ENZYMIC DEGRADATION OF PECTIC ACID

V. Mode of Action of Fungal Saccharifying Polygalacturonase

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Carrot exo-polygalacturonase (CPG) degrades pectic acid removing galacturonic acid unit from the chain end of the molecule. In a previous paper (8), the following evidence was presented for this mode of action:

1) When CPG was incubated with pectic acid, galacturonic acid alone was detected by paper chromatography from the beginning. Oligogalacturonides were not observed even at the end of the reaction.

2) Amount of galacturonic acid, determined by the naphtoresorcinol method after paper chromatography, and loss of pectic acid estimated by calcium pectate method were in good agreement with the quantities calculated from the reducing power of the reaction mixture measured by the Willstätter-Schudel method.

3) CPG degraded citrus pectic acid incompletely leaving a limit polygalacturonide comparable to the β-limit dextrin obtained from starch (4, 8). Higher degradation limits were obtained with acid soluble and acid insoluble pectic acid than with the citrus pectic acid.

4) Addition of tomato endo-PG increased the activity of CPG toward citrus pectic acid at a rate greater than the sum of the component rates and the degree of hydrolysis exceeded the limit achieved by CPG alone.

5) By the CPG preparation digalacturonic acid was degraded far slower than pectic acid. This rejects the possibility that pectic acid may first be hydrolyzed to digalacturonic acid and then the latter converted to glalacturonic acid by the CPG preparation.

Similar mode of action has been proposed by some workers (1, 2, 9) for fungal saccharifying PG. But evidence drawn by them from qualitative paper chromatographic examination does not appear to be conclusive, though this mode of action has received wide acceptance for the fungal saccharifying PG. Furthermore, it has been reported that complete hydrolysis of pectic acid is achieved with the fungal saccharifying PG. This is quite different from our result on CPG action. As an explanation for this discrepancy two possibilities may be considered.

1) Fungal saccharifying PG may be different from CPG in the mode of action.

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a) Pectic acid molecules may contain some anomalous linkages which are split by the fungal saccharifying PG but not by CPG.
b) The fungal saccharifying PG may split inner linkages of pectic acid molecule as well as terminal one, that is to say, this enzyme may not be a true exo-PG.

2) Fungal saccharifying PG preparation used by the above workers may have contained endo-PG as impurity.

In this study the saccharifying PG of Sclase was purified by the chromatography on DEAE-cellulose columns and its mode of action was compared with that of CPG.

MATERIALS AND METHODS

Crude Preparation of Fungal Saccharifying PG. From Sclase* [strong, 200g, (PGu)g=5,760,000] a large portion of endo-PG was removed away by ammonium sulfate precipitation and adsorption on Duolite CS-101 according to the method of Endo (2). The effluent from the Duolite CS-101 column, after pervaporation and dialysis against 0.02 M acetate buffer, pH5 [50 m*l*, (PGu)m*l*=480], was placed on a column (2.2×10 cm) of DEAE-cellulose (0.93 meq./g. Brown Co.), previously equilibrated with the

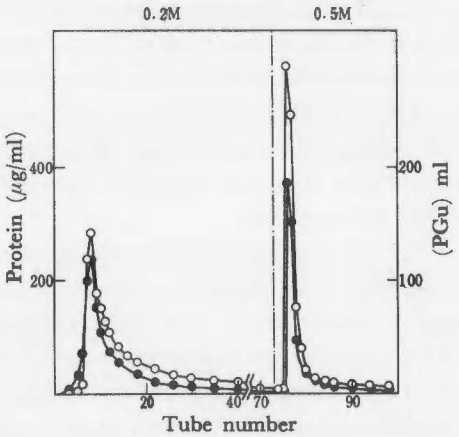


Fig. 1. DEAE-Cellulose chromatography of the eluate from a Duolite CS-101 column. Fraction size, 5 m*l*. Assay conditions: pectic acid, 0.5%; acetate buffer, pH 4.65, 0.01 M; 27°C. ○ (PGu)m*l*, ● protein concentration. (PGu)m*l*; μmol. of bonds split (determined by reducing power measurement) per m*l* of enzyme solution per hr.

