

ENZYMIC DEGRADATION OF PECTIC ACID

XII. Structure of the Deoxysugar Produced from Unsaturated Oligo- and Polygalacturonic Acids by one of the Exopolygalacturonases of *Aspergillus niger*

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Previous papers (1, 2) reported a new type of exopolygalacturonase produced by *Aspergillus niger*. By the action of this enzyme galacturonic acid and an unknown compound are formed from 4, 5-unsaturated digalacturonic acid. The unknown compound produced can be separated from galacturonic acid by DEAE-cellulose chromatography. The present paper describes the results of a study made on the chemical structure of this unknown compound.

The exopolygalacturonase of *Aspergillus niger* hydrolyzes also unsaturated pectic acid to yield an unknown compound and galacturonic acid. This unknown compound proved in this study to be identical with the above unknown compound from 4, 5-unsaturated digalacturonic acid.

MATERIALS AND METHODS

The exopolygalacturonase hydrolyzing 4, 5-unsaturated galacturonidic linkage. According to the method previously described (1), a mycelial extract was treated with Duolite CS-101 and chromatographed on a DEAE-cellulose column. The eluate containing the enzyme hydrolyzing 4, 5-unsaturated galacturonidic linkage [fraction No. 8 (1)] was collected and chromatographed once more on a DEAE-cellulose column (2). The fractions having the enzymic activity were collected and, after being concentrated under reduced pressure, dialyzed against 0.02 M acetate buffer, pH 4.6.

Substrates

4, 5-Unsaturated digalacturonic acid. By the method previously described (3), this was prepared as crystalline calcium salt and used after removal of calcium ion and neutralization with sodium hydroxide.

Unsaturated acid-soluble pectic acid. This was prepared by treating pectinic acid with 0.05 N sodium hydroxide at 100°C for 5 minutes (4). As the unsaturated acid-soluble pectic acid so prepared contained a small amount of acid-soluble pectic acid, it (0.2 %) was incubated with carrot exopolygalacturonase (5) in 0.02 M acetate buffer at pH 4.6 and 27°C to degrade this impurity. The mixture was then freed from carrot exopolygalacturonase by treating it with Duolite CS-101 and chromatographed on a DEAE-cellulose column (6). From the fractions eluted with 0.1 M carbonate-bicarbonate buffer, pH 9.5, unsaturated acid-soluble pectic acid was precipitated as sodium salt by the addition of ethanol

and dried at room temperature. Its average degree of polymerization was found to be 15.2.

4-Deoxy-5-keto-D-fructuronic acid (3-deoxy-D-glycero-2,5-hexodiulosonic acid). According to the method previously described (7), a crude solution of saccharifying pectate transeliminase was prepared from the cells of *Erwinia aroideae*. It was allowed to act on pectic acid (0.5 %) in 0.02 M phosphate buffer, pH 7.5, at 30°C for about 40 hours. The reaction mixture was treated with cation exchange resin, neutralized with barium hydroxide and, after removal of precipitate, concentrated to about one-fiftieth of the volume. To the concentrate was added ethanol to a concentration of 20 % and the precipitate formed was removed away. By adding ethanol to the supernatant to give a final concentration of 90 %, a pale yellow precipitate was obtained. Besides 4-deoxy-5-keto-D-fructuronic acid (7) this precipitate contained an appreciable amount of galacturonic acid and a little unknown substance. 4-Deoxy-5-keto-D-fructuronic acid was prepared from the precipitate by removing these contaminants by DEAE-cellulose chromatography. Its properties were compared with those reported by Preiss and Ashwell (8, 9).

