Studies on Pectic Enzymes of Microorganisms

Part I Isolation of Microorganisms which Specifically Produce one of Several Pectic Enzymes*

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To pick out potent strains which specifically produce one of several pectic enzymes, endo- and exo-polygalacturonase, pectin esterase, macerating, and apple juice clarifying activities were examined with regard to 344 strains of mold (containing 71 strains of phytopathogenic mold) grown on a bran culture medium and 56 strains of shakingly cultured yeast. As the result of screening, *Aspergillus saitoi* and *Penicillium islandicum* were isolated as potent specific producers of endo-polygalacturonase. And the composition of pectic enzymes of mold was found to be rather genus or species specific. So far as examined in crude enzyme systems, there was no parallelism between any one of pectic enzyme activities and apple juice clarifying or macerating activities.

INTRODUCTION

It is well known that many microorganisms produce several pectic enzymes in complex forms. And the composition of pectic enzymes varies among species of microorganisms, so the authors thought it was worth while to pick out microorganisms which could specifically produce one of several pectic enzymes. If such a potent specific producer of a pectic enzyme could be obtained, it would be much more easy to purify the enzyme from the crude preparation of the specific producer than from the other strains' preparations which contained several pectic enzymes.

In the course of conducting measurements of several pectic enzyme activities of microorganisms, the authors thought it was also worth while to examine whether the composition of pectic enzymes was genus or species

specific or not.

As the main commercial use of pectic enzymes is the clarifying agent for cloudy fruits juices, the clarifying activity of each microorganisms was also examined.

It is the purpose of this paper to inform the isolation of specific producers of endo-polygalacturonase and the characteristic feature of the composition of pectic enzymes of various genera of mold. As for the nomenclature and the abbreviation of names of pectic enzymes, Demain and Phaff's proposition¹⁾ was ordinarily adopted.

MATERIALS AND METHODS

Microorganisms.

All the strains of mold and yeast used in this experiment were subcultured from the collection of our laboratory.

Preparation of Crude Enzyme Solutions.

Molds to be tested were inoculated to a bran culture medium in 100 ml Erlenmeyer flasks. The bran culture medium was composed of 5 g of wheat bran, 1 g of rice bran, and 7 ml of distilled water, and

* A part of this report was presented at the Annual Meeting of the Agricultural Chemical Society of Japan, Sendai, April 29, 1962.

1) A.L. Demain and H.J. Phaff, *Wallerstein Lab. Comm.*, **20**, 119 (1957).

FIG. 1. Standard Curve for Endo-PG Assay.

FIG. 3. Standard Curve for Macerating Activity.

FIG. 2. Standard Curve for Pectinesterase Assay.

FIG. 4. Standard Curve for Apple Juice-clarifying Activity.

sterilized at 120°C for 20 min. Microorganisms were cultured for 3 or 4 days at 26.5°C in case of *Penicillia* and plant pathogenic molds, or at 30°C in case of other molds. Exocellular pectic enzymes in the bran culture medium were extracted overnight at room temperature with 40 ml of distilled water in the presence of 1 ml of toluol as a preservative, and then filtered off with filter paper. Clear filtrates obtained are referred to as crude enzyme solutions hereafter.

For culturing yeasts, an arbitrary medium was employed, which contained 2% of glucose, 0.5% of peptone, 0.5% of pectin N.F., 0.1% of KH_2PO_4 , 0.04% of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.1% of yeast extracts.

Aliquots of 10 ml of the medium were transferred to 2×20 cm test tubes plugged with sterile cotton. After three days' shaking culture at 30°C, cells were removed by centrifugation at 3,500 r.p.m. for 15 min. The culture filtrates were used as crude enzyme solutions.

Evaluation of Polygalacturonase Activity.

Steel and Yang's modified technique²⁾ of the cup-plate method devised by Dingle et al.³⁾ was adopted for polygalacturonase assay with further slight modification of placing paper disks moistened with the crude enzyme solution on a plate in stead of placing drops of the enzyme solution. The pH of the plate was adjusted at 4.2 with potassium biphthalate-NaOH buffer. Paper disks were 6 mm in diameter, and made of Tōyō filter paper No. 2. The standard curve obtained by plotting the diameter of clear zone formed against the log. per cent of the concentration of pectinase (Takamine Lab., Clifton, New Jersey, U.S.A.) gave a straight line over a wide range of concentration (Fig. 1).

