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PECTIC AND CELLULOLYTIC ENZYMES IN SOYBEANS*

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Key Word Index—*Glycine max*; Leguminosae; soybean enzymes; pectic enzymes; cellulolytic enzymes; endopolygalacturonase; endopolymethylgalacturonase.

Abstract—The dominant cell wall degrading enzymes detected in extracts from 6-day-old, dark grown, Lee soybean seedlings carried out random (endo) and hydrolytic cleavages of pectic acid, pectin and methylcellulose. The pH optima of these activities were 6·0, 6·5 and 5·7 respectively. Monogalacturonic acid and another unidentified product accumulated during the degradation of polygalacturonic acid.

INTRODUCTION

Little work has been done on the identification of cell wall degrading enzymes in soybeans or other legumes. Most past studies [1–6] concerned the role of enzymes in the invasion of legumes by rhizobia and enzymes were identified only by their substrate specificity. Enzymes which split the glycosidic bond of polygalacturonic acid (pectic acid), polymethylgalacturonic acid (pectin), and cellulolytic substances are generally classified into groups [7]. Classification is based on substrate specificity and whether the substrate molecule is split in a random (endo) or terminal (exo) manner. Pectic enzymes, those degrading pectic acid and pectin, are further subdivided into hydrolytic and transeliminase enzymes.

Enzymes that attack in a random manner are distinguished from those which attack in a terminal manner by two methods. This involves comparing the per cent degradation of the substrate with the per cent decrease in viscosity. A low degradation rate, 1–5% at 50% decrease in viscosity, suggests a random attack [8–10]. A higher rate, 20–25% at 50% decrease in viscosity suggests a terminal attack [11]. Also with a terminal degradation the decrease in substrate molecular length with time is arithmetic. Thus substrate viscosity, when expressed as viscosity number, decreases in linear manner as well. With random degradation the decrease in molecular length and viscosity is exponential [12,13]. A transeliminase may be distinguished from a hydrolytic enzyme by testing for a product which absorbs light strongly at 235 nm [14]. The β-4,5 unsaturated reaction product absorbs strongly at this wavelength, whereas the saturated hydrolytic product does not.

The study reported here concerns identification of the major pectic and cellulolytic depolymerase enzymes of the soybean, *Glycine max*.

RESULTS AND DISCUSSION

Initial experiments were performed to determine the optimum pH for the depolymerase activity of soybean extracts on pectic acid, pectin, and methylcellulose. Maximum activity was observed at about pH 5·7 with methylcellulose, 6·0 with pectic acid, and 6·5 with pectin as substrate. Later work with all three substrates was performed at pH 5·5 because gel formation with pectic acid and pectin frequently interferred with the viscosity assay at pHs greater than this.

Studies on cleavage sequence indicate that the major pectic enzymes in soybean extracts attack substrates in a random rather than teminal manner. The theoretical basis for this determination is illustrated in Fig. 1. With a random attack the drop in viscosity with time would be logarithmic and a straight line would be produced when viscosity number is plotted against a logarithmic time axis [12,13]. With a terminal attack the drop in viscosity with time is linear and a curved line, similar to that shown is produced.

When data from the action of soybean extracts on common cell wall components was plotted in a

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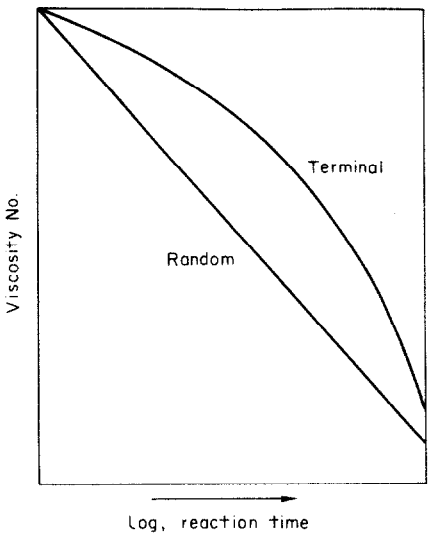

Fig. 1. Theoretical viscosity changes occurring with time for random and terminal cleavage of a polymer.

similar manner the curves presented in Fig. 2 were obtained. With pectic acid and pectin as substrate straight lines were obtained. This is characteristic of random cleavage.

Viscosity changes with methylcellulose as substrate failed to match either of the patterns presented in Fig. 1. The changes in viscosity observed approach the random pattern more closely than the terminal pattern. It seems more likely that methylcellulose is degraded by a random enzyme. A loss of enzyme activity, due to substrate molecular weight decreases or to reaction product accumulation, might produce such a curve with either type of cleavage. These data might also be explained in terms of a 'weak link' attack by a random cleaving enzyme [13]. Such a degradation depends upon links, within the polymer, that are more susceptible to attack than others. Variations in the extent of monomer methylation or the presence of extraneous groups could account for such a depolymerization of methyl-cellulose.

Enzyme denaturation during the above assays could produce misleading data; thus controls were run to determine enzyme stability during the viscosity assay. Stability was demonstrated by comparing the activity of increasingly dilute enzyme preparations assayed for increasingly long reaction times.

