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Investigation of Enzymatic Degradation of Pectin Polysaccharides under Limiting Conditions

N. Yu. Selivanov*, I. V. Sorokina, O. G. Selivanova, O. I. Sokolov, and V. V. Ignatov

Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, pr. Entuziastov 13, 410049 Saratov, Russia; fax: (452) 970-383; E-mail: selivanov@ibppm.sgu.ru

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Abstract—The dynamics of changes in spectra of oligosaccharide fragments formed during enzymatic degradation of plant pectins at low enzyme/substrate ratio was studied. It is shown that degradation of deesterified pectin molecules is a discrete and determined process manifested in establishment of a stable polysaccharide spectrum. It is noted that introduction of chemical modifications into the polysaccharide substrate structure preserves the discreteness of the polymer molecule fragmentation but changes the spectrum of formed oligosaccharide fragments. It is supposed that degradation is defined by the spatial (three-dimensional) organization of the polysaccharide molecule.

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Pectins are specific plant polysaccharides. As a rule, a pectin polysaccharide molecule is characterized by a high extent of polymerization (n = 300-800) and consists of parts having different composition and structure (linear homogalacturonan, branched rhamnogalactouronan I, and rhamnogalactouronan II with a high content of neutral carbohydrates both in the main and side chains) [1-3]. The homogalacturonan structure is conserved, with only slight variations being noted in individual plant species [1]. The main difference was found in the content and distribution of the carboxyl group modifications in the polysaccharide molecule.

It has been found that carbohydrate structures with middle and low extent of polymerization (oligosaccharides) exhibit biological activity, whereas high molecular weight carbohydrate polymers are usually information-inert [4, 5]. The main form of activity is induction of various physiological processes [6]. This suggests that oligosaccharides are important extracellular signal regulators in plants. Thus, short oligosaccharides with polymerization extent of 2-4 stimulate ethylene biosynthesis that regulates fruit ripening [7, 8], whereas oligomers with polymerization extent of 7-12 induce biosynthesis of secondary metabolites and protective enzymes (chitinase, β-1,3-glucanase, etc.) [6]. Oligogalacturonides with polymerization extent of 10-14 are involved in regulation of morphogenesis; for example, they induce formation of flowers in a culture of thin-layer tobacco explants and inhibit root formation in a culture of the tobacco leaf explants [9, 10]. Oligosaccharides formed during enzymatic degradation of the cell wall homogalacturonan are involved in plant growth regulation [11].

In most cases, oligogalacturonides are active at very low concentrations (10^{-7} M), and fragments with polymerization extent of 12-14 exhibit the highest activity [12]. At the same time, oligogalacturonides exhibit "anti-auxin" activity against explants of plant organs and whole plants at a rather high concentration (10^{-4} M) [13], whereas in suspension cell cultures such activity is registered at a concentration of 10^{-7} M [6].

Formation of functionally active molecules and their subsequent degradation in plant cells and tissues happen during enzymatic processing mediated by a complex of pectolytic enzymes of different specificity, including exo- and endopectinases as well as a number of lyases and esterases [14]. Deesterified regions of homogalacturonan serve as substrates for endopolygalacturonases of plant and microbial origin. The deesterified oligogalacturonides exhibit biological activity [1]. It is assumed that deesterification and change in distribution of the carboxyl group methylation in the pectin polymer molecule is one of basic mechanisms of regulation of degradation. This important function is carried out by a group of pectin-methyl esterases [15-17]. As a whole, the availability of

* To whom correspondence should be addressed.

the homogalacturonan molecule as a regular polymer substrate for enzymatic degradation is mainly defined by substrate specificity of pectolytic enzymes.

However, the described model of enzymolysis and its regulation does not answer a very important question: is formation of physiologically active oligosaccharides of a certain degree of polymerization a regulated and directed process, or is it a process of nondirectional chaotic degradation of carbohydrate polymers? It is not clear what defines the size of formed oligosaccharides in the course of enzymatic degradation of a highly deesterified pectin polymer. Answers to these questions will help us to understand the mechanisms of formation of oligosaccharides exhibiting different biological activities.