Chromatography of Galacturonides.

To 5 ml of 1% of polygalacturonic acid solution, which was previously adjusted to pH 4.2 with final 0.05 M acetate buffer, 0.2 ml of the crude enzyme solution was added. The enzymation was carried out at 30°C. Aliquots of 15 μl of the mixture were applied to a Tōyō filter paper No. 50 (40×40 cm) at one hour intervals from 1 to 5 hrs. The paper was developed with butanol-acetic acid-water (5:2:3)⁴⁾. Aniline phthalate or brom phenol blue was used as color reagents.

Evaluation of Pectinesterase Activity.

The indicator test devised by Kertesz⁵⁾ was adopted so as to be able to treat many samples at a same time, although this method was rather rough than alkali titrimetric method.

0.2 ml of the crude enzyme solution was added to 5 ml of 1% citrus pectin N.F. (Sunkist Growers, Inc., Ontario, Calif., U.S.A.) solution which was previously adjusted to pH 5.6 in the presence of methyl red. The reaction mixture was re-adjusted to pH 5.6 with appropriately diluted NaOH or HCl solutions. The time required for the development of pink color was followed. The standard curve obtained by plotting logarithm of the time against the log. per cent of the concentration of pectinase (Takamine Lab.) solution gave a straight line over a range of concentration of pectin esterase (Fig. 2).

Evaluation of Macerating Activity.

A potato tuber slice, 12.5 mm in diameter, 1.5 mm thick, was placed on the bottom of a flat-bottomed test tube, which contained 1 ml of the crude enzyme solution. The time required for the maceration of the slice at 30°C was followed by gently pressing the slice with a flat-headed glass rod. The relationship between the concentration of pectinase (Takamine Lab.) and the time required for maceration is shown in Fig. 3.

Evaluation of Apple Juice Clarifying Activity.

Apple juice clarifying activity was determined as follows. To 10 ml of apple juice (variety of apple: Kōgyoku, pasteurized at 60°C for one hour, pH: 3.50, s.g.: 1.037 at 25°C, total acid: 0.434% as malic acid, pectin: 0.13% as anhydrogalacturonic acid), 0.5 ml of an appropriately diluted enzyme solution was added. The time required for flocculation of suspending materials in apple juice at 40°C was recorded. The relationship between the concentration of pectinase (Takamine Lab.) and the time required for flocculation is shown in Fig. 4.

RESULTS AND DISCUSSION

The Determination of the Type of Polygalacturonases (PGs).

The chromatograms of end-products of polygalacturonase were found to be classified in two, i.e., endo-PG and exo-PG patterns as

2) W.F. Steele and H.Y. Yang, *Food Technol.*, **14**, 121 (1960).

3) J. Dingle, W.W. Reid and G.L. Solomons, *J. Sci. Food Agr.*, **4**, 149 (1953).

4) J. Ozawa, *J. Agr. Chem. Soc. Japan*, **26**, 505 (1952).

5) Z.I. Kertesz, "The Pectic Substances", Interscience Pub., 1951, p. 361.

stated by Saitô⁶⁾ In the endo-PG pattern, mono-, di-, tri-, and tetra-galacturonic acids were observed with the elapse of time.

The trimer and tetramer were faded away with further incubation.

On the other hand, in the exo-PG pattern, monomer was the sole end-product except intermediates remained at start line.

The formation of large clear zones on the plate may reasonably be attributed to the action of endo-PG and in fact this was almost the case. Then the rough determination of type and intensity of polygalacturonases of various microorganisms was done as follows:

1) Strains which Produce only Endo-PG.

If a large clear zone was observed in the plate and the chromatogram showed endo-PG pattern, only endo-PG would overwhelmingly exist.

2) Strains which Produce Endo- and Exo-PG.

If the chromatogram was exo-PG pattern

in spite of the appearance of a large clear zone in the plate, both endo- and exo-PG would exist and the latter would be very active so that oligomers were immediately splitted down to monomer.

