl groups determined titrimetrically and the content of reducing groups estimated by the Somogyi method[14]. Digalacturonic acid employed for exo-D-galacturonanase activity determination and oligogalacturonic acids of polymerization degree 3—8 were prepared from a partial acid hydrolysate of pectic acid by a separation gel chromatography on Sephadex G-25 (ref.[15]) and desalting on Sephadex G-10. Purity of the individual oligogalacturonic acid was checked by thin-layer chromatography on silica gel using commercial Silufol (Kavalier, Votice) plates, ethyl acetate–pyridine–acetic acid–water (5 : 5 : 1 : 3) being the developing system. The individual oligogalacturonic acids were detected with aniline–phthalate reagent and identified according to the $\log R/(1 - R)f$) values as a function of polymerization degree[16]; the reference substance was D-galactopyranuronic acid. Further criterion for the purity of oligogalacturonic acids was the ratio of carboxyl groups estimated titrimetrically and the content of reducing groups determined by the Somogyi method[14].

Methods

The endo-D-galacturonanase activity was determined from the measurement of the increment of reducing groups during the enzyme reaction by a spectrophotometric method according to[14]. The substrate was 0·5% solution of sodium pectate of pH 4·6 in a 0·1M acetate buffer. The exo-D-galacturonanase activity was estimated from the measurement of the reducing groups increment during the enzyme reaction by the same method[14] using digalacturonic acid as a substrate at pH 4·4. These substrates were used in a 1 mol l^{-1} concentration. The series of substrates (0·5 ml each) was, in general, mixed with the enzyme solution (0·5 ml) and incubated in various time intervals at 30°C. The reducing group content was determined from a calibration curve for D-galacturonic acid. The initial velocities v_i, of the enzyme reaction were calculated by a graphic extrapolation from experimental data of the reaction velocity upon time and expressed in kat per 1 kg of the protein.

The activities of endo- and exo-D-galacturonanases were differentiated on the basis of analysis of the reaction products by thin-layer chromatography on silica gel[16], as well as on the basis of action of the enzyme preparation on digalacturonic acid, which is a specific substrate for exo-D-galacturonanase.

For viscometric activity determination of exo-D-galacturonanase, solution of the enzyme (1 ml) was incubated with 0·5% galacturonic acid (10 ml) and acetate buffer of pH 4·9 (9 ml) at 30°C. The viscosity was measured with an Ubbelohde viscometer in one-hour intervals from 0—24 h. In the same time intervals also the liberated reducing groups were determined; the 50% decrease of viscosity was correlated with the amount of liberated reducing groups (with the per cent of the cleaved bonds).

RESULTS AND DISCUSSION

The enzyme was won from the ripe bananas (*Musa sapientum* var. GROS MICHEL) from Columbia. These were ice-dried and homogenized and extracted with an Ultra-Turax TP 18/2 apparatus. Of various extraction procedures[6] the best results concerning the yield of exo-D-galacturonanase were obtained when using water (100 ml per 100 g of bananas) in three instaments. The extract was worked up by centifugation at 6 000*g*, a fractional precipitation with ammonium sulfate (15–90 % saturation), dialysis and ice-drying. The product obtained in this way was separated by ultracentrifugation using the Amicon accessory and a UM 50 membrane which enabled to remove the high-molecular gel-forming polysaccharides. The activity of exo-D-galacturonanase was represented by a fraction of $\overline{M}_r$ 50 000. This product desalted by a gel filtration through Sephadex G-25 medium and ice-dried represented the enzyme crude product T_1, which was further purified ona DEAE Sephadex A-50 column equilibrated with a 0·05M phosphate pH 6·0 buffer. The separation was effected by a progressive concentration and pH gradient. The course of separation was monitored by absorbance at 280 nm. The successive elution by phosphate buffer solutions afforded four fractions (Fig. 1). The respective