* This is a commercial pectinase preparation (Sankyo Co. Ltd.) made from the culture of *Coniothyrium diplodiella*.

same buffer. Chromatography was done by stepwise elution with 0.2 and 0.5 M acetate buffer, pH 5 (Fig. 1). The first peak (175 m*l*) was further chromatographed as will be described later. The ratio of saccharifying to liquefying of the first peak was higher than that of the second (Fig. 4). Recovery of PG of the first peak from the starting Sclase was 0.75%.

Pectic acid was prepared from Citrus Pectin (Nippon Kako Co., Ltd.) by the method previously described (8).

Paper Chromatography. Reaction mixtures (0.1 m*l*) were spotted on the origin of the strip (Toyo No. 52 filter paper, 2 cm wide) and chromatograms run with the ascending technique in n-butanol•acetic acid•water (4:1:2). The spots were made visible with aniline-HCl reagent. By using this reagent as little as 1μg of galacturonic acid could be detected.

Protein concentration was determined by the Folin-Lowry method modified by Hagiwara (3), with use of bovine plasma albumin as a standard.

RESULTS

DEAE-Cellulose Chromatography of Fungal Saccharifying PG Preparations

a. The crude preparation of fungal saccharifying PG described in the Materials and Methods section, after being concentrated and dialyzed against 0.05 M acetate buffer, pH 5 [4 m*l*, (PGu)m*l*=1120], was placed on a column of DEAE-cellulose (2×6 cm), previously equilibrated with the same buffer. The column was eluted with an increasing concentration gradient of acetate buffer, pH 5 (concentrated buffer vessel, 0.4 M, 250 m*l*; mixer, 0.05 M, 250 m*l*). As shown in Fig. 2, a single and symmetrical peak was obtained. This was divided into three parts, A (90 m*l*), B (14 m*l*) and C (90 m*l*).

b. After concentration and dialysis A [3.6 m*l*, (PGu)m*l*=365] was further chromatographed on a DEAE-cellulose column (2×4.8 cm) with a gradient of acetate buffer, pH 5 (concentrated buffer vessel, 0.4 M, 150 m*l*; mixer, 0.05 M, 150 m*l*). The symmetrical peak obtained (Fig. 3) was divided into three parts, A_1 (84 m*l*), A_2 (51 m*l*) and A_3 (65 m*l*). The peak of protein emerged earlier than that of PG activity; there seems to be little correlation between the protein concentration of the fractions and their PG activity. Recovery of PG from the starting Sclase was 0.04% for A and 0.0035% for A_1.

Saccharifying and Liquefying PG Activity of A, B and C

Degradation of pectic acid by A, B and C was followed by measuring reducing power and viscosity of the incubation mixtures. As can be seen from Fig. 4, the order of ratios of saccharifying to liquefying activity was A>B>C.

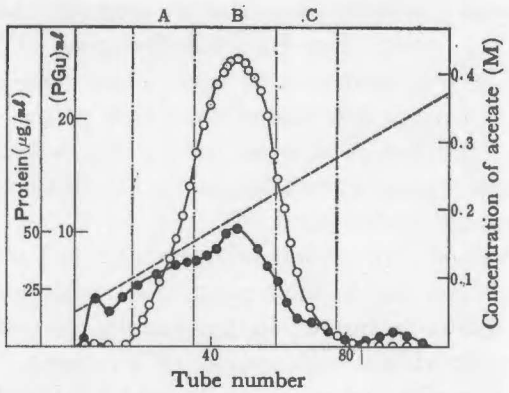


Fig. 2. DEAE-Cellulose chromatography of the crude preparation of fungal saccharifying PG. Fractions of 5 m*l* were collected. Assay conditions were the same as described for Fig. 1. ○ (PGu)m*l*, ● protein concentration, ------ acetate concentration.