Investigation of the mechanism of exopectinase activity clarifies formation of small oligosaccharides (n = 2-4). In this case, the oligosaccharide size is defined by substrate specificity and the size of the binding site of the enzyme active center [18, 19].

In most works investigating enzymatic degradation of pectin polysaccharides, the products formed during "deep" or comprehensive hydrolysis of a polysaccharide polymer are analyzed. In most of these cases, degradation is carried out at a high enzyme concentration [20].

In the case of exhaustive enzymolysis by endopectinase, the restriction of degradation and determination of fragmentation are defined by the amount and the character of intramolecular distribution of esterified and deesterified regions in the homogalacturonan molecule [19].

In the case of complete deesterification of a pectin molecule, the main factor that defines the extent of polymer fragmentation is substrate specificity of the enzymes, their ability to recognize as substrate the polysaccharide fragments with the polymerization extent not below a critical level that defines the constant of enzyme affinity. It was shown by exhaustive hydrolysis of pectin by exo- and endopectinases that the end-product distribution by polymerization extent is characterized by high extent of homology with domination of mono-, di-, and trioligosaccharides [18, 21, 22].

Here we describe the results of investigation of oligosaccharide formation during the initial stages of enzymolysis of deesterified pectin, as well as the effect on this process of artificial chemical modifications of the polymer.

MATERIALS AND METHODS

Analytical methods. Gel chromatography of poly- and oligosaccharides was carried out on a column (1.6 × 72 cm) of TSK-Gel Toyopearl-HW 55F in 30 mM sodium phosphate buffer, pH 7.2. Anion-exchange chromatography of enzymolysis products was carried out on a Mono-Q HR5/5 column in an FPLC system (Pharmacia, Sweden) in 20 mM Bis-tris-propane-HCl buffer, pH 7.2, in a 0-0.5 M NaCl gradient. Carbohydrates were detected using a Uvicord SII (LKB, Sweden) at λ = 226 nm.

Enzyme purification. A commercial preparation of pectinase (EC 3.2.1.15) of *Aspergillus niger* (Fluka, Switzerland) was purified by anion-exchange chromatography on a Mono-Q HR5/5 column in an FPLC chromatographic system (Pharmacia). The sample was preliminarily transferred into 20 mM piperazine-HCl buffer, pH 5.5, using a PD-10 column (Pharmacia). Protein was eluted in a 0-0.7 M NaCl gradient. The eluate was detected photometrically at λ = 280 nm. Polygalacturonase activity of fractions was determined quantitatively by the dinitrosalicylic method [23, 24]. Qualitative determination of pectinase isoforms in fractions was performed by enzymography. Electrophoresis was carried out under standard conditions in polyacrylamide gel plates (17 × 12 × 0.1 cm) using the buffer solution system of Laemmli [25]. The separating gel contained 12% acrylamide and 0.5 mg/ml deesterified citrus pectin. After electrophoresis, the gel was incubated overnight in 0.5 M sodium acetate buffer, pH 4.5, at 37°C, washed in bidistilled water, and stained with 0.05% Ruthenium Red solution for 60 min. Enzyme activity was revealed as light zones of various intensity against the colored background.

Preparation of deesterified pectin. A commercial preparation of citrus pectin (Sigma, USA) containing 80% galacturonic acid (esterification extent 9%) was purified by reprecipitation with three volumes of 96% ethanol. Demethoxylation was carried out in 0.1 M NaOH under stirring for 30 min at room temperature [26]. The resulting solution of deesterified pectin was neutralized with 0.5 M citric acid to pH 6.0 and precipitated with three volumes of 96% ethanol. The precipitate was separated, dehydrated with acetone, and air-dried at 50°C.

