Pectolytic Enzymes of Exo-Types

Part I. Oligogalacturonide Transeliminase of a *Pseudomonas*[†]

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A bacterium identified as *Pseudomonas* sp. was found to be a better source of oligogalacturonide transeliminase (OGTE) than *Erwinia aroideae*.

The OGTE of *Pseudomonas* sp. differed from that of *Erwinia aroideae* in the following respects: (1) The activity was maximal with tetramer and followed by trimer, dimer and polymers. (2) The OGTE of *Pseudomonas* sp. degraded the saturated uronides as rapidly as, or a little more rapidly than, the corresponding unsaturated uronides. (3) Calcium ion stimulated considerably the OGTE activity.

Both oxidized and reduced acid-soluble pectic acids were resistant to the action of the OGTE.

With the purified enzyme preparation, 4-deoxy-5-keto-D-glucuronic acid was the end product of the OGTE action on oligo- and polygalacturonides. 4,5-Unsaturated galacturonic acid is probably the intermediate in the formation of 4-deoxy-5-keto-D-glucuronic acid.

Many enzymes are known which split the glycosidic linkages in the pectic substances. They may be classed as follows:

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1) H. J. Phaff and A. L. Demain, *J. Biol. Chem.*, **218**, 875 (1956).

2) F. Uchino, Y. Kurono and S. Doi, *Agr. Biol. Chem.*, **30**, 1066 (1966).

3) C. W. Nagel and R. H. Vaughn, *Arch. Biochem. Biophys.*, **93**, 344 (1961).

4) M. P. Starr and F. Moran, *Science*, **135**, 920 (1962).

5) P. Albersheim, H. Neukom and H. Deuel, *Helv. Chim. Acta*, **43**, 1422 (1960).

6) R. D. Edstrom and H. J. Phaff, *J. Biol. Chem.*, **239**, 2403, 2409 (1964).

Although the enzymes of exo-types are economically not so important as those of endo-types as juice-clarifying and plant-tissue-macerating agents, the use of the enzymes of these types such as galacturonopolygalacturonases of carrot⁷⁾ and Sclase (*Coniothyrium diploidiella*),⁹⁾ and OGTE (see below) must provide an indispensable tool for the structural analysis of the pectic substances. Furthermore, they play a significant role in the metabolism of the pectic substances: they, in cooperation with the enzymes of endo-types, produce monosaccharides from the pectic substances. In our laboratory a study has been undertaken to enlarge the knowledge about the pectolytic enzymes of exo-types.

It has been known that *Erwinia carotovora*¹⁸⁾ and *Erwinia aroideae*¹⁹⁾ produce OGTE, which attacks preferentially the short-chain uronides and cleaves the first glycosidic linkage from the reducing end of the substrate molecule. In this study it was found that a *Pseudomonas* also produced an OGTE. This OGTE, how-

ever, differed in some properties from that of *Erwinia*. The present paper describes the purification and properties of the OGTE of *Pseudomonas* sp.

MATERIALS AND METHODS

Crude enzyme solution. The strain S2 used in this study, classified in the genus *Pseudomonas*, was isolated from soil and grown under the same conditions used for cultivation of *Erwinia aroideae*.^{12,14)} The cells were harvested after 20 hr growth and acetone-ized in the usual manner. The acetone-ized cells (2 g) were suspended in 100 parts of 0.01 M phosphate buffer, pH 7.0, and allowed to stand overnight at 4°C. After centrifugation the supernatant solution was dialyzed against 0.01 M phosphate buffer, pH 7.0, for three days. The dialyzed solution (enzyme preparation 1) was added to a Duolite CS-101 column (4×20 cm) which had been equilibrated to pH 7.0 with 0.01 M phosphate buffer and the column was washed with the same buffer. The effluent showing OGTE activity was collected (enzyme preparation 2) and added to a DEAE-cellulose column (4×10 cm) equilibrated previously with 0.01 M phosphate buffer, pH 7.0, followed by 400 ml of the same buffer. After the eluent had been changed to 0.2 M sodium chloride in the same buffer, the OGTE began to emerge from the column. The eluates having OGTE activity were collected and dialyzed against 0.01 M phosphate buffer, pH 7.0, for two days. To the dialyzed solution (enzyme preparation 3) was added an equal volume of calcium phosphate gel suspension containing 10 mg, dry weight, per ml. The mixture was held at 4°C for 30 min with occasional stirring and centrifuged. The supernatant solution (enzyme preparation 4) was concentrated by pervaporation and dialyzed against 0.01 M phosphate buffer, pH 7.0 (crude enzyme solution, about 20 ml).