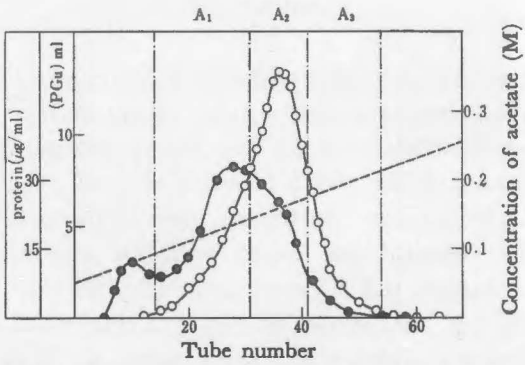


Fig. 3. DEAE-Cellulose chromatography of A. Fractions of 5 ml were collected. Assay conditions were as described for Fig. 1. ○ (PGu)m*l*, ● protein concentration, ------ acetate concentration.

Paper chromatography of the incubation mixtures of B and C showed the presence of digalacturonic acid besides galacturonic acid. In the incubation mixture of A, no reducing substance other than galacturonic acid was detected throughout the reaction.

These results suggest that all the parts of the above peaks are heterogeneous and not free of endo-PG, though they are the components of symmetrical peaks.

Saccharifying and Liquefying PG Activity of A_1, A_2, A_3 and CPG

Ratio of saccharifying and liquefying activity toward pectic acid was different among A_1, A_2 and A_3, the order of the ratios being $A_1 > A_2 > A_3$ (Fig. 5). The ratio of A_1 was almost the same as that of CPG. Paper chro-

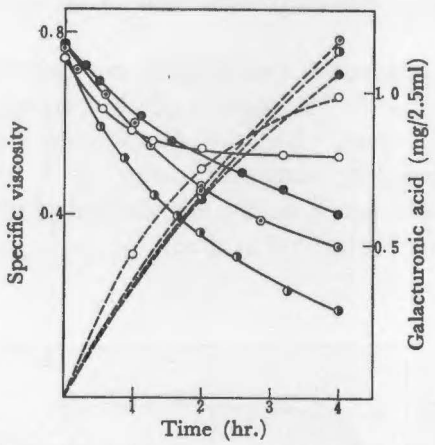


Fig. 4. Saccharifying and liquefying activity of A, B and C toward pectic acid. Pectic acid (final concentration, 0.3%) was incubated with enzyme at 27°C in 0.015 M acetate buffer, pH 4.65. Reducing power was determined by a modification of the Willstätter-Schudel method and expressed as mg of galacturonic acid per 2.5 m*l* of the reaction mixture. ○—○ Specific viscosity, A; ●—● specific viscosity, B; ⊙—⊙ specific viscosity, C; ◑—◑ specific viscosity, eluate with 0.5 M acetate buffer described in Fig. 1: ○···○ reducing power, A; ●···● reducing power, B; ⊙···⊙ reducing power, C; ◑···◑ reducing power, eluate with 0.5 M acetate buffer (Fig. 1).

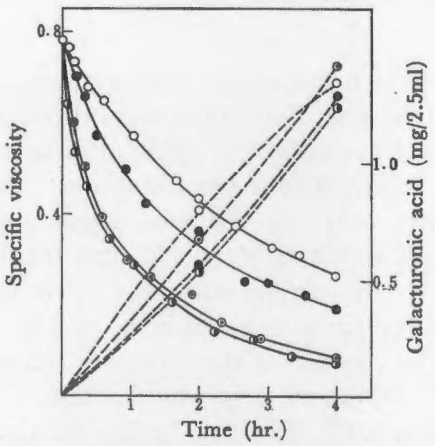


Fig. 5. Saccharifying and liquefying PG activity of A_1, A_2, A_3 and CPG. Incubation was as described for Fig. 4. ●—● Specific viscosity, A_1; ⊙—⊙ specific viscosity, A_2; ◑—◑ specific viscosity, A_3; ○—○ specific viscosity, CPG: ●···● reducing power, A_1; ⊙···⊙ reducing power, A_2; ◑···◑ reducing power, A_3: ○···○ reducing power, CPG.

matography revealed no digalacturonic acid in all the incubation mixtures examined.