Preparation of pectin partially modified by glucosamine. Deesterified pectin (100 mg) was dissolved in 20 ml bidistilled water, 16 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Fluka, Switzerland) and 27 mg of β-D-glucosamine hydrochloride were added, and the mixture was titrated with 0.5 M HCl to pH 4.7. The reaction was carried out at room temperature for 4 h under stirring. The assigned pH value was maintained using 0.5 M HCl solution. When the reaction was over, the mixture was precipitated with three volumes of 96% ethanol and kept overnight at 4°C. The precipitate was separated by centrifugation and washed with acetone. The sample was air-dried at 50°C. The glucosamine content in the product was determined in an AAA T 339 amino acid analyzer (Mikroteknika Praha, Czech Republic) after acid hydrolysis of the sample with 2 M solution of trifluoroacetic acid at 100°C for 4 h. The glucosamine content in the sample was 4% (w/w), which corresponds to modification of 3.8% of the galacturonic acid residues in the polymer.

Enzymatic hydrolysis of pectin preparations. Pectin polysaccharide (10 mg) was dissolved in 2.5 ml 0.04 M sodium acetate buffer, pH 4.5, 27 μl of purified enzyme preparation (activity 2.66 U/ml) in 0.02 M piperazine-HCl buffer, pH 5.5, were added, and the mixture was stirred and incubated at 37°C. Aliquots were taken at 1, 2, and 3 h. The reaction was stopped by boiling, samples were stored at −20°C, and the products of enzymolysis were analyzed by anion-exchange chromatography.

RESULTS AND DISCUSSION

At early stages of the pectin polymer degradation by endopectinase, the site of enzyme attack is not defined by the polymer or its fragment primary structure as in the case of exopectinase degradation [18]. Three-dimensional structure of the polymer molecule, formed due to inter- and intramolecular interactions of galacturonic acid residues, may be a factor regulating the availability of different sites of this molecule to the enzyme action [22, 27, 28]. To reveal the effect of this factor, we studied the spectrum of oligosaccharide fragments formed under the action of a purified isoform of pectinase of *A. niger* at early stages of enzymolysis of two model pectin polymers, deesterified citrus pectin and its lowly substituted conjugate with glucosamine.

Preparation of model pectin polymers—pectinase substrates. Since pectinase (EC 3.2.1.15) specifically hydrolyzes α-1,4-bonds of only deesterified sites of pectin polymer, the citrus pectin deesterified to provide for even availability of all polysaccharide sites to enzyme action was used as a substrate. Deesterification was carried out by the "mild" alkaline hydrolysis of ether bonds, which preserves a sufficiently high extent of the pectin molecule polymerization upon uniform remoteness of O-methyl groupings of the galacturonic acid carboxyls [26]. As shown by chromatographic analysis, the molecular mass of the deesterified homogalacturonan was 150 kD.

To evaluate the effect of substrate restrictions on the character of enzymatic degradation of the pectin molecule, artificial chemical modifications of carboxyl groups were introduced into the pectin polymer structure. To achieve this, a fraction of the galacturonic acid residues were conjugated to an amine-containing ligand. In this case, the carboxyl important for enzyme binding was neutralized and an additional side substituent was introduced into the linear polymer structure. The joined ligand was glucosamine. Conjugation was carried out using the carbodiimide condensation technique that makes it possible to cross-link chosen components at amino- and carboxy-groups under mild conditions with amide bond formation [29]. Polysaccharide preparations in which only 3.8% of galacturonic acid residues are ligated by glucosamine were used in this work.

In the case of artificial modifications of pectin polymer by chemical reactions, the incorporation of ligand molecules is supposed to be spontaneous. As a result, the arrangement of sites available for the enzyme may significantly differ from the "natural" distribution of deesterified sites formed in the pectin molecule by pectin-methyl esterases. Besides, spatial organization of the polymer molecule may be altered due to a change in its charge and blocking some of the carboxyl groups. Similar aggregates do not appear in nature, but the presence of the amide bond is able to provide for preservation of structural and physicochemical properties both of the ligand and carbohydrate polymer [30].

Purification of pectinase. Most commercially available pectinase preparations are not very suitable for fine enzymatic investigations because they are mixtures of enzyme isoforms and are significantly contaminated by other proteins and stabilizing additives and also often exhibit a different glycosidase activity [18, 19].