Yeast endopolygalacturonase (YPG). YPG was prepared from the culture liquid of *Saccharomyces fragilis* in the same manner as described previously.²⁰⁾

Pectic acid. Commercial pectinic acid was saponified with pectin esterase under the same conditions as described previously²¹⁾ and treated with 0.05 N

7) C. Hatanaka and J. Ozawa, *Agr. Biol. Chem.*, **28**, 627 (1964).

8) S. Hasegawa and C. W. Nagel, *Arch. Biochem. Biophys.*, **124**, 513 (1968).

9) C. Hatanaka and J. Ozawa, *Nippon Nogeikagaku Kaishi*, **43**, 67 (1969).

10) C. Hatanaka and J. Ozawa, *ibid.*, **43**, 77, 85 (1969).

11) C. Hatanaka and J. Ozawa, *Agr. Biol. Chem.*, **33**, 116 (1969).

12) C. Hatanaka and J. Ozawa, *Nippon Nogeikagaku Kaishi*, **43**, 764 (1969).

13) K. Okamoto, C. Hatanaka and J. Ozawa, *Agr. Biol. Chem.*, **27**, 596 (1963).

14) K. Okamoto, C. Hatanaka and J. Ozawa, *ibid.*, **28**, 331 (1964).

15) K. Okamoto, C. Hatanaka and J. Ozawa, *Nippon Nogeikagaku Kaishi*, **38**, 237 (1964).

16) J. D. MacMillan and R. H. Vaughn, *Biochemistry*, **3**, 564 (1964).

17) J. D. MacMillan, H. J. Phaff and R. H. Vaughn, *ibid.*, **3**, 572 (1964).

18) F. Moran, S. Nasuno and M. P. Starr, *Arch. Biochem. Biophys.*, **125**, 734 (1968).

19) C. Hatanaka and J. Ozawa, *Agr. Biol. Chem.*, **34**, 1618 (1970).

20) C. Hatanaka and J. Ozawa, *Nippon Nogeikagaku Kaishi*, **42**, 645 (1968).

sodium hydroxide for 90 min at 0°C. The mixture was then neutralized with acetic acid and to this was added an equal volume of ethanol. The resulting precipitate was washed with ethanol (50%, five time; 80%, twice; 99%, twice) and ether (twice), and dried at 35°C.

Acid-soluble pectic acid and oligogalacturonides. These were prepared by the method described previously.^{12,14)} The average degree of polymerization of the acid-soluble pectic acid was found to be 9.5.

Oxidized and reduced acid-soluble pectic acids. These were made from the acid-soluble pectic acid described above. The acid-soluble pectic acid was oxidized with hypiodite according to the micro Willstätter-Schudel method²²⁾ or reduced with sodium borohydride under the conditions similar to those described by McCready and Seegmiller.²³⁾

Assay of enzyme activity. Unless otherwise stated, the OGTE activity was determined by measuring the increase in the reducing power. The reaction mixture for the determination of the units of the OGTE contained 0.1% unsaturated digalacturonic acid, 0.03 M phosphate buffer, pH 7.0, and a suitable concentration of the enzyme. After 30 min of incubation at 35°C, the reducing power of the mixture was determined by the micro Willstätter-Schudel method.²²⁾ One unit of the OGTE is defined as that amount of enzyme required to split 1 μ mole of bonds per hr. As described later, the degradation product of unsaturated digalacturonic acid by the OGTE is probably 4-deoxy-5-keto-D-glucuronic acid. In the iodometry the keturonic acid requires per mole about 4.5 times as much iodine as does galacturonic acid.¹⁰⁾ On the basis of this finding the moles of bonds split by the OGTE were calculated from the amount of the iodine consumed by the keturonic acid in the reaction mixture.