Analytical Methods

Paper chromatography. This was done on strips of Toyo Roshi No. 52 filter paper at room temperature. The following solvent systems were used: A, n-butanol-acetic acid-water (4 : 1 : 2); B, n-butanol-acetic acid-water (50 : 12 : 25); C, ethyl acetate-acetic acid-water-formic acid (18 : 3 : 4 : 1); D, n-butanol-formic acid-water (8 : 3 : 2). The spray reagents used were: for sugars, aniline hydrochloride; for acid, bromophenol blue. Chromatograms were developed by the descending method in case where comparisons were made of chromatographic mobilities of unknown compounds and 4-deoxy-5-keto-D-fructuronic acid. In the other cases the ascending method was used.

Chromatography on DEAE-cellulose columns. To a DEAE-cellulose column (0.9 meq. /g, 2 × 50~70 cm) prepared in 0.01 M acetate buffer, pH 5.0, was added 1~5 ml of 1 % sample solution and the column eluted with the same buffer. Approximately 5 ml per tube was collected and the course of the elution was followed by estimating sugars and making the thiobarbituric acid test (10). The sugar estimations were made by the phenol-sulfuric acid (11) and carbazole methods (12) in which the observed readings were compared with calibration curves previously prepared with galacturonic acid.

Periodic acid oxidation. Samples and their reduced derivatives (2 μmoles per ml) were oxidized with periodic acid at 20°C (10). After 20 minutes or 10 minutes (in case of 4-deoxy-5-keto-D-fructuronic acid) β-formyl pyruvic acid (10), formaldehyde (13) and glycolic acid (14) were determined in reaction mixtures. The reduction of the samples was done with sodium borohydride according to the method of McCready and Seegmiller (15).

RESULTS

Degradation of unsaturated digalacturonic acid and unsaturated acid-soluble pectic acid by the exopolygalacturonase. Hydrolysis of unsaturated digalacturonic acid and unsaturated acid-soluble pectic acid by the exopolygalacturonase action was followed by determining galacturonic acid and the unknown compound (Fig. 1). In each case conditions for the reaction were: substrate,

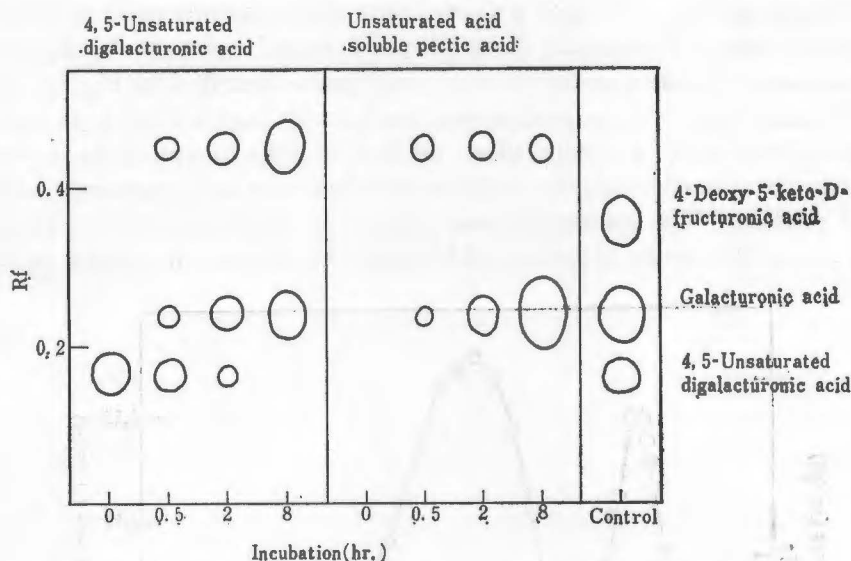


Fig. 1. Paper chromatograms of products formed from 4,5-unsaturated digalacturonic acid and unsaturated acid-soluble pectic acid by exopolygalacturonase. Assay conditions: substrate, 0.1 %; acetate buffer, 0.05 M, pH 4.6 (4,5-unsaturated digalacturonic acid), pH 4.0 (unsaturated acid-soluble pectic acid); enzyme, about 5 units per ml; temperature, 35°C. Paper chromatography: reaction mixture applied, 0.05 ml; solvent, A; development, upward irrigation, room temperature, 16 hr.