Fractionation of a commercial pectinase preparation (EC 3.2.1.15) from *A. niger* (Fluka) with initial activity of 1.52 U/mg resulted in eight fractions exhibiting pectolytic activity (Fig. 1). The isolated fractions differed by the types of activity or by conditions of its expression (table). Three types of enzyme isoform were detected by enzymography: low-mobility highly active (fractions *1-3*), low-mobility low activity (inert) (fractions *4-6*), and high-mobility (fractions *7* and *8*). It should be noted that under the enzymography conditions endopeptidase activity of the enzyme is revealed more intensely, whereas the

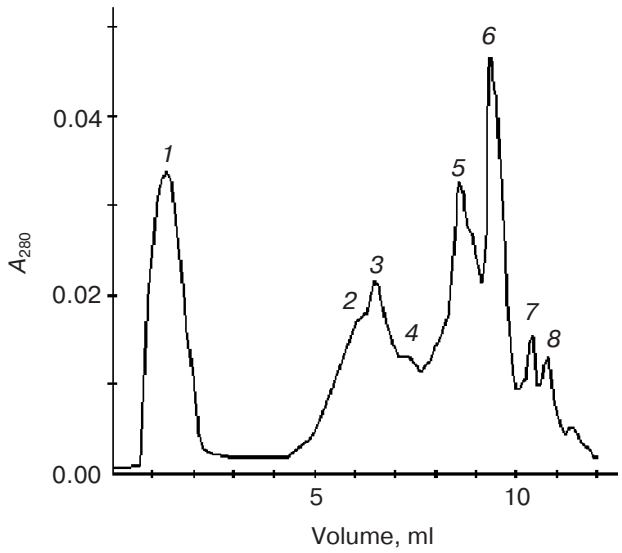

Fig. 1. Fractionation of a commercial preparation of pectinase from *A. niger* on a Mono-Q column. *1-8*) Fractions exhibiting pectinase activity. Sample concentration 13.3 mg/ml, volume 1.0 ml. Elution was carried out in a 0-0.7 M NaCl gradient in 0.02 M piperazine-HCl buffer, pH 5.5. Flow rate 0.5 ml/min. The eluate was followed at λ = 280 nm with a Uvicord SII (LKB).

Quantitative characteristics of pectinase activity of the enzyme chromatographic fractions by the dinitrosalicylic method

Protein fraction number	Specific activity, U/ml	Total activity of fraction, U
1	2.66	3.19
2	1.01	1.82
3	1.95	1.56
4	1.44	0.58
5	1.24	0.87
6	2.45	1.72
7	0.74	0.22
8	0.19	0.06

Note: U, μmol/min.

dinitrosalicylic method determines total hydrolase activity (exo- and endopeptidase). The comparison of the data shows that fraction *1* contains polypeptides not adsorbed on the column and is represented by endopectinase exhibiting the highest activity in both tests. Fractions *4-6* are evidently represented by exoglycosidases, because their specific activity is high in determination by the dinitrosalicylic method and is slightly revealed by enzymography. Fraction *1* with activity of 2.66 U/ml was used below.

Enzymolysis. Since α-1,4-glycoside bonds between galacturonic acid residues are the main target for the enzyme, the substrate and enzyme amounts were calculated so that 8-10% of glycoside bonds should undergo degradation in 1 h. In this case, the concentration of glycoside bonds in all samples was 20-25 mM. To achieve spontaneous and equally probable polymer cleavage, the initial molar ratio of enzyme and polymer substrate was chosen at the level of 1 : 50. In this case molar ratio of enzyme and the amount of glycoside bonds was 1 : $(4.5 \cdot 10^4)$. The enzymolysis conditions were determined based on 100% accessibility of glycoside bonds to the enzyme. Enzymolysis was restricted by the time of reaction.

