PURIFICATION, CHARACTERIZATION AND MODE OF EFFECT OF ANOTHER *endo*-D-GALACTURONANASE FROM *Aspergillus niger*

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Received November 11th, 1979

A mixture of pectolytic enzymes was isolated from a commercial preparation of Pectinex Ultra. *endo*-D-Galacturonanase (EC 3.2.1.15) was obtained from this mixture of enzymes, produced by *Aspergillus niger* cultures, by affinity chromatography using cross-linked pectic acid as a support and by subsequent chromatography on a Sephadex G-100 column. One form of endo-D-galacturonanase only of approximate molecular weight of 35 000 was detected in the purified product by electrophoresis in polyacrylamide gel. The enzyme showed maximal activity and stability at pH 4·9 and 40°C. The mode of degradation of a high molecular weight substrate and the per cent of the glycosidic bonds cleaved (6%) at a viscosity decrease by 50% indicate an endo type action pattern. *endo*-D-Galacturonanase was characterized by the mode of cleavage and the kinetic constants K_m and V for oligogalacturonic acids and polygalacturonic acid. On the basis of the knowledge of the kinetic constants and the degradation products the mode of action of the enzyme and the extent of its active site are discussed.

The importance and practical applicability of pectolytic enzymes have stimulated during the recent years an increasing interest of basic research in the characterization and examination of the properties of the pectolytic enzyme which is most important from the viewpoint of practical use, of *endo*-D-galacturonanase. Attention has been focused on novel isolation procedures and on the mode of action of this enzyme on naturally occurring and defined substrates. The characterization of the optimal conditions of the action and specificity permits the categorization and classification of these enzymes to be performed. This research provides information on the different mode of action of the individual *endo*-D-galacturonanases and as its final result may be expected the elucidation of the mechanism of the catalytic reaction. *endo*-D-Galacturonanases represent a good model for these studies since there are differences not only between the individual enzymes from different species but also between enzymes produced by the same biological individual[1-5]. The most marked differences have been observed in the effect of these enzymes on defined oligomeric substrates where the ability, mode, and rate of degradation are factors according to which the individual *endo*-D-galacturonanases can be distinguished and classified[6,7].

The aim of this study was to purify the *Aspergillus niger endo*-D-galacturonanase from a so far unexplored source, to obtain information on certain molecular features of this enzyme, to characterize the optimal conditions and mode of its action on poly- and oligomeric substrates, and to partly define the size of its active site. Pectinex, a commercial preparation was assayed in our Laboratory from the viewpoint of its

properties for industrial application. The favorable results of these experiments stimulated our interest in the isolation and studies of *endo*-D-galacturonanase (poly-1-4-α-D-galacturonide glycosohydrolase, EC 3.2.1.15) from this preparation.

EXPERIMENTAL

Material

The preparation Pectinex Ultra, Schweizerische Ferment AG 4056 Basel (Switzerland) containing a complex of pectolytic enzymes, *i.e.* pectin esterase, *endo*-D-galacturonanase, and *exo*-D-galacturonanase was used for the purification of *endo*-D-galacturonanase. Sodium pectate (polyuronide content 75·5%, molecular weight 27 000) used as a substrate for the determination of the activity of *endo*-D-galacturonanase was prepared from apple pectin by repeated alkaline deesterification by 0·1M-NaOH at pH 10 and 22°C. The deesterification product was precipitated after the pH of the reaction solution had been decreased to 2·5 by hydrochloric acid. Pectin (esterification degree 60%) was prepared from commercial citrus pectin according to Olsen and coworkers[8].

The oligogalacturonic acids (polymerization degree 2—7) were prepared from a partial acid hydrolysate of pectic acid by gel chromatography on Sephadex G-25 Fine and desalting on Sephadex G-10 according to a procedure described before[9]. The purity of the individual oligogalacturonic acids was tested chromatographically on a thin layer of silica gel (Silufol, Kavalier) in the mixture 1-butanol–formic acid–water (2 : 3 : 1) according to Koller and Neukom[10]. The oligogalacturonic acids were detected by aniline phthalate and identified according to their log ($R_f/1 - R_f$) values which linearly depend on the polymerization degree of oligogalacturonic acids[11]. D-Galactopyranuric acid was used as a reference sample. As another criterion of the purity of oligogalacturonic acids served the ratio of the content of carboxylic groups to the content of reducing groups[12]. 4,5-Dehydrogalacturonosyl-D-oligogalacturonides (4,5-unsaturated oligogalacturonic acids) were prepared by β-eliminative degradation of highly esterified pectin (esterification degree 95%) effected at pH 6·8 by heating in a boiling water bath.