RESULTS

Purification of the OGTE

To the crude enzyme solution described in

the Materials and Methods section one-tenth volume of 0.5 M sodium chloride was added and the mixture was placed on a DEAE-Sephadex A-50 column (2×7 cm) equilibrated previously with 0.05 M sodium chloride in 0.01 M phosphate buffer pH 7.0. Elution was carried out with a linear concentration gradient of sodium chloride in phosphate buffer; the mixing chamber contained 200 ml of 0.05 M sodium chloride in 0.01 M phosphate buffer, pH 7.0, and the reservoir 200 ml of 0.5 M sodium chloride in the same buffer. Fractions of 5 ml were collected and the protein concentration was estimated by measuring absorption at 280 m μ (Fig. 1). Tubes 20~30 were collected and, after dialysis, concentrated at 4°C by pervaporation. The concentrate was dialyzed against 0.01 M phosphate buffer, pH 7.0. Unless otherwise stated, the dialyzed solution (enzyme preparation 5) was used as the purified enzyme in the following experiments. The results of purification of the enzyme are shown in Table I. This purification produced a 31-fold over-all increase in the specific activity.

Properties of the OGTE

Effect of pH on the OGTE activity. When saturated and unsaturated digalacturonic acids

FIG. 1. Chromatography of the Crude Enzyme Solution on a DEAE-Sephadex A-50 Column.

○—○ protein concentration, ●—● enzyme activity, ---- concentration of sodium chloride in 0.01 M phosphate buffer of pH 7.0.

21) C. Hatanaka and J. Ozawa, *Nippon Nogeikagaku Kaishi*, **42**, 698 (1968).

22) C. Hatanaka, *ibid.*, **41**, 448 (1967).

23) R. M. McCready and C. G. Seegmiller, *Arch. Biochem. Biophys.*, **50**, 440 (1954).

TABLE I. SUMMARY OF PURIFICATION PROCEDURE

Protein was determined by the Folin-Lowry method, as modified by Hagiwara.²⁴⁾

Enzyme preparation	Volume (ml)	Total unit	Total protein (mg)	Specific activity (unit/ml)	Recovery of enzyme (%)
1. Cell-free extracts	218	154.78	344.20	0.45	100
2. Duolite CS-101 effluent	263	131.50	265.60	0.50	85.0
3. DEAE-cellulose eluate	252	63.00	66.78	0.94	40.7
4. Calcium phosphate gel supernatant	436	52.32	31.42	1.67	33.8
5. DEAE-Sephadex A-50 eluate	18.5	41.07	2.93	14.02	26.5

FIG. 2. pH-activity Curves.

Assay conditions: substrate, 0.1%; phosphate buffer, 0.05 M; enzyme, about 0.15 unit per ml; incubation, for 1 hr at 35°C. Enzyme activity was expressed as ml of 0.002 N-I₂ consumed per ml of reaction mixture per hour. ○—○ 4,5-unsaturated digalacturonic acid, ◐—◐ digalacturonic acid, ●—● acid-soluble pectic acid.

were used as substrates, the pH optima were found to be at about 7.0. With acid-soluble pectic acid as substrate, it was at about 6.4 (Fig. 2).

Activities of the OGTE toward oxidized and reduced acid-soluble pectic acids. As shown in Fig. 3(A), both oxidized and reduced substrates were not degraded by the OGTE (enzyme preparation 5). Similar results were obtained even when the cell-free extract with-

FIG. 3. The OGTE Activities toward Acid-soluble, Oxidized Acid-soluble and Reduced Acid-soluble Pectic Acids.