Analysis of changes in the oligosaccharide spectrum during enzymolysis. The efficiency of enzymolysis of the polymer was evaluated by chromatography. Analysis of products by gel filtration showed that degradation of both model polymers is efficient and in 60 min it results in formation of clearly differentiated peaks corresponding to the high molecular weight (>50 kD) and low molecular weight (6-10 kD) products. Enzymolysis of deesterified pectin is characterized by a higher extent of destruction of the initial polymer and homogeneity of the formed low molecular weight products compared to the results of the degradation of the modified substrate (Fig. 2). Ion-exchange chromatography on a strong anionite (Mono-Q HR5/5 column) was used for more detailed analysis of enzymolysis products.

The chosen way of analyzing the enzymatic degradation products with separation of oligosaccharide fragments by charge is advantageous over the traditionally used method of separation by polymerization extent (Fig. 2) because it exhibits higher resolving power and enables efficient separation of the medium and high polymer fragments (Figs. 3 and 4). In this case, the monomer is

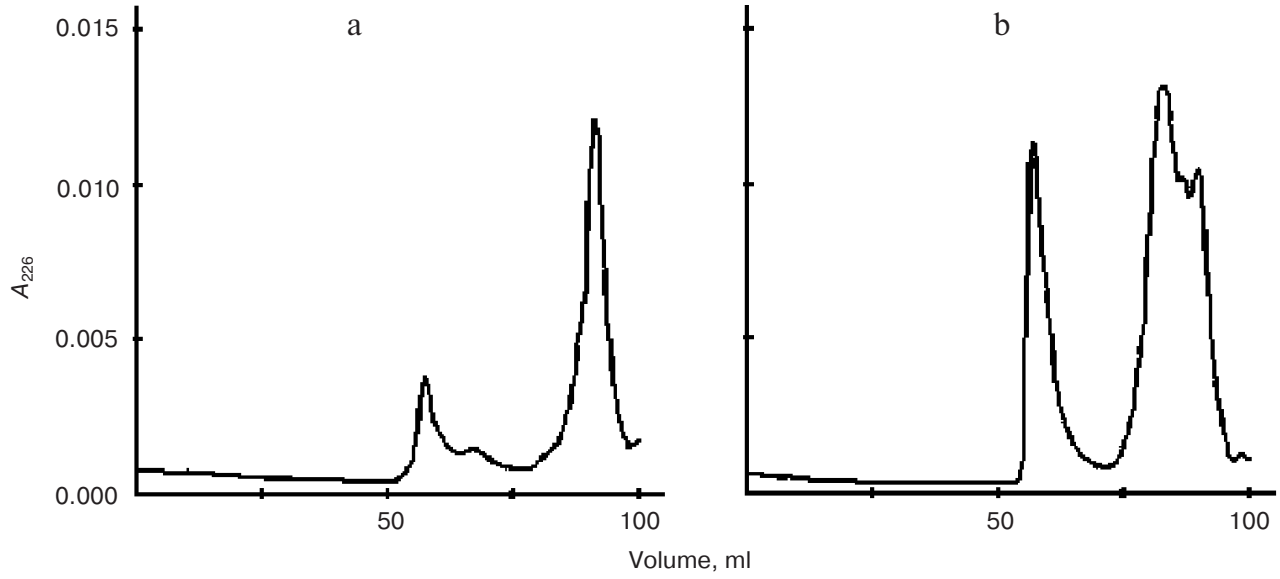

Fig. 2. Gel filtration of enzymolysis products on a Toyopearl TSK-Gel HW 50 F column (1.6 × 74 cm). a) Deesterified citrus pectin; b) deesterified citrus pectin partially modified by glucosamine. Enzymolysis time, 60 min; 1.5 ml solution contained 1.6 mg sample; elution in 0.03 M sodium phosphate buffer, pH 7.2; flow rate, 30 ml/h; detection on a Uvicord SII at λ = 226 nm.

eluted from the column in the void volume. It is important that in the ion-exchange chromatography of most charged poly- and oligosaccharides of pectin and algin nature the process of separation is defined by both the charge interaction and adsorption parameters of these components, which also define their ability for intermolecular interaction.