Cross-linked pectic acid was prepared by Dr L. Kuniak, Institute of Chemistry, Slovak Academy of Sciences[13], from sodium pectate after alkaline deesterification of citrus pectin (Genu Pectin Type B Kobenhaven Pectinfabrick, Denmark).

Methods

The activity of *endo*-D-galacturonanase was determined in terms of the increase of reducing groups during the enzymatic reaction according to the method of Somogyi[14] using sodium pectate as substrate (0·5% solution of sodium pectate in 0·1M acetate buffer at pH 4·9). In a typical assay 0·5 ml samples of substrate were mixed with 0·5 ml of the enzyme solution and incubated for varying periods at 30°C. The activity was expressed in catals. The specific activity was defined as the number of catals per 1 kg of protein. In the viscosimetric assay of the activity of *endo*-D-galacturonanase 1 ml of the enzyme solution was incubated with 10 ml of 1% polygalacturonic acid and 9 ml of acetate buffer at pH 4·9 in a vessel thermostated at 30°C. The viscosity was measured in a Ubbelohde viscosimeter at 5 min intervals over the period of 0—60 min. Together with the viscosity measurement the liberated reducing groups were determined at the same intervals. A 50% viscosity decrease was correlated with the per cent of reducing groups (with the per cent of cleaved bonds). The assay of the cleavage products (with sodium pectate and oligogalacturonic acids as substrates) was carried out by thin-layer chromatography on silica gel in the system 1-butanol–formic acid–water (2 : 3 : 1) (ref.[10]);

the products were detected by the aniline phthalate reagent. The initial reaction rate was calculated by graphical extrapolation of the experimental data as a function of time and was expressed in catals per kg of protein. The determination of the Michaelis constant and of the maximal degradation rates by *endo*-D-galacturonanase was effected according to Lineweaver and Burk[15] using polygalacturonic acids and oligogalacturonic acids over the concentration range 0·1—1% of uronide and 0·025—0·5 mg of enzyme per 1 ml of reaction mixture. The incubation was carried out for 10—60 min at 30°C. To determine the value of V and of the apparent K_m always 18 experimental values of v and s were treated by linear regression. The values of K_m and V were calculated from constants a and b of the regression equation. The Michaelis constant is expressed in mol l^{-1}, the maximal rate in catals per 1 kg of protein. The approximate molecular weight of *endo*-D-galacturonanase was determined by thin-layer chromatography on Sephadex G-150 Superfine, equilibrated with 0·9% NaCl, in the apparatus manufactured by Pharmacia, Uppsala (Sweden); ribonuclease A, chymotrypsinogen, ovalbumin, and human serum albumin were used as standards. The proteins were detected by 3M bromophenol blue on paper replicas prepared on Whatman No 3MM paper. The activity of D-galacturonanase was detected on replicas prepared with the same paper which had been impregnated by 1% solution of sodium pectrate in 0·1M sodium acetate. After 20 min of incubation the paper was stained with 0·5% aqueous solution of ruthenium red. Sites at which *endo*-D-galacturonanase activity was localized appeared as a white spot on a red background. Discontinuous electrophoresis was performed according to Orstein[16] and Davis[17]. The proteins were stained with amido black 108; the activity of D-galacturonanase was detected according to Lisker and Rettig[18] by incubation of the gel in 1·25% solution of sodium pectate and by staining with ruthenium red. The proteins were determined according to Lowry and coworkers[19].

RESULTS

Purification of endo-D-*Galacturonanase*

The Pectinex Ultra preparation (30 g) was dissolved in 2 000 ml of water at 4°C with stirring. The insoluble portion was centrifuged off at 8 000 ***g***. Crude preparation L_1 was obtained by salting out the protein fraction from the supernatant, saturated with ammonium sulfate to 0·9, saturation by ethanol (four volumes of ethanol per one volume of supernatant), dialysis, lyophilization, and desalting of the lyophilisate on a Sephadex G-25 Medium column. The activity of *endo*-D-galacturonanase was effected by ultrafiltration through a UM 100 Amicon membrane; the ultrafiltration resulted in the separation of high molecular weight contaminants. Product L_2 thus obtained was subsequently purified by affinity chromatography on pectic acid, cross-linked by epichlorohydrine, which by its structure simultaneously acted as the insoluble support and as the substrate analog.