3) Strains which Produce only Exo-PG.

When a crude enzyme solution showed chromatographically the exo-PG pattern and the formation of the clear zone was small or none, only exo-PG would exist in the solution. The earlier the appearance of monomer in the chromatogram, the higher in exo-PG activity.

Genus Specificity of Pectic Enzyme Composition.

The number of the strains which showed pectic enzyme activities and which had high activities above given levels in each genus is listed in Table I. The composition of pectic enzymes of following genera or species, i.e., Black *Aspergilli*, *Penicillium chrysogenum* and mutants, *Pen. islandicum*, *Rhizopus*,

TABLE I. THE NUMBERS OF THE STRAINS WHICH SHOWED PECTIC ENZYME ACTIVITIES AND WHICH HAD RELATIVELY HIGH ACTIVITY IN EACH GENUS

Genus	Numbers treated	Endo-PG		Exo-PG	PE		M.A. ²⁾		A.C.A. ³⁾	
		Detcd. ¹⁾	Above 1,000 unit/ml	Detcd	Detcd.	Above 4.0 unit/ml	Detcd.	Above 5.0 unit/ml	Detcd.	Above 2.0 unit/ml
<i>Aspergillus</i> (Black)	108	85	19	20	67	7	15	0	94	12
<i>Aspergillus</i> (Others)	28	18	2	5	12	4	12	0	22	6
<i>Pen. chrysogenum</i> and mutants	49	16	2	4	20	0	49	6	49	24
<i>Pen. islandicum</i>	5	5	5	0	0	0	0	0	2	0
<i>Penicillium</i> (Others)	28	13	1	6	7	2	14	0	17	2
<i>Rhizopus</i>	33	28	0	3	21	2	12	0	28	2
<i>Monascus</i>	9	0	0	0	0	0	0	0	0	0
<i>Absidia</i>	6	6	0	0	4	0	0	0	1	0
<i>Mucor</i>	3	3	0	0	3	0	1	0	3	0
<i>Neurospora</i>	2	1	0	0	1	0	1	0	1	1
<i>Monilia</i>	2	0	0	0	0	0	0	0	0	0
<i>Fusarium</i> *	10	7	1	0	3	0	6	3	6	0
<i>Sclerotinia</i> *	3	2	1	0	2	2	2	0	3	2
Other phytopathogenic molds	58	41	2	22	24	3	20	3	32	1
Total	344	225	33	60	164	20	132	12	258	50

* Phytopathogenic, 1) Detcd.: Detected, 2) M.A.: macerating activity, 3) A.C.A.: apple juice-clarifying activity.

6) H. Saitô, *J. Agr. Chem. Soc., Japan*, **28**, 869 (1957); H. Saitô, *J. Gen. Appl. Microbiol.*, **1**, 38 (1955).

Mucor, *Monascus*, *Absidia*, *Monilia*, and *Sclerotinia*, was fairly similar within genus, although several exceptions were observed in each case.

Then it can be said that the composition of pectic enzymes of molds was genus or species specific at least with respect to the genera or species mentioned above so far as grown on the bran culture medium. The characteristic feature of the composition of the enzymes of each genus or species will be summarized as follows:

1) Black *Aspergilli* (*Asp. niger* and Kuro-Koji group) generally had high endo-PG and apple juice-clarifying activities. Highest endo-PG activity was observed in this group. As for the other activities, conspicuous feature was not observed.

2) All the strains of *Pen. chrysogenum* and their mutants tested had high apple juice-clarifying and macerating activities. It may be a matter of course that phytopathogenic

Fusarium had high macerating activity. But it has not been reported that non-phytopathogenic *Pen. chrysogenum* group had high macerating activity. It is also characteristic that *Pen. chrysogenum* group had very low endo-PG and pectinesterase activities.

3) All the strains of *Pen. islandicum* indicated very high endo-PG activity. Other pectic enzyme activities were very low or none.

4) Although the characteristic feature of pectic enzyme composition could not be clearly pointed out, *Rhizopus* and *Mucor* generally had relatively low activity of each pectic enzyme.

5) *Monilia* and *Monascus* did not produce any pectic enzyme when grown on the bran culture medium so far as examined.