0.1 %; acetate buffer, 0.05 M; enzyme, about 5 units (1) per ml of reaction mixture, temperature, 35°C. pH was maintained at the optimum of the exopolygalacturonase activity, namely, at about 4.6 and 4.0 with unsaturated digalacturonic and acid-soluble pectic acids as substrates, respectively. In both cases the unknown compound as well as galacturonic acid was noticed on paper chromatograms from the beginning of the reaction. With unsaturated digalacturonic acid as substrate both products were formed in equal proportions. In case of unsaturated acid-soluble pectic acid the unknown compound was predominant over galacturonic acid at the beginning, but the proportion was inverted as the reaction proceeded. Hereafter the unknown compound will be referred to as X: the unknown compound from unsaturated digalacturonic acid will be called Xd and that from unsaturated acid-soluble pectic acid Xp.

Hasegawa and Nagel (16) reported that a compound supposed to be 4,5-un-

saturated galacturonic acid was produced by acid hydrolysis of unsaturated digalacturonic acid. When the paper chromatograms of the enzyme hydrolyzate described above were sprayed with quinine sulfate reagent [0.01 % quinine sulfate in 95 % ethanol containing 0.04 N sulfuric acid (17)], any spot showing ultra-violet absorption was not found except the spot of 4, 5-unsaturated digalacturonic acid. This indicates the absence of 4, 5-unsaturated galacturonic acid in the enzyme hydrolyzate.

Chromatography of X and 4-deoxy-5-keto-D-fructuronic acid on DEAE-cellulose columns. Unsaturated digalacturonic acid and unsaturated acid-soluble pectic acid were incubated under the same conditions as described for Fig. 1. After about 15 hours the incubation mixture was treated with Duolite CS-101 previously equilibrated with 0.02 M acetate buffer, pH 3.3, in order to remove the enzyme. The mixture was freed from cation with ion exchange resin and concentrated under reduced pressure. The concentrate was chromatographed on a DEAE-cellulose column as described under Materials and Methods. Fig. 2 shows the elution pattern

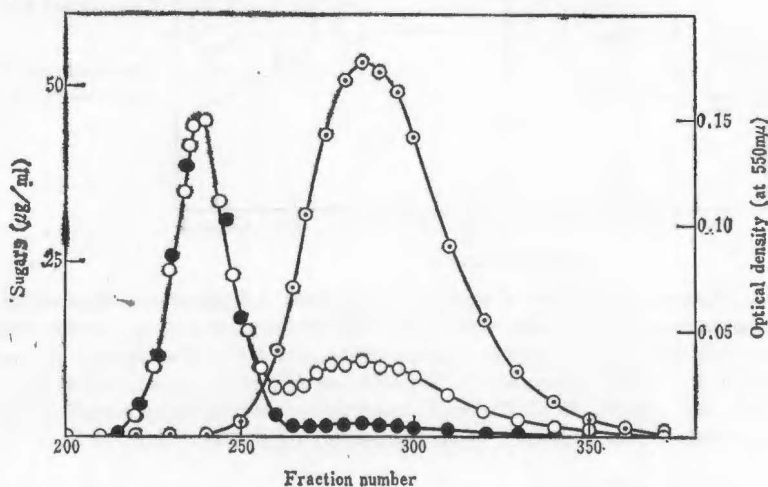


Fig. 2. Chromatography on a DEAE-cellulose column of products formed from 4, 5-unsaturated digalacturonic acid by the exopolygalacturonase. Assay conditions were the same as described for Fig. 1 except for the reaction time of about 15 hr. $\odot$ — $\odot$ Optical density at 550 m μ in the thiobarbituric acid test, $\circ$ — $\circ$ sugar amount estimated by the phenol-sulfuric acid method, $\bullet$ — $\bullet$ sugar amount estimated by the carbazole method.