Assay conditions: substrate, 0.1%; phosphate buffer, 0.02 M, pH 6.4; enzyme, about 0.6 unit per ml; incubation, at 35°C. Enzyme activity was expressed as ml of 0.002 N-I₂ consumed per ml of reaction mixture per hour. ○—○ acid-soluble pectic acid, ●—● oxidized acid-soluble pectic acid, ×—× reduced acid-soluble pectic acid. (A) enzyme preparation 5, (B) enzyme preparation 1.

out purification (enzyme preparation 1) was incubated with these substrates [Fig. 3(B)]. When aliquots (about 0.05 ml) of the reaction mixtures (22 hr of incubation) of both enzyme preparations were chromatographed on Toyo Roshi No. 52 paper with *n*-butanol-acetic acid-water (5:2:3), no degradation products could be detected. These results indicate that the OGTE attacks the first glycosidic linkage from the reducing end of the substrate molecule.

24) B. Hagiwara, "Koso Kenkyuho," Vol. I, ed. by S. Akabori, Asakura Shoten, 1955, p. 166.

Degradation of reduced acid-soluble pectic acid by the combined action of the OGTE and YPG. Reduced acid-soluble pectic acid was degraded by a combination of the OGTE and a small amount of YPG. Although the rate was very slow at the early stage of the reaction, the reaction proceeded rapidly thereafter (Fig. 4).

FIG. 4. Degradation of Reduced Acid-soluble Pectic Acid by a Combination of the OGTE and YPG.

○—○ OGTE (about 0.2 unit/ml) + YPG (about 0.04 unit²⁵/ml), ●—● YPG (about 0.04 unit/ml), ○—○ OGTE (about 0.2 unit/ml). Other conditions were the same as described for Fig. 3.

This must have been caused by the rapid degradation of the normal oligogalacturonic acids which had been produced by YPG from the reduced acid-soluble pectic acid.

Ratio of the OGTE activities toward oligo- and polygalacturonides. Table II shows the relative activities of the OGTE toward oligo- and polygalacturonides. The activity was maximal with tetragalacturonic acid and followed by trimer, dimer and polymers. Pectic acid was degraded very slowly (approximately 1/208 the rate of tetragalacturonic acid).

Erwinia OGTE, as reported previously, degraded the unsaturated uronides more rapidly than the corresponding saturated uronides. Such was not the case, however, with the OGTE of *Pseudomonas* (Table II).

Reaction products. Table III summarizes the results of paper chromatographic analysis of reaction products obtained from various substrates. When the purified enzyme (preparation 5) was allowed to act on pectic acid and acid-soluble pectic acid, only one spot corresponding to 4-deoxy-5-keto-D-glucuronic acid^{25,26} was detected. With trigalacturonic acid as substrate, di- and mono-galacturonic

TABLE II. RATIO OF THE OGTE ACTIVITIES TOWARD OLIGO- AND POLYGALACTURONIDES
The reaction mixture containing 0.03 M phosphate buffer was incubated for 1 hr at 35°C.

Substrate	Substrate concentration	pH of reaction mixture	Enzyme concentration (unit/ml)	Glycosidic bond split (μ mole/ml)	Relative rate ^{a1}
Pectic acid	0.2%	6.4	0.90	0.013	1
Acid-soluble pectic acid	"	"	0.18	0.173	66.54
Acid-soluble pectic acid	3 mM	6.4	0.075	0.071	66.54
Tetragalacturonic acid	"	7.0	"	0.222	208.07
Trigalacturonic acid	"	"	"	0.164	153.71
Unsaturated trigalacturonic acid	"	"	"	0.138	129.35
Digalacturonic acid	"	"	"	0.080	75.92
Unsaturated digalacturonic acid	"	"	"	0.076	71.20

^{a1} Expressed as relative rates of bonds split by the same amount of enzyme.

25) C. Hatanaka and J. Ozawa, *Nippon Nogeikagaku Kaishi*, **43**, 139 (1969).

26) J. Preiss and G. Ashwell, *J. Biol. Chem.*, **238**, 1571, 1577 (1963).