6) *Absidia* did not show apple juice clarifying activity in spite of producing endo-PG and pectinesterase.

7) *Sclerotinia* had very strong pectinesterase and showed rather high apple juice-

TABLE II. POTENT PRODUCERS OF SEVERAL PECTIC ENZYMES

Enzymes	Strains	Endo-PG	Exo-PG	PE	Mac. ¹⁾	A. C. A. ²⁾
Endo-PG	<i>Asp. saitoi</i> 7-6	20,000	—	0.25	0.8	1.4
	<i>Asp. saitoi</i> 4-3	6,500	—	0.75	—	1.4
	<i>Asp. nakazawai</i> 11-8	6,200	—	1.80	—	1.7
	<i>Pen. islandicum</i> Sopp 777	4,000	—	—	—	<0.1
	<i>Pen. islandicum</i> Sopp 1212	4,000	—	—	—	<0.1
	<i>Pen. islandicum</i> Sopp 1270	3,000	—	—	—	0.5
PE	<i>Sclerotinia arachmidis</i> PP-3-5	1,200	—	22.00	1.2	3.6
	<i>Sclerotinia sclerotiorum</i> S-27	400	—	19.00	0.6	12.5
	<i>Pen. sp.</i> P-Y-2	3,000	—	13.00	2.1	1.4
	<i>Asp. awamori</i> 22-6	1,000	—	10.00	2.1	4.4
	<i>Asp. nakazawai</i> 11-2	weak	—	9.00	—	2.6
Mac. ¹⁾	<i>Cercospora cruenta</i> C-10	2,000	—	1.70	5.0	5.0
	<i>Pen. sp.</i> P-9-1	—	+	?	5.0	3.6
	<i>Pen. chrysoginum</i> Q 176	weak	—	—	5.0	3.0
	<i>Gibberella fujikuroi</i> PP-1-10	1,100	—	4.00	5.0	2.6
	<i>Fusarium oxysporum</i> IAM 5062	300	—	1.80	5.0	1.2
	<i>Fusarium buffigenum</i> F-4	2,000	—	0.80	5.0	0.5
A.C.A. ²⁾	<i>Sclerotinia sclerotiorum</i> S-27	400	—	19.00	0.6	12.5
	<i>Pen. sp.</i> P-7-4	weak	±	0.25	1.2	8.5
	<i>Pen. sp.</i> P-8-1	weak	—	0.34	5.0	8.5
	<i>Asp. foetidus</i> IAM 2085	?	—	4.00	0.6	8.5
	<i>Asp. niger</i> A-9-9	1,500	—	6.40	0.6	6.4
Exo-PG	<i>Rhizopus thermosus</i> R-2-7	weak	+	0.48	—	<0.1
	<i>Helminthosporium sesamum</i> PP-2-7	weak	+	0.35	—	—
	<i>Asp. flavus</i> A-7-5	230	+	6.20	2.1	5.0

1) Mac.: Macerating enzyme activity.

2) A.C.A.: Apple juice-clarifying activity.

All the figures listed are expressed in unit/ml.

clarifying activity.

Potent Producers of Each Pectic Enzyme.

Among potent producers of each enzyme, the highest five or six strains are listed in Table II.

1) Producers of endo-PG.

Black *Aspergilli*, especially *Asp. saitoi*, and *Pen. islandicum* were the most potent producers of endo-PG. Several investigators selected *Asp. niger* as an endo-PG producer^{6,7)}. *Asp. saitoi* and *Asp. nakazawai* are closely related to *Asp. niger*.

It has not been reported that *Pen. islandicum* showed the high endo-PG activity, when grown on the bran culture medium. Both Black *Aspergilli* and *Pen. islandicum* did not produce detectable exo-PG. The activities of PE and macerating action, moreover, were very low or undetectable. Then these strains may be called as the specific producers as far as pectic enzymes are concerned.

2) Producers of pectinesterase (PE).