This mode of separation made use of the known fact that the interaction of pectolytic enzymes (*endo*-D-galacturonanase, *exo*-D-galacturonanase, and pectin esterase) under the pH which is optimal for *endo*-D-galacturonanase results in selective binding of *endo*-D-galacturonanase to cross-linked pectic acid whereas the remaining pectolytic enzymes as well as the contaminants present in the enzyme preparation remain in the incubation medium. *endo*-D-Galacturonanase is strongly attached to cross-

-linked pectic acid. It can be displaced from the support by several procedures. We used the procedure described by Rexová-Benková and coworkers[20] making use of the pH-dependence of the formation and decomposition of the E-S complex.

Product L_2 (400 mg) was applied to a column (4·6 × 35 cm) of cross-linked pectic acid equilibrated with 0·1 M acetate buffer at pH 4·9. The column was eluted by the equilibrating solution. *exo*-D-Galacturonanase, pectin esterase, protein contaminants, colored components, and about 50% of the *endo*-D-galacturonanase activity originally applied emerged in the hold-up volume. This protein mixture was rechromatographed on another column of cross-linked pectic acid under identical conditions as those used for product L_2. *endo*-D-Galacturonanase was no longer bound to cross-linked pectic acid during the rechromatography and this indicated that the capacity of the support was adequate for the separation of product L_2. *endo*-D-Galacturonanase bound was displaced from the column by 0·1M acetate buffer at pH 6·0. The course and the result of the separation are shown in Fig. 1. By using this procedure specific *endo*-D-galacturonanase was obtained which after desalting on Sephadex G-25 and lyophilization represents product L_3 and 46% of the quantity of *endo*-D-galacturonanase originally applied. When subjected to polyacrylamide gel elctrophoresis this product showed the presence of one *endo*-D-galacturonanase form only and of two protein

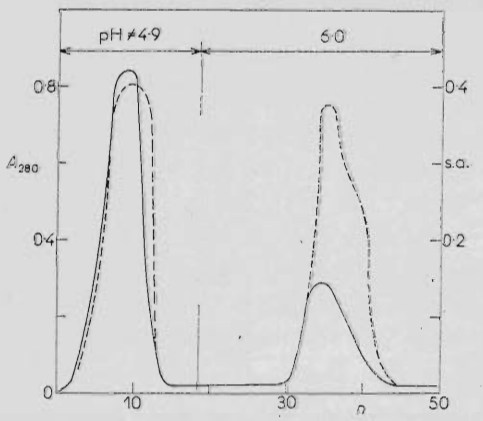

FIG. 1

Chromatography of crude product L_2 on cross-linked pectic acid. Column dimensions 4·6 × 35 cm, first peak eluted by 0·1M acetate buffer, pH 4·9, second peak eluted by 0·1M acetate buffer, pH 6·0. Elution rate 14 ml/30 min, full line absorbance at 280 nm, broken line specific activity, *n* number of fractions

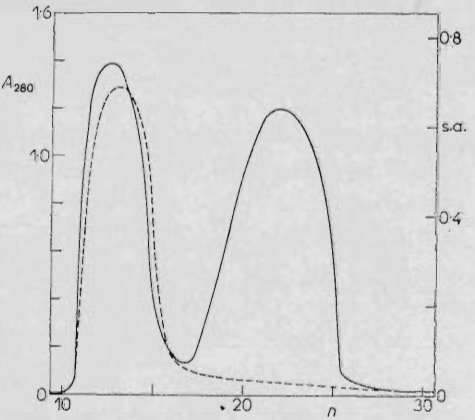

FIG. 2

Gel chromatography of fraction L_3 on Sephadex G-100. Column dimensions 1·4 × × 90 cm, eluted by 0·1M acetate buffer in 0·5M-NaCl, pH 4·9. Elution rate 6 ml/30 min, full line absorbance at 280 nm, broken line specific activity, *n* number of fractions

contaminants lacking pectolytic activity. Product L_3 was subsequently purified on Sephadex G-100 equilibrated with 0·1M acetate buffer at pH 5·0, containing 0·5M--NaCl. The *endo*-D-galacturonanase activity was detected in the first fraction only (Fig. 2). The desalting of this product on a column of Sephadex G-25 medium and lyophilization afforded final product L_4. Two protein zones were detected in this product by polyacrylamide gel electrophoresis; one zone only showed *endo*-D-galacturonanase activity. The results of the purification of the enzyme are given in Table I.