TABLE III. PAPER CHROMATOGRAPHIC ANALYSIS OF DEGRADATION PRODUCTS FROM OLIGO- AND POLYGALACTURONIDES

Assay conditions: substrate, 0.2%; phosphate buffer, 0.02 M, pH 7.0 (oligogalacturonides) or pH 6.4 (polygalacturonides); enzyme, about 0.2 (oligogalacturonides) or about 0.5 unit (polygalacturonides) per ml; incubation, for 22 hr at 30°C.

About 0.03 ml of the reaction mixture was chromatographed on Toyo Roshi No. 52 paper with *n*-butanol-acetic acid-water (5 : 2 : 3). Spots were made visible by spraying with the reagents as described previously.¹⁹⁾

Enzyme preparation	Substrate	Products				
		Digalacturonic acid	Unsaturated digalacturonic acid	Galacturonic acid	4-Deoxy-5-keto-D-glucuronic acid	4-Deoxy-5-keto-D-fructuronic acid
5	Pectic acid	—	—	—	†	—
	Acid-soluble pectic acid	—	—	—	†	—
	Trigalacturonic acid	†	—	+	†	—
	Digalacturonic acid	+	—	†	†	—
	Unsaturated trigalacturonic acid	—	†	—	†	—
	Unsaturated digalacturonic acid	—	†	—	†	—
1	Pectic acid	—	—	—	+	+
	Digalacturonic acid	+	—	†	†	†
	Unsaturated digalacturonic acid	—	+	—	†	†

acids were produced besides the above keturonic acid. Digalacturonic acid yielded equal amounts of galacturonic acid and the keturonic acid. Unsaturated trigalacturonic acid was degraded to a mixture of unsaturated digalacturonic acid and the keturonic acid, whereas unsaturated digalacturonic acid yielded only the keturonic acid.

In the case of the reaction mixtures of the cell-free extract without purification (enzyme preparation 1), 4-deoxy-5-keto-D-fructuronic acid^{25, 26)} as well as 4-deoxy-5-keto-D-glucuronic acid was produced from pectic acid and unsaturated digalacturonic acid. With digalacturonic acid as substrate, galacturonic acid was detected, in addition to both keturonic acids.

Relation of enzyme concentration to the OGTE activity. The relation of enzyme concentration to the OGTE activity is given in Fig. 5. The enzyme activity was determined by measuring both the increase in reducing power and the increase in optical density at 232 m μ in the reaction mixtures. The OGTE ac-

tivities toward di- and trigalacturonic acids and acid-soluble pectic acid were proportional to the enzyme concentration under the conditions of the experiments.

Provisional evidence for accumulation of 4,5-unsaturated galacturonic acid as intermediate. As indicated in Table III the degradation products from di- and trigalacturonic acids and acid-soluble pectic acid were 4-deoxy-5-keto-D-glucuronic acid and galacturonic acid, besides these two the partially degraded substrates being detected in some cases. When the paper chromatograms of the reaction mixtures were examined under ultraviolet lamp after spraying the quinine sulfate reagent,²⁷⁾ we could not detect any spot showing ultraviolet absorption. As can be seen from Fig. 5, however, the increased absorption at 232 m μ suggests that some unsaturated compound was produced in the reaction mixtures. From the

27) R. D. Edstrom and H. J. Phaff, *J. Biol. Chem.*, **239**, 2409 (1964).

FIG. 5. Relation of Enzyme Concentration to the OGTE Activities toward Di- and Trigalacturonic Acids and Acid-soluble Pectic Acid.

Assay conditions: substrate, 0.1%; phosphate buffer, 0.03 M, pH 7.0 (di- and trigalacturonic acids) or pH 6.4 (acid-soluble pectic acid); incubation, for 1 hr at 35°C. ○—○ increase in reducing power (0.002 N-I₂-consumption, ml/ml), ●—● increase in optical density at 232 mμ (× 1/11). (A) digalacturonic acid, (B) trigalacturonic acid, (C) acid-soluble pectic acid.

results given in Table III it is clear that one mole of 4,5-unsaturated digalacturonic acid yields two moles of 4-deoxy-5-keto-D-glucuronic acid. The formation of the keturonic acid from both unsaturated and saturated galacturonic acid residues of 4,5-unsaturated digalacturonic acid suggests that the first product of the OGTE action on the substrates is 4,5-unsaturated galacturonic acid. Therefore, the unsaturated compound which accumulates in the reaction mixtures is probably 4,5-unsaturated galacturonic acid. The rate of its accumulation varied according to the experimental conditions and was proportional to the enzyme concentration (Fig. 5).