Further evidence that pectic acid is degraded by a random attack was obtained by comparing per cent decrease in viscosity with per cent degradation. These data are shown in Fig. 3. The low degradation rate, less than 3% at 50% decrease in viscosity, suggests a random attack [8–10]. A ter-

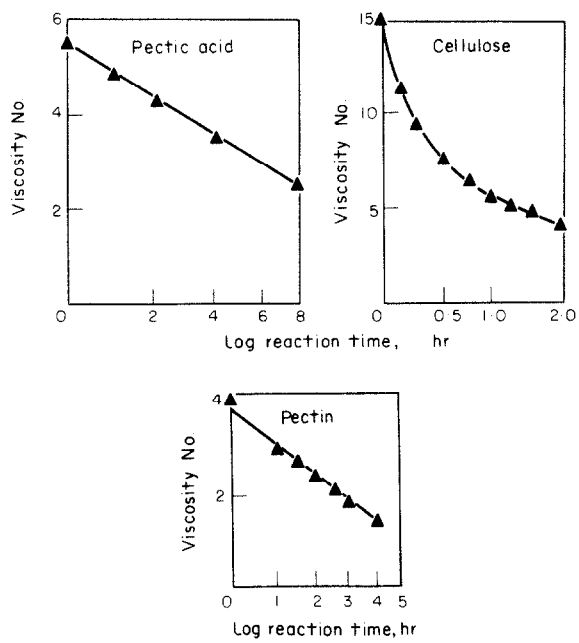

Fig. 2. Viscosity changes observed when pectic acid, pectin, and methyl cellulose solutions are treated with soybean seedling extract.

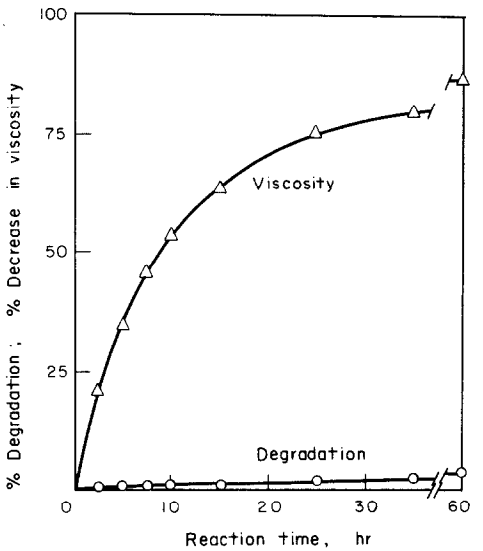

Fig. 3. Relation between per cent decrease in viscosity and per cent degradation in the enzymatic breakdown of pectic acid.

minal attack would result in a 20–25% degradation at 50% decrease in viscosity [11]. Low degradation rates were also observed with pectin and methylcellulose as substrate suggesting that these two are primarily degraded in a random manner.

The glycosidic bond of pectic substances may be broken by either a hydrolytic or transelimination reaction. The transeliminative degradation produces a β-4-5 unsaturated product which absorbs light strongly at a wavelength of 235 nm [14]. The hydrolytic product does not absorb strongly at this wavelength. Failure of reaction mixtures to show increases in absorbancy at 235 nm suggests a hydrolytic cleavage of both pectic acid and pectin. If the degradation of either substrate proceeded wholly by a transelimination reaction then absorbance increases of 4·9 units with pectic acid or 4·5 units with pectin would have been expected at 24 hr reaction time.

Paper chromatographic studies suggest that monogalacturonic acid is a product of the action of soybean extracts on pectic acid. Two spots were detected following the chromatography of the reaction mixture; one at R_f 0·30, corresponding to galacturonic acid and a second, unidentified, at R_f 0·39. The results suggest the presence of an enzyme in soybean extracts capable of releasing some of the monomer, but do not necessarily imply that it totally degrades pectic acid to monomeric units. Short chain oligogalacturonic acids, which have R_f values lower than that of monogalacturonic acid, were not detected. Monogalacturonic acid was detected only with the most active enzyme preparations and only then when the reaction mixture was incubated for 40 hr or more. No reaction products were detected with pectin or methyl-cellulose as substrate.

The results indicate that the major pectic and cellulolytic depolymerase enzymes in soybean extracts are random and hydrolytic in nature. If enzymes which degrade in a terminal manner were present their activity was small compared to those which degrade in a random manner. Thus the principal enzymes present appear to endocellulase, endopolygalacturonase, and perhaps endopolymethylgalacturonase. The involvement of endopolymethylgalacturonase can be questioned as no attempt was made to determine if pectin was being degraded by this enzyme or by the two step reaction catalyzed by pectinmethylesterase and endopolygalacturonase.

EXPERIMENTAL

Dark grown, 6-day-old, Lee soybean seedlings were used as an enzyme source. Seeds were surface sterilized by soaking in 95% EtOH for 5 min, 5·25% NaClO soln for 10 min, then rinsed with sterile H_