Properties of Purified endo-D-*Galacturonanase*

The effect of pH on the rate of enzyme degradation and the stability of the enzyme as a function of pH are shown in Fig. 3. The enzyme shows a relatively narrow range of pH-stability. It is labile in strongly acidic, neutral, and alkaline media. Its pH-optimum of activity lies at pH 4·9.

The effect of temperature on the effect and stability of *endo*-D-galacturonanase is demonstrated in Fig. 4. The optimum temperature for the action of the enzyme is 40°C. When exposed to increased temperature even for short periods the enzyme loses a substantial part of its activity.

Effect of endo-D-*Galacturonanase on Defined Polymers and Oligomer Substrates*

The initial rate of pectin degradation of ED = 60%, of polygalacturonic acid of Dp = 142 and Dp = 10, and of oligogalacturonic acids of Dp = 3–7 are given in Table II. It can be seen from the initial rate values of degradation of the substrates

TABLE I

Purification of *endo*-D-galacturonanase. L_0 commercial preparation Pectinex Ultra, L_1 product precipitated by ammonium sulfate and ethyl alcohol, L_2 product obtained after ultrafiltration through Amicon, L_3 fraction bound to cross-linked pectic acid, obtained by elution by acetate buffer at pH 6·0, L_4 fraction with activity of *endo*-D-galacturonanase, obtained by gel filtration on Sephadex G-100

Product	Yield g	Protein content %	Specific activity	Relative activity	% of original activity
L_0	30·00	21·0	0·25	1	100
L_1	3·16	43·8	0·44	1·7	53
L_2	1·63	62·5	0·92	3·6	50
L_3	0·76	70·0	1·82	7·2	18
L_4	0·57	82·6	2·33	9·3	17

TABLE II

Initial rate of degradation of polymer and oligomer substrates by *endo*-D-galacturonanase of *Aspergillus niger* from Pectinex Ultra

Substrate	cat. kg^{-1}
Polygalacturonic acid	1·77
Pectin, esterification degree 60%	0·03
Oligogalacturonic acid $D_p = 10$	1·21
Heptagalacturonic acid	1·00
Hexagalacturonic acid	0·61
Pentagalacturonic acid	0·44
Tetragalacturonic acid	0·33
Trigalacturonic acid	0·012

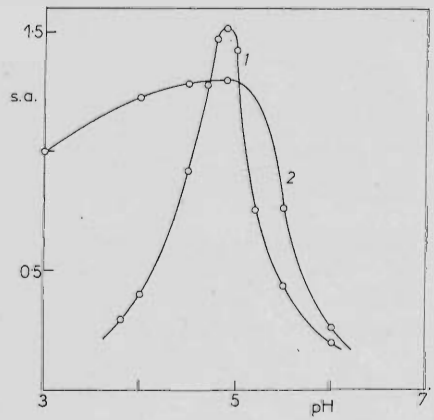

Fig. 3

Effect of pH on activity of endo-D-galacturonanase. *a* Effect of pH on rate of reaction catalyzed by *endo*-D-galacturonanase: substrate 0·5% solution of sodium pectate in 0·1M acetate buffer; *b* effect of pH on stability of *endo*-D-galacturonanase, assayed by incubation of enzyme in 0·1M acetate buffer of corresponding pH for 60 min. The activity was determined with sodium pectate as substrate after the adustment of pH to 4·9