Chromatographic analysis of products of enzymolysis of the deesterified pectin revealed pronounced discreteness of polymer destruction. The process of degradation is characterized by practically complete destruction of the initial polymer and formation of two dominating peaks of high molecular weight highly charged components. The time-dependent dynamics of the process under the chosen conditions is shown as a gradual change of the formed fragment ratio. In this case, the quantitative degradation of one of the highly charged fragments is observed along with an increase in the fraction of the lowly charged ones (Fig. 3).

The dynamics of enzymolysis at early stages of enzymatic degradation of deesterified pectin polymers and those partially modified by glucosamine is characterized similarly. Introduction of artificial modifications into the structure of pectin polysaccharides changes the process of fragmentation, which is revealed in changes of spectra of formed oligosaccharide fragments and in increased heterogeneity of their composition (Fig. 4). Some of the substrate does not undergo degradation and formed oligosac-

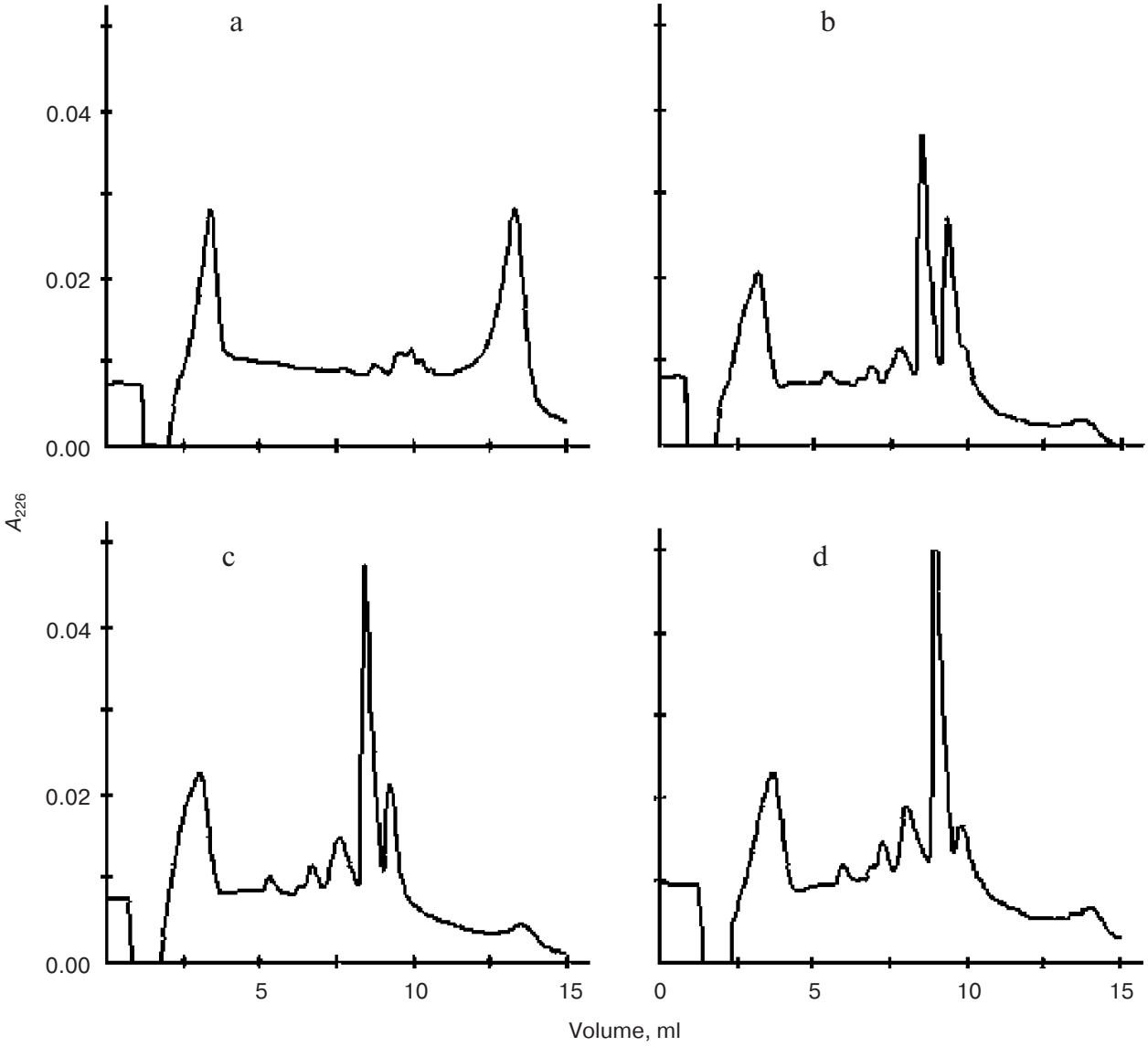

Fig. 3. Anion-exchange chromatography of pectin oligosaccharides produced by limited enzymolysis of deesterified citrus pectin on a Mono-Q HR5/5 column in an FPLC system. Enzymolysis time was 0, 60, 120, and 180 min ((a)-(d), respectively); 200 µl solution contained 800 µg sample; elution in 0-0.5 M NaCl gradient in 0.02 M Bis-tris-propane-HCl buffer, pH 7.2; flow rate, 0.5 ml/min; detection using a Uvicord SII at $\lambda = 226$ nm.

charide fragments have a lower charge along with increased amount of separated fractions. In this case, the spectrum of oligosaccharide fragments changes similarly, but two fractions of highly charged high molecular weight fragments undergo sequential degradation.

Analysis of the data shows that a stable set of oligosaccharide components of different charge is formed under conditions of limited enzymolysis of both substrates. The character of spectral changes is indicative of the existence of structural determination of fragmentation of these pectin polymers (Figs. 3 and 4). Introduction of glucosamine modifications enhances the discreteness of polymer fragmentation, which can be explained by facilitation of the initial step of this process by changing structural organization of the molecule.

It is important to note that dimensional characteristics and quantitative distribution of oligosaccharides formed during limited enzymolysis clearly differ from the distribution well described in the literature and obtained upon chemical and deep enzymatic hydrolysis of carbohydrate polymers, the peculiarity of which is the prevalence of monosaccharides and low molecular weight oligosaccharides [22]. It is possible that the process is defined by properties of the initial polymer chain and changes in details after introduction of artificial modifications. In this case, fragmentation of a highly polymeric