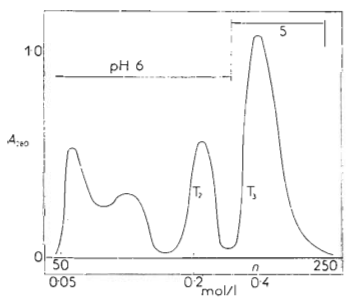

FIG. 1

Separation of the crude product T_1 by chromatography on a DEAE Sephadex A-50 packed column (3·5 × 60 cm). Elution volume 14 ml per 30 min, A_{280} absorbance at 280 nm, *n* number of fractions, *c* concentration of the phosphate buffer solution

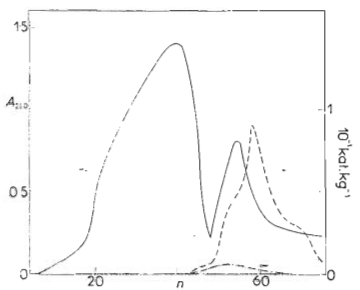

FIG. 2

Gel chromatography of combined fractions T_2 and T_3 on Sephadex G-100. The column (1·4 × 90 cm) was eluted with 0·1M acetate pH 5·0 buffer in 0·5M-NaCl. Elution volume 6 ml per 30 min. ——— Absorbance at 280 nm, – – – – activity of endo-D-galacturonanase, – · – · – activity of exo-D-galacturonanase, *n* number of fractions

fractions obtained by chromatography on DEAE Sephadex A-50 were desalted on Sephadex G-25 medium and their enzyme activity was determined. The activities of exo-D-galacturonanase and endo-D-galacturonanase were established with fractions eluted with 0·2M and 0·4M phosphate buffers (products T_2 and T_3). The activity determination of both products using equal amounts of ice-dried products with respect to the protein content, did not show substantial difference in specific activity of exo-D-galacturonanase. Therefore, fractions T_2 and T_3 were combined and the enzyme was purified by separation on a Sephadex G-100 column equilibrated with an acetate pH 6·0 buffer containing 0·5M-NaCl. Two fractions were obtained, the second of which displayed the activity of both exo- and endo-D-galacturonanase (Fig. 2). Desalting of this product using Sephadex G-25 medium gave product T_4. Aiming to separate exo-D-glalacturonanse from endo-D-galacturonanase, this fraction was further separated on Scphadex G-75 respecting the above-mentioned conditions (Fig 3). Three fractions were obtained: the first without D-galacturonanase activity, the second with only exo-D-galacturonanase activity (T_5) and the third revealed the endo-D-galacturonanase activity contaminated with that of exo-D-galacturonanase.

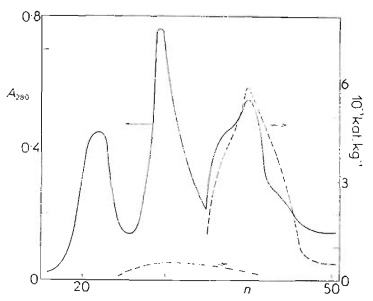

FIG. 3

Gel chromatography of fraction T_4 on Sephadex G-75. The column (1·4 × 90 cm) was eluted with a 0·1M acetate pH 5·0 buffer in 0·5M-NaCl. Elution volume 6 ml per 30 min. ——— Absorbance at 280 nm, – – – activity of endo-D-galacturonanase, –·–·– activity of exo-D-galacturonanase, *n* number of fractions

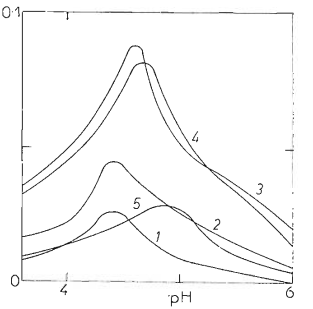

FIG. 4

The effect of pH on the velocity of reaction catalyzed by exo-D-galacturonanase. Substrates: *1* digalacturonic acid, *2* tetragalacturonic acid, *3* hexagalacturonic acid, *4* heptagalacturonic acid in a 0·1M acetate buffer (*c* 1 μmol/1 ml of reaction mixture), *5* a 0·5% sodium pectate solution in a 0·1M acetate buffer