Effects of calcium chloride concentration on the OGTE activity. Figure 6 shows that the OGTE activity was considerably stimulated by calcium ion, the maximal activity being obtained at about 0.2 mM or 0.5 mM with unsaturated digalacturonic acid or acid-soluble pectic acid, respectively.

DISCUSSION

The OGTE of *Pseudomonas* sp. was found

FIG. 6. Effects of Calcium Chloride on the OGTE Activities toward 4,5-Unsaturated Digalacturonic Acid and Acid-soluble Pectic Acid.

Assay conditions: substrate, 0.1%; phosphate buffer, 0.02 M, pH 7.0 (4,5-unsaturated digalacturonic acid, ○—○) or pH 6.4 (acid soluble pectic acid, ●—●); enzyme, about 0.05 unit per ml; incubation, for 1 hr at 35°C. Enzyme activity was expressed as ml of 0.002 N-I₂ consumed per ml of reaction mixture per hour.

to be different from that of *Erwinia aroideae* in the following respects:

(1) The rates of its activity toward oligo-

and polygalacturonides were in the following order: tetramer > trimer > dimer > polymer. In the case of the OGTE of *Erwinia aroideae*, the order was dimer > trimer > tetramer > polymer.

(2) The OGTE of *Pseudomonas* sp. degraded the saturated uronides a little more rapidly than, or as rapidly as, the corresponding unsaturated uronides. The OGTE of *Erwinia aroideae* decomposed the latter much faster than the former.

(3) Calcium ion stimulated considerably the OGTE activity of *Pseudomonas* sp., while it did not affect that of *Erwinia aroideae*.

Both oxidized and reduced acid-soluble pectic acids were resistant to the action of the OGTE of *Pseudomonas* sp. By a combination of the OGTE and a small amount of YPG, however, reduced acid-soluble pectic acid was degraded, although the rate was very slow at the early stage of the reaction. These results indicate that the OGTE attacks the first glycosidic linkage from the reducing end of the substrate molecule.

Even when the cell-free extract without purification (enzyme preparation 1) was incubated with oxidized or reduced acid-soluble pectic acid, no reaction could be observed. This finding together with the chromatographic results (Table III) shows that *Pseudomonas* sp. appears to produce no pectolytic enzyme except the OGTE. As this organism, furthermore, is richer in the enzyme activity than *Erwinia aroideae*, the former is a better source

of OGTE than the latter.

On incubating enzyme preparations from *Erwinia aroideae* with oligo- and polygalacturonides, 4-deoxy-5-keto-D-fructuronic acid was identified in previous studies as the end product (In addition, galacturonic acid or the partially degraded substrates were detected. Such was the case in the following two cases). When the crude enzyme preparation from *Pseudomonas* sp. (enzyme preparation 1) was made to act on the same substrates, 4-deoxy-5-keto-D-glucuronic and 4-deoxy-5-keto-D-fructuronic acids were formed in almost the same amount. With the purified enzyme preparation (enzyme preparation 5) the end product was 4-deoxy-5-keto-D-glucuronic acid. When the paper chromatograms of the reaction mixtures in the last case were examined under ultraviolet lamp after spraying the quinine sulfate reagent, no spot showing ultraviolet absorption could be detected. The increased absorption at 232 m μ , however, suggests that an unsaturated compound, possibly 4,5-unsaturated galacturonic acid, is produced in the reaction mixture. The formation of 4-deoxy-5-keto-D-glucuronic acid from both 4,5-unsaturated and saturated galacturonic acid residues of 4,5-unsaturated digalacturonic acid suggests that the first product of the OGTE action on the substrates may be 4,5-unsaturated galacturonic acid. The mechanism by which 4-deoxy-5-keto-D-glucuronic acid is formed is under further investigation.