Two strains of *Sclerotinia* showed highest PE activity. The fact that *Sclerotinia sclerotiorum* showed high PE activity was already reported⁸⁾. *Sclerotinia hydrophilum*, another tested strain of *Sclerotinia*, however, had very low activity. These PE producers were accompanied by other pectic enzymes, especially endo-PG. Then it will be difficult to obtain the strain which specifically produce only PE. The growth rate of *Sc. sclerotiorum* on the bran and potato decoction media was very slow. On the other hand, *Sc. arachnidis* grew rather rapidly on either medium.

3) Producers which had high macerating activity.

The number of strains which showed the macerating activity above 5.0 unit/ml was twelve (*Pen. chrysogenum* and its mutants: 6, *Fusarium*: 3, *Gibberella*: 1, *Cercospora*: 2). The presence of macerating enzyme different

from known pectic enzymes was already reported⁹⁾.

But it is still obscure whether only the macerating enzyme causes the maceration of plant tissues or not, although the macerating activity of *Pen. chrysogenum* Q176 seemed to be independent from PG and PE. There was no parallelism between macerating activity and each of other pectic enzyme activities.

4) Producers which showed high juice-clarifying activity.

Sclerotinia sclerotiorum was the most potent producer of apple juice-clarifying activity.

It is very interesting that there was no parallelism between apple juice-clarifying activity and any one of pectic enzyme activity as already pointed out by several investigators.^{10~12)} This non-parallelism was also distinct in the column of producers which had high macerating activity. Recently Endô¹³⁾ discovered that only two enzymes, i.e., endo-PG and PE could cooperatively cause the clarification of cloudy apple juice. Then, on an equal basis of PE activity, it is expected that a certain parallelism will exist between endo-PG and apple juice-clarifying activities. Such a calculation was tried, for example, in the column of PE producers in Table II, but again non-parallelism was observed. This may be partly because of the measurements of enzymic activities at different pH (endo- and exo-PG: pH 4.2, PE: starting pH was 5.6, apple juice-clarifying activity: pH 3.5) and partly because of the possible participation of other enzymes or factors taking part in the clarification than endo-PG and PE.

5) Producers of exo-PG.

As the chromatographical method was

9) A. Kaji, This Journal, **23**, 131 (1959).

10) M.A. Joslyn, S. Mist, and E. Lambert, *Food Technol.*, **6**, 133 (1952).

11) C.E. Neubeck, *J. Assoc. Offic. Agr. Chemists*, **42**, 374 (1959).

12) A. Endo, This Journal, **25**, 389 (1961).

13) A. Endô, Proceeding of 15th Symposium on Enzyme Chemistry, Osaka, 1963, p. 126.

7) R. Tuttobello and P.J. Mill, *Biochem. J.*, **79**, 51 (1961).

8) E. Echandi and J.C. Walker, *Phytopathol.*, **47**, 303 (1957).

adopted to detect exo-PG activity, the absolute activity of the enzyme could not be expressed.

Helminthosporium sesamum PP-2-7, *Rhizopus thermosus* R-2-7 and *Asp. flavus* A-7-5, however, showed very strong exo-PG activity on chromatograms, accompanied by very small amount of endo-PG and PE.

The presence of exo-PG did not accelerate the macerating and apple juice-clarifying activities.

Results on Yeasts.

For culturing yeasts, rather rich medium was employed for fear that tested yeasts should not grow in poor media and not express any pectic enzyme activity. Among 56 strains of yeasts tested, covering 12 genera, six strains showed weak activity of only endo-PG. They were *Torula lactis* LH-1, and LH-9, *Rhodotorula diffluens* Nov. Comb. No. 0612, *Rhodotorula gelatinosa* Nov. Comb. No. 0385,

Frohberg yeast BH-1-1, and *Saccharomyces fragilis* NRRL Y-610. The fact that *Sacch. fragilis* produced the most active endo-PG is consistent with the results of earlier workers^{14,15}. The six strains were exactly specific producers of endo-PG, but the activity of the enzyme was as low as about 100 unit per ml of crude enzyme solution with *Sacch. fragilis*, which was the most potent producer of endo-PG among tested yeasts.

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14) B.S. Luh and H.J. Phaff, *Arch. Biochem. Biophys.*, **32**, 212 (1951).

15) M. Furuichi and T. Okamoto, *J. Agr. Chem. Soc., Japan*, **28**, 703 (1954).