This purification process afforded the final product T_5 21-times repurified when compared with the crude T_1. The dependence of exo-D-galacturonanase activity upon pH shows Fig. 4. The best medium for the action of the enzyme is acidic, the pH optimum being dependent on the chain length of the degraded substrate. For di- and tetragalacturonic acids it was 4·4, for hexa- and heptagalacturonic acids 4·6 and for polygalacturonic acid 4·9. The change in pH optimum in relation to the degraded substrate was ascertained[17] when studying the effect of exo-D-galacturonanase product by *Erwinia aroideae* upon defined oligomeric substrates; in accordance with our results it was found that the pH optimum is shifted towards lower values with shortening the molecule chain. A like behaviour revealed also the purified oligogalacturonide transeliminase produced by the *Pseudomonas* strain[18] and exo-D-galacturonanase of *Verticilium alboatrum*[19].

The optimum effect of the enzyme is at 50°C, and this undergoes a rapid inactivation at temperatures higher than 60°C. The purified enzyme does not fully degrade polygalacturonic acid, the limit degradation after a 60 h-hydrolysis represents 51% of the cleaved glycosidic bonds; this is associated with the fact that the action of exo-D-galacturonanase is restricted to α-1-4 bonds between D-galacturonate units. Terminal mode of action of banana exo-D-galacturonanase was also manifested by the change of viscosity upon its action on a polymeric substrate. A 50% decrease of viscosity corresponded with a 39% cleavage of glycosidic bonds (Fig. 5). The activity of banana exo-D-galacturonanase depends, in accordance with other enzymes of higher plants having terminal mode of action, on the polymerization degree of the substrate. Another common feature of the isolated enzyme with other exo-D-galacturonanases of plant origin is that it slower cleaves polygalacturonic acid as a partially degraded substrate. The different behaviour in the action of exo-D-

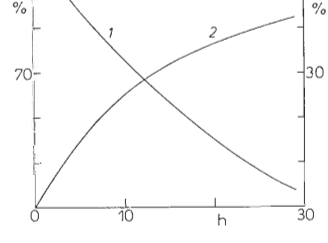

FIG. 5

The effect of exo-D-galacturonanase on the viscosity decrease in correlation with the amount of liberated reducing groups. *1* The decrease of viscosity upon time (in per cent), *2* the increment of reducing groups expressed by the per cent of cleaved glycosidic bonds. Substrate: 0·1% sodium pectate in 0·1M acetate buffer of pH 4·9

-galacturonanases so far isolated from higher plants and of banana exo-D-galacturonanase is backed by the fact that higher polymers of polymerization degree 10 to 22 are the best substrates for all hitherto described exo-D-galacturonanases[7-9,11], whereas the optimum substrate for the banana enzyme is the lower oligomer of polymerization degree 6 (Table I); this enzyme also cleaves low oligogalacturonans relatively well. This fact well correlates with one of the conceptions on physiological function of exo-D-galacturonanase of higher plants[20], the degrading effect of which is assumed after the action of pectinesterase on pectin and thus the modified substrate is affected by endo-D-galacturonanase; oligomers formed by a random cleavage are further hydrolyzed by exo-D-galacturonanase up to D-galacturonic acid.