of the enzyme hydrolyzate of unsaturated digalacturonic acid. The compound in the first peak was characterized as galacturonic acid on the basis that the results obtained with the phenol-sulfuric acid and carbazole methods coincided with each other and that it gave no thiobarbituric acid test. The second peak giving a positive thiobarbituric acid test was considered as X. In the phenol-sulfuric acid reaction color of X was considerably weak as compared with that of galacturonic acid and, in the carbazole reaction, X gave only a faint color. The second peak,

tubes 275~340, was treated with cation exchange resin and concentrated under reduced pressure (Xd and Xp).

A crude 4-deoxy-5-keto-D-fructuronic acid solution (barium salt, see Materials and Methods), after being treated with cation exchange resin, was chromatographed on a DEAE-cellulose column in the same manner as described for the preparation of X. The elution curve obtained is shown in Fig. 3. A small peak of

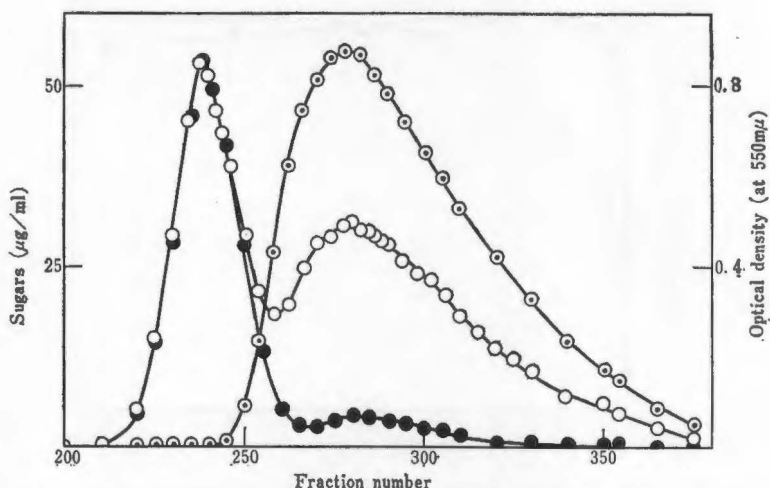


Fig. 3. Purification of 4-deoxy-5-keto-D-fructuronic acid by DEAE-cellulose column chromatography. Symbols are the same as described for Fig. 2.

an unidentified substance which appeared before the main peak, tubes 35~40, was omitted from the figure. It is clear that the first main peak is galacturonic acid and the second 4-deoxy-5-keto-D-fructuronic acid. Tubes 277~350 were pooled and, after being treated with cation exchange resin, concentrated under reduced pressure.

Paper chromatography of X and 4-deoxy-5-keto-D-fructuronic acid. According to the method of Preiss and Ashwell (9) paper chromatography was done by downward irrigation with solvents B and C. The results obtained are shown in Table 1. When solvent B was used, X moved faster than 4-deoxy-5-keto-D-fructuronic acid. The reverse was the case with solvent C.

TABLE 1
Paper chromatography of X and 4-deoxy-5-keto-D-fructuronic acid

Sugars	Solvent A (cm/16 hr.)	Solvent B (cm/5 hr.)
Xd	25.3	11.1
Xp	25.1	11.8
4-Deoxy-5-keto-D-fructuronic acid	22.9	16.4
Galacturonic acid	19.3	10.7

Rate of periodic acid oxidation of X and 4-deoxy-5-keto-D-fructuronic acid. By the method of Weissbach and Hurwitz (10) X and 4-deoxy-5-keto-D-fructuronic acid ($0.1 \mu\text{mole}$ each) were oxidized with periodic acid and, after a fixed time, the thiobarbituric acid test was made with the reaction mixture. As shown in Fig. 4, 4-deoxy-5-keto-D-fructuronic acid was oxidized faster than X.