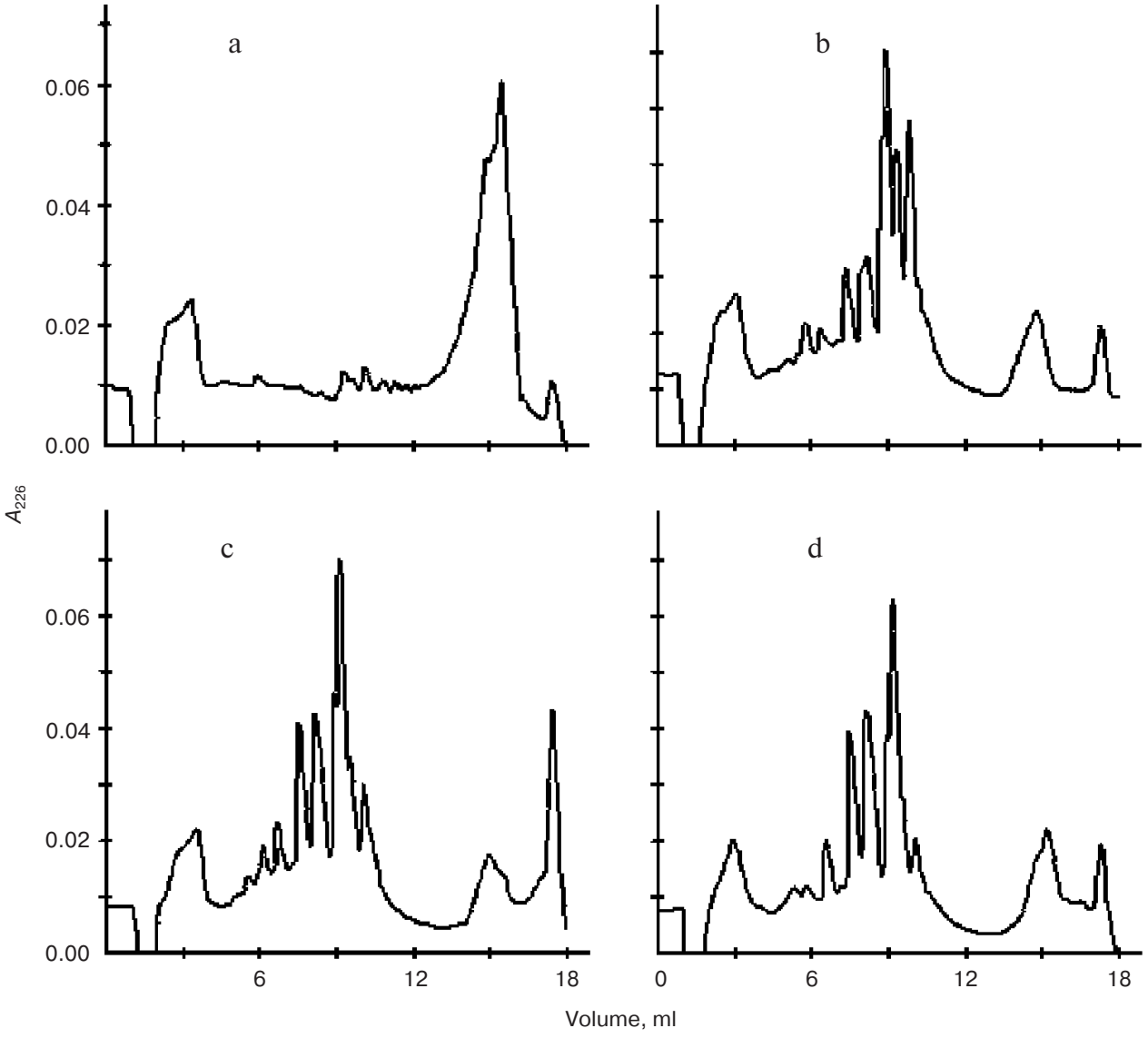

Fig. 4. Anion-exchange chromatography of oligosaccharides obtained by limited enzymolysis of deesterified citrus pectin partially modified by glucosamine on a Mono-Q HR5/5 column in an FPLC system. Enzymolysis time was 0, 60, 120, and 180 min ((a)-(d), respectively); 200 µl solution contained 800 µg sample; elution in 0-0.5 M NaCl gradient in 0.02 M Bis-tris-propane-HCl buffer, pH 7.2; flow rate, 0.5 ml/min; detection using a Uvicord SII at $\lambda = 226$ nm.

pectin molecule at the initial stage probably depends on its three-dimensional configuration. The most accessible exposed sites—"outer hairpins" of a friable ball or globule—first undergo enzymatic attack.

It should be noted that during enzymolysis in the pectin-polymer–endopectinase system the polymer substrate concentration increases in geometric progression, which can be also a factor limiting degradation of separate fragments due to the concentration excess of the substrate. On the background of high competition of substrate molecules, the establishment of substrate selection is possible under these conditions, i.e. generation of fragments or conformations with a higher affinity to the enzyme, which results in more rapid cleavage.

Limited enzymolysis of pectin polysaccharides may be revealed in different forms. In particular, the effect of spatial organization of pectin polymers on their fragmentation can significantly increase under conditions of high substrate concentrations and its immobilization observed in the plant cell wall *in vivo*. Structural restrictions can be decisive in formation of biologically active oligosaccharides.

Our results show that under low enzyme concentrations selective degradation of high molecular weight fragments of pectin polysaccharides takes place. The process of pectin molecule degradation proceeds discretely and determinedly with formation of a stable spectrum of oligosaccharides. Introduction of chemical modifications into the polysaccharide substrate structure preserves the discreteness of its fragmentation but changes the spectrum of formed oligosaccharide fragments. It is supposed that the determinedness of degradation is caused by three-dimensional organization of the polysaccharide molecule, which is defined by intramolecular bonds of the pectin molecule, which are changed upon partial modification of its carboxyls.

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