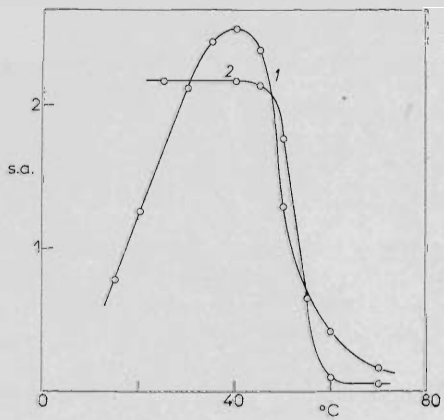

FIG. 4

Effect of temperature on activity of *endo*-D-galacturonanase. *a* Dependence of *endo*-D-galacturonanase activity on temperature: 0·5% solution of sodium acetate buffer, pH 4·9, as substrate; *b* effect of temperature on stability of *endo*-D-galacturonanase. Assayed by incubation of enzyme in 0·1M acetate buffer, pH 4·9, at corresponding temperature for 30 min. Sodium pectate as substrate

examined that the enzyme prefers the deesterified substrate to the esterified substrate, that polygalacturonic acid is the optimal substrate, and that the tetrasaccharide is the last substrate degraded at a relatively high rate.

The decrease of the viscosity of polygalacturonic acid during the enzyme hydrolysis in correlation to the number of the reducing groups liberated is shown graphically in Fig. 5. To a 50% decrease of the substrate solution correspond 6% of glycosidic bonds cleaved.

Examination of Products of Degradation by endo-D-*Galacturonanase*

When polygalacturonic acid was treated with isolated *endo*-D-galacturonanase all oligogalacturonides were obtained as degradation products. The following products (identified by thin-layer chromatography on silica gel) were obtained when the enzyme was allowed to act on the individual oligogalacturonic acids at the pH optimum, temperature of 30°C for 20 min on tetra- to hexagalacturonic acid, for 80 min on trigalacturonic acid, and for 240 min on digalacturonic acid:

Substrate*	Degradation products
$(GalpUA)_7$	$(GalpUA)_6$; $(GalpUA)_5$; $(GalpUA)_4$ $(GalpUA)_3$; $(GalpUA)_2$; GalpUA
$(GalpUA)_6$	$(GalpUA)_5$; $(GalpUA)_4$; $(GalpUA)_3$ $(GalpUA)_2$; GalpUA
$(GalpUA)_5$	$(GalpUA)_4$; $(GalpUA)_3$; $(GalpUA)_3$ GalpUA
$(GalpUA)_4$	$(GapUA)_3$; GalpUA
$(GalpUA)_3$	$(GalpUA)_2$; GalpUA
$(GalpUA)_2$	–

The enzyme reaction catalyzed by *endo*-D-galacturonanase as a function of the polymer and oligomer substrates is given in Table III.

DISCUSSION

The commercial preparation of Pectinex Ultra (30 g) afforded 570 mg of purified *endo*-D-galacturonanase of total activity 1 096 catal containing 82·6% of proteins. Polyacrylamide gel electrophoresis showed the presence of one form of *endo*-D--galacturonanase only of approximate molecular weight 35 000. The final *endo*-D--galacturonanase preparation contained a small quantity of one enzymatically inactive protein. (The presence of the inactive protein components in the final purified

* $(GalpUA)_n$ oligogalacturonic acid of polymerization degree *n*.

product is most likely related to the slightly basic character of this component which was probably affected by the ion-exchange character of the support[32].) The enzyme showed maximum stability and activity at pH 4·9 and 40°C. The enzyme purified was characterized in terms of its effect on the polymer substrate; all oligogalacturonides were determined as products of reaction. When the polymer substrate was used and the viscosity decreased by 50% about 6% of the internal glycosidic bonds were cleaved; this indicates a random manner of cleavage. The absorbance at 235 nm did not change during the action of the enzyme and this indicates that the cleavage of the glycosidic bonds proceeds *via* a mechanism of acid hydrolysis. We observed in experiments with *endo*-D-galacturonanase from Pectinex Ultra that the initial rate

TABLE III

Kinetic constants of *endo*-D-galacturonanase from Pectinex Ultra

Substrate	K_m mol l^{-1}	V_{max} cat. kg^{-1}
Polygalacturonic acid	$(0{\cdot}34 \pm 0{\cdot}001) \cdot 10^{-3}$	$1{\cdot}73 \pm 0{\cdot}06$
$(GalpUA)_6$[a]	$(0{\cdot}70 \pm 0{\cdot}009) \cdot 10^{-3}$	$1{\cdot}35 \pm 0{\cdot}11$
$(GalpUA)_5$	$(0{\cdot}89 \pm 0{\cdot}008) \cdot 10^{-3}$	$0{\cdot}86 \pm 0{\cdot}05$
$(GalpUA)_4$	$(1{\cdot}82 \pm 0{\cdot}05) \cdot 10^{-3}$	$0{\cdot}48 \pm 0{\cdot}03$
$(GalpUA)_3$	$(3{\cdot}67 \pm 0{\cdot}08) \cdot 10^{-3}$	$0{\cdot}10 \pm 0{\cdot}14$

[a] $(GalpUA)_n$ oligogalacturonic acid of polymerization degree *n*.