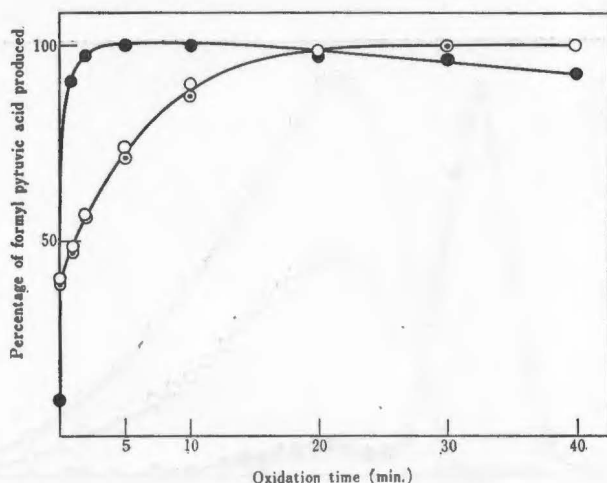


Fig. 4. Rate of periodic acid oxidation of X and 4-deoxy-5-ket-D-fructuronic acid. Xd (O—O), Xp (⊙—⊙) and 4-deoxy-5-keto-D-fructuronic acid (●—●) ($0.1 \mu\text{mole}$ each) were tested in the thiobarbituric acid assay. Periodic acid oxidation was carried out at 20°C .

The time of periodic acid oxidation required to obtain maximum color production was about 30 minutes for X. In contrast it was only several minutes for 4-deoxy-5-keto-D-fructuronic acid and prolonged oxidation, on the contrary, decreased color production.

Periodic acid oxidation products of X and 4-deoxy-5-keto-D-fructuronic acid. Table 2 shows the results obtained in analyses of the periodic acid oxidation products of X and 4-deoxy-5-keto-D-fructuronic acid, and their reduced derivatives. As was expected 4-deoxy-5-keto-D-fructuronic acid gave one molar equivalent of β -formyl pyruvic acid and glycolic acid each, and a little formaldehyde. In contrast X yielded mainly β -formyl pyruvic acid with only small amounts of other products. From reduced X and 4-deoxy-5-keto-D-fructuronic acid β -formyl pyruvic acid did not arise. In stead of this product an equivalent amount of formaldehyde was produced. Glycolic acid formed from 4-deoxy-5-keto-D-fructuronic acid was detected by the following method. The reaction mixture, after removal of iodine-containing compound by treating with strontium carbonate, was extracted with ether containing sulfuric acid. Ether layer was examined by paper chromatography in solvents A and B.

TABLE 2
Periodic acid oxidation products of X and 4-deoxy-5-keto-D-fructuronic acid

Sugars	β -Formyl pyruvic acid		Formaldehyde		Glycolic acid
	Before reduction	After reduction	Before reduction	After reduction	
Xd	2.00*	0.04	0.07	2.03	0.30
Xp	1.98	0.03	0.09	1.99	0.38
4-Deoxy-5-keto-D-fructuronic acid	1.93	0.04	0.20	1.91	1.99
Mannit	—	—	2.00*	—	—
Glycolic acid	—	—	—	—	2.00*

Concentration of the test solutions was 2 μ moles per ml. Mannit (1 μ mole per ml) and glycolic acid (2 μ moles per ml) were used for controls.

* These values were taken as 2.00.