Action of A_1 on Unsaturated Acid Insoluble and Acid Soluble Pectic Acid

In contrast to CPG (6), A_1 could hydrolyze unsaturated acid insoluble and acid soluble pectic acid. But the degradation limits were lower than those of the corresponding saturated pectic acids (Fig. 6). Especially unsaturated acid soluble pectic acid could be hydrolyzed to only a limit as low as onefourth that of saturated acid soluble pectic acid.

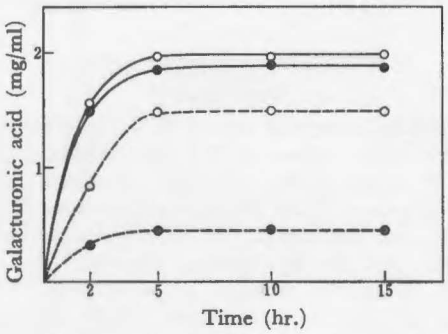


Fig. 6. PG activity of A_1 toward saturated and unsaturated pectic acids. Pectic acids (final concentration, 0.2%) was incubated with enzyme at 27°C in 0.015 M acetate buffer, pH 4.65. Reducing power was expressed as mg of galacturonic acid per m*l* of the reaction mixture. ○—○ saturated acid insoluble pectic acid, ○···○ unsaturated acid insoluble pectic acid, ●—● saturated acid soluble pectic acid, ●···● unsaturated acid soluble pectic acid.

A new enzyme* which hydrolyzes the unsaturated pectic acids at the non-reducing end of the molecules is present in the original PG preparation (Sclase). The products are saturated pectic acids and an unknown reducing compound which has no double bond and must have been derived from unsaturated galacturonic acid unit. If this enzyme were present in A_1 also, unsaturated pectic acid would be degraded presumably to the same limit as that obtained with the corresponding saturated pectic acids. But no acitivity of this enzyme could be detected in A_1 (Table 1). Paper chromatography also showed the absence of the unknown compound.

Activity of A_1 and CPG toward Digalacturonic Acid

A_1 was incubated in 0.015 M acetate buffer, pH 5, at 27°C with digalacturonic acid (0.5%) and with acid soluble pectic acid (0.25%), increasing dilutions of the enzyme solution being used in the latter case. The initial rate of hydrolysis of digalacturonic acid was found to be identical with that

* The properties of this enzyme will be described elsewhere.

TABLE 1

Action of A_1 on saturated and unsaturated pectic acid

Enzyme solution	Unsaturated acid soluble pectic acid		Saturated acid soluble pectic acid	
	Absorption at 230 mμ	Galacturonic acid μg/ml	Absorption at 230 mμ	Galacturonic acid μg/ml
CPG	0.251*	2.6*	0.011*	6.5*
CPG	0.246	3.0	0.009	343.5
CPG·YPG	0.245	81.3	—	—
A_1	0.243	66.9	0.010	376.1
Crude preparation of fungal saccharifying PG	0.065	241.3	—	—

Incubation of substrate and enzyme (A_1) was at 27°C in 0.02M acetate buffer, pH 5, the substrate concentration being about 0.05%. After about 15 hr. the incubation mixture was diluted to 5-fold. Optical density of the diluted mixture was measured at 230 mμ and galacturonic acid formed determined by the naphtoresorcinol method.

* Zero time.

of the acid soluble pectic acid incubated with a 1:2.8 dilution of the enzyme solution. Thus the percentage of the rate of attack of digalacturonic acid to acid soluble pectic acid could be calculated to be 36. By the same experiment it was found to be 11 for CPG.