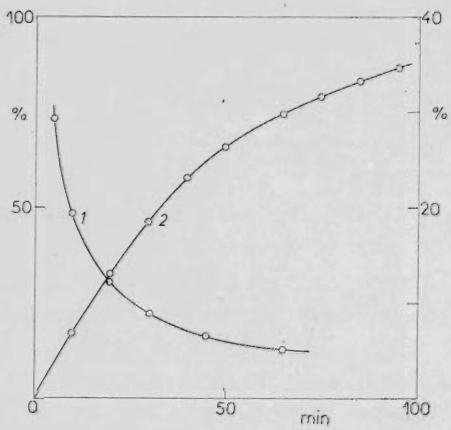

FIG. 5

Effect of *endo*-D-galacturonanase on viscosity decrease in correlation with number of reducing groups liberated. *1* Decrease of viscosity in % with time; *2* increase of reducing groups expressed in % of reducing groups cleaved: 1% solution of sodium pectate in 0·1M acetate buffer as substrate.

of pectin degradation of esterification degree 60% is 50 times lower than with polygalacturonic acid.

The examination of the effect of *endo*-D-galacturonanases on the polymer substrate, which represents a very heterogeneous material, provides information on the basic features of their effect only yet in no case permits us to get an insight into the mode of their degradation and the mechanism of the reaction. A more detailed picture will emerge from experiments with homogeneous, defined substrates of various polymerization degrees where the branching of the molecule and the presence of neutral saccharides are without effect and the degradation products can exactly be determined. We assayed therefore the effect of purified *endo*-D-galacturonanase on di- to heptagalacturonic acid, characterized the degradation products, determined the initial rates of the degradation of substrates and the kinetic constants K_m and V.

The action of the enzyme on the individual oligogalacturonic acids leads to the following degradation products: digalacturonic acid was not degraded by the enzyme. trigalacturonic acid was cleaved to the disaccharide and D-galactopyranuric acid, tetragalacturonic acid was cleaved in one manner only, namely to the trisaccharide and D-galactopyranuric acid. The products of cleavage of penta-, hexa-, and heptagalacturonic acid are all the lower oligogalacturonides and the monosaccharide. The manner of degradation was not dependent on the initial concentration of substrates in any case.

It follows from the initial rate of degradation of tri- to heptagalacturonic acid that the hydrolysis rate decreases with the decreasing length of the chain of oligomer substrates. Tetragalacturonic acid is the lowest oligosaccharide which is still degraded at a relatively high rate. The trisaccharide is cleaved very slowly, approximately 30-times more slowly than the tetrasaccharide and 167-times more slowly than the polymer substrate. The value of the maximum degradation rate V decreases with the decreasing length of the oligomer substrates; the value of the apparent Michaelis constant K_m simultaneously increases. As regards the V-values there is a big gap between the tri- and tetrasaccharide whereas the difference between the tetra- and the pentasaccharide was smaller.

It follows from the character of the products of degradation of the individual oligogalacturonic acids that the *endo*-D-galacturonanase present in the preparation Pectinex Ultra differs in its mode of action from the *endo*-D-galacturonanase isolated from Pectinase Koch-Light[21] and from the *endo*-D-galacturonanase of *Aspergillus niger* isolated by Koller[22]. The main difference is in the specific cleavage of tetragalacturonic acid and in the alternative modes of cleavage of penta- and hexagalacturonic acid. Judging by the mode of action belong to the group of endo-D-galacturonanases degrading by mode *I* (ref.[7]) the *endo*-D-galacturonanase of *Sacharomyces fragilis*[23,24], *Aerocylindrium*[25], and the extracellular *endo*-D-galacturonanase of *Aspergillus niger*[6,26].