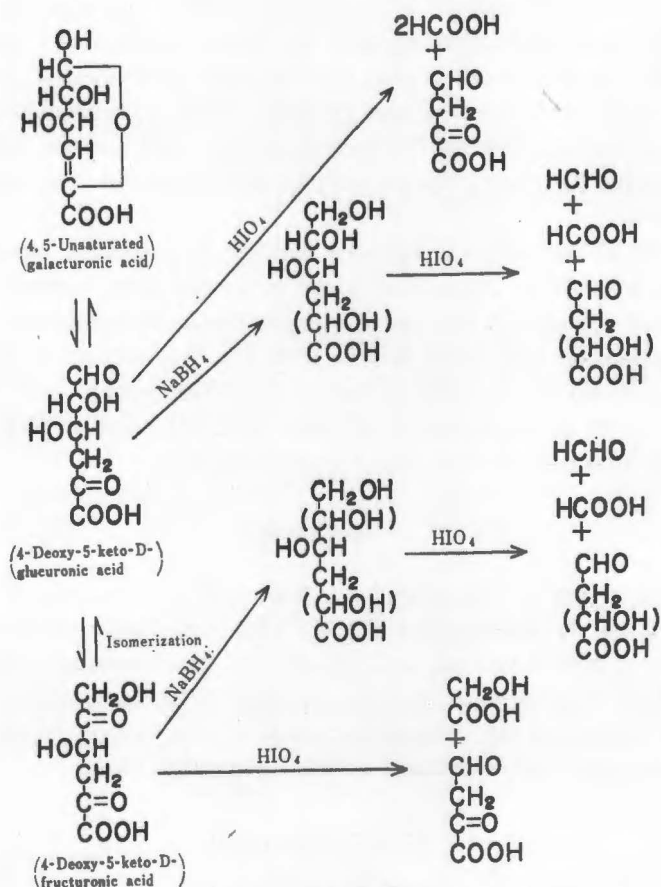


Fig. 5. Periodic acid oxidation products.

DISCUSSION

Hasegawa and Nagel (16) reported that, on hydrolysis with 1 N hydrochloric acid at 100°C, unsaturated digalacturonic acid gave 4, 5 unsaturated galacturonic acid together with galacturonic acid. Preiss and Ashwell (8) obtained 4-deoxy-5-keto-D-glucuronic acid (4-deoxy-L-threo-5-hexoseulose uronic acid) and 4-deoxy-5-keto-D-fructuronic acid as products of pectic acid degradation with an enzyme preparation of a pseudomonad. However, 4, 5-unsaturated galacturonic acid which was expected to arise could not be detected in the enzymic digest. These authors considered that this compound was converted non enzymically into 4-deoxy-5-keto-D-glucuronic acid owing its instability. 4, 5-Unsaturated galacturonic acid was not detected also in case where unsaturated digalacturonic and pectic acids were degraded by the exopolygalacturonase of *Aspergillus niger*.

When the exopolygalacturonase of *Aspergillus niger* was made to act on unsaturated digalacturonic acid or unsaturated pectic acid an unknown product was found in the hydrolyzate. The unknown product prepared from unsaturated digalacturonic acid was called Xd and that from unsaturated acid-soluble pectic acid Xp. By treatment with Duolite CS-101 and DEAE-cellulose chromatography both Xd and Xp were separated and purified. They were identical in properties, and characterized as 4-deoxy-5-keto-D-glucuronic acid on the following basis: chromatographic behavior; rate of periodic acid oxidation; products of periodic acid oxidation.

From the above results it followed that the non-reducing terminal residue of unsaturated acid-soluble pectic acid which is formed from pectinic acid by alkali-treatment is identical with the non-reducing monosaccharose moiety of 4, 5-unsaturated digalacturonic acid which results from the degradation of pectic acid with pectate trans-eliminase, and that 4-deoxy-5-keto-D-glucuronic acid arises not only from the enzymic transelimination of pectic acid (8) but also from the enzymic hydrolysis of unsaturated oligo- and polygalacturonides.

SUMMARY

Previous papers of this series have dealt with a new type of exopolygalacturonase produced by *Aspergillus niger*. This exopolygalacturonase was capable of hydrolyzing both saturated and unsaturated galacturonidic linkages. In this paper, evidence was presented that the structure of the deoxysugar produced from unsaturated oligo- and polygalacturonic acids by the exopolygalacturonase action was consistent with that of 4-deoxy-5-keto-D-glucuronic acid.

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