DISCUSSION

When the crude saccharifying PG preparation from Sclase was chromatographed on DEAE-cellulose columns by using an increasing concentration gradient of acetate buffer, a single and symmetrical peak was obtained. But three parts of the peak, which was called for convenience A, B and C, from the fastest to the slowest, were different in the ratio of saccharifying to liquefying activity, the ratios decreasing in that order. In addition, paper chromatography indicated the presence of digalacturonic acid in the incubation mixtures of B and C. When A was rechromatographed in the same manner and the symmetrical peak obtained was divided into three parts, A_1, A_2 and A_3, their ratios of saccharifying to liquefying activity were also different from each other, the order being $A_1 > A_2 > A_3$. A probable explanation of these results is as follows:

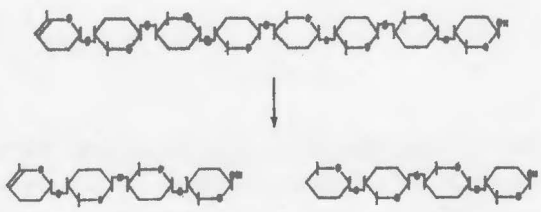
The crude fungal saccharifying PG preparation consists of a saccharifying and a liquefying PG. On DEAE-cellulose chromatography these two enzymes overlap to give a single and symmetrical peak, owing to the interaction between saccharifying PG or other substances and liquefying PG.

But resolutions, though being poor, are nevertheless obtained between them, the ratio of saccharifying to liquefying activity becoming lower with fraction number.

From the above results, it is considered that even A_1, the purest saccharifying PG preparation, contaminates some liquefying PG. But difference in ratio of saccharifying to liquefying PG activity between A_1 and CPG being slight, the amount of contaminating liquefying PG in A_1 must be very small.

Neutral sugars are normal constituents of pectin (5), and pectic acids commonly used as substrate for PGs contain unsaturated pectic acid (6). In contrast to the low degradation limit by CPG action, more than 80% glycosidic hydrolysis of the pectic acid from Citrus Pectin was obtained with A_1. Furthermore, unsaturated acid insoluble and acid soluble pectic acids, which were resistant to CPG action, were hydrolyzed by A_1, though the degradation limits were low as compared with those of the corresponding saturated pectic acids. These results may be explained in two ways.

(a) A_1 consists of an exo- and an endo-PG. The exo-PG attacks pectic acid from the non-reducing end of the molecule but is incapable of hydrolyzing unsaturated pectic acids. The unsaturated pectic acids are hydrolyzed by the endo-PG into saturated and unsaturated polygalacturonides. The former are susceptible to the exo-PG but the latter resistant to the same enzyme.



(b) A_1 contaminates an enzyme which hydrolyzes unsaturated pectic acids at the non-reducing ends, the products being saturated pectic acids and an unknown compound. The former are degraded by the exo-PG.

The results shown in Table 1 exclude the latter possibility (b). The former mechanism (a) is supported by all the results obtained in this study.

It has been reported by several workers that, unlike CPG, an exo-PG produced by fungi hydrolyzes pectic acid completely. The paper chromatograms reported on the products of enzymic action on pectic acid also differs from those of CPG (2,9). These discrepancies must have resulted from the contamination of fungal exo-PG preparations with endo-PG. In the present study we failed in preparing a pure fungal exo-PG. But the above results indicate the presence of the fungal exo-PG which hydrolyzes

pectic acid incompletely, releasing galacturonic acid unit from the non-reducing chain end.

McCready and Seegmiller (7) reported that the percentage of the rate of attack of digalacturonic acid to acid soluble pectic acid by Pectinol 100 was 5. In this study it was found to be 36 for A_1 and 11 for CPG.

SUMMARY

It has been reported by some workers that an exo-PG is produced by fungi and complete glycosidic hydrolysis of pectic acid is achieved with this enzyme. The present results, however, indicate the presence of a fungal exo-PG which degrades pectic acid incompletely. The reported complete hydrolysis with fungal exo-PG preparations is supposed to have been caused by the contamination with endo-PG. CPG seems to be the only exo-PG that was ever obtained in the form free from endo-PG.

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