Three velocity phases[21] were observed when investigating the kinetics of cleavage of D-galacturonan with endo-D-galacturonanase. The first, the most rapid phase involves cleavage of polymeric molecules and higher oligogalacturonans up to pentamer. The second, by 44-times slower phase, is associated with the cleavage of a tetrasaccharide and the last phase with cleavage of trisaccharide. Basing upon the degradation rate of oligogalacturonans with banana exo-D-galacturonanases, one is entitled to assume that this enzyme acts on degradation products originating primarily in the second and third velocity phases by the action of banana endo-D-galacturonanase. A little different mode of action indicates the exo-D-galacturonanase of peaches[20], the action of which was manifested at the maximum measure on the degradation products originating already in the first velocity phase by the action of endo-D-galacturonanase present in peaches; this enzyme, nevertheless, hydrolyzes well the degradation products of the second and third velocity phases.

TABLE I

Initial velocities (v_i) of degradation of galacturonic acids by banana exo-D-galacturonanase

Acid	v_i kat. kg^{-1}
Digalacturonic	0·0048
Trigalacturonic	0·0060
Tetragalacturonic	0·0078
Pentagalacturonic	0·0090
Hexagalacturonic	0·0102
Heptagalacturonic	0·0080
Octagalacturonic	0·0074
Oligogalacturonic DP ~ 10	0·0058
Polygalacturonic	0·0046

On the basis of Michaelis constant values found when investigating the action of plant exo-D-galacturonanase on oligomeric substrates[8,9,20] it was possible to assume a different behaviour of this enzyme towards substrates of various chain length; in this case, when enzymes with a random and a terminal modes of action are in coaction in plants, the exo-D-galacturonanases exhibited a higher affinity toward lower oligosaccharides than in that case, when only exo-enzyme was present in the plant.

The presented conclusions constitute a hypothesis on the basis of experimental data so far available; the generalization is incomplete for the restricted number of higher plants in which the presence and properties of D-galacturonanases were examined. Nonetheless, they indicate that for the knowledge of the function of exo-D-galacturonanases in higher plants also the detailed study of action and mechanism of action of individual plant endo-D-galacturonanases is necessary.

REFERENCES

1. Von Lossecke H. W.: *Bananas*, 2nd Ed. Interscience, New York 1960.
2. Adams M. R.: Trop. Sci. *20*, 11 (1978).
3. Hultin H. O., Levine A. S.: Arch. Biochem. Biophys. *10*, 396 (1963).
4. Hultin H. O., Levine A. S.: J. Food Sci. *30*, 917 (1965).
5. Hultin H. O., Sun B., Bulger J.: J. Food Sci. *31*, 320 (1966).
6. Markovič O., Heinrichová K., Lenkey B.: This Journal *40*, 769 (1975).
7. Hatanaka C., Ozawa J.: Agr. Biol. Chem. *28*, 627 (1964).
8. Heinrichová K.: This Journal *42*, 3214 (1977).
9. Pressey R., Avants J. K.: J. Food Sci. *40*, 937 (1975).
10. Bartley J. M.: Phytochemistry *17*, 213 (1978).
11. Pressey R., Avants J. K.: Plant. Physiol. *52*, 252 (1973).
12. Demain A. L., Phaff H. J.: Wallerstein Lab. Commun. *20*, 19 (1957).
13. McCready R. M., Seegmiller C. G.: Arch. Biochem. Biophys. *50*, 440 (1954).
14. Somogyi M.: J. Biol. Chem. 195, *19* (1952).
15. Rexová-Benková Ľ.: Chem. Zvesti *24*, 50 (1970).
16. French D., Wild D. M.: J. Amer. Chem. Soc. *75*, 2612 (1963).
17. Hatanaka C., Ozawa J.: J. Agr. Chem. Soc. Jap. *43*, 764 (1969).
18. Hatanaka C., Ozawa J.: Agr. Biol. Chem. *35*, 1617 (1971).
19. Mussel H. W., Strouse B.: Can. J. Biochem. *50*, 625 (1972).
20. Pressey R., Avants J. K.: Phytochemistry *14*, 957 (1975).
21. Rexová-Benková Ľ., Markovič O.: Advan. Carbohyd. Chem. Biochem. *33*, 323 (1976).

Translated by Z. Votický.