The active center of the enzymes catalyzing the degradation of polymer substrates

by the *endo* mechanism is assumed to involve a certain number of binding subcenters which are geometrically complementary to the monomer units of the polymer substrate[27-29]. Each subcenter can bind the monomer unit of the substrate molecule in a certain orientation. The affinity of the enzyme for the substrate and the mode of its degradation are functions of the length of the substrate molecule, *i.e.* of the number of monomer units and of the number of subcenters in which the interaction takes place. The correctness of this concept has been verified experimentally with lysozyme[30].

From the viewpoint of this interpretation and on the basis of the knowledge of the mode of degradation and the number of productive complexes of *endo*-D-galacturonanase from Pectinex Ultra and the values of initial degradation rates determined with the individual oligogalacturonides, we assume that the enzyme isolated by us has an active center similar to that described for the *endo*-D-galacturonanase from *Aspergillus niger*[6,26]. According to this model the active center of the enzyme is composed of four subcenters the catalytic groups being situated between the first and second subcenter. To verify this assumption it would be necessary to determine the kinetic constants K_m and V also for the homologous series of higher oligogalacturonic acids and to complement the kinetic constants by a study of the degradation of the modified analogs of the individual oligogalacturonides.

Even though it would appear from the mode and extent of degradation of the oligomer substrates that the *endo*-D-galacturonanase from the commercial preparation of Pectinex Ultra has similar properties to the extracellular *endo*-D-galacturonanase of *Aspergillus niger*[6], certain differences were observed in the behavior of these two enzymes. These are the pH and temperature optimum and the stability of these two enzymes. The two crude enzyme preparations also showed a different behavior when purified by affinity chromatography on cross-linked pectic acid. When a mixture of extracellular pectolytic enzymes produced by *Aspergillus niger*[26] was allowed to interact at the pH-optimum with cross-linked pectic acid practically all of the *endo*-D-galacturonanase present in the mixture applied to the support was bound. Only a part of *endo*-D-galacturonanase (46%) was bound, similarly to *endo*-D-galacturonanase from *Goetricium candidum*[31], from our preparation even under the optimum conditions of the formation and decomposition of the enzyme–substrate complex and even when the capacity of the support was sufficient. We studied therefore also the properties of the *endo*-D-galacturonanase which was not bound to cross-linked pectic acid and was present together with *exo*-D-galacturonanase, pectin esterase, contaminant proteins, and the colored components in the fraction emerging in the elution volume of the equibrating buffer. The results of these experiments led to the conclusion that the *endo*-D-galacturonanase not bound to the support is not identical to purified *endo*-D-galacturonanase; they differ in the pH-optimum of enzymatic effect (4·0–4·2) and in electrophoretic mobility. The fact that the bond of the complex *endo*-D-galacturonanase–cross-linked pectic acid mediated by the

binding site of the enzyme depends on the size of the active site of the enzyme[26,32] and on the cross-linkage degree of the support could explain the difference in the behavior of these two enzymes during affinity chromatography; this is even more probable since in this study one type only of the support of cross-linkage degree (DSC.L) 0·2–0·25, $V = 3{\cdot}5$ ml g^{-1} was used and the conditions of the interaction of the enzyme with the affinity support did not change.

Another characteristic of purified *endo*-D-galacturonanase represents its behavior which is different if the enzyme is present in a complex with the remaining pectolytic enzymes and different if it is in pure state. The crude enzyme preparation Pectinex Ultra, a complex of enzymes has a high stability and shows a strong activity toward naturally occurring substrates, yet also a high specific activity of D-galacturonanase toward model substrates (own unpublished results). Purified *endo*-D-galacturonanase is thermolabile, sensitive to the pH of the reaction medium even at pH-values very close to the optimum. It is negatively affected also by every purification procedure This can explain the fact that the specific activity of the enzyme increased ten times only after it had been purified to a high degree (82·6% of protein).

The results obtained indicate that even enzymes produced by the same biological individual show differences in properties which most likely are related to the conditions of cultivation and growth of the microorganism as well as to the origin of the enzyme (extra-intracellular). It is therefore necessary to characterize each individual enzyme as regards its properties and mechanism, mode and extent of action to learn the conditions of its optimal exploitation.

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Translated by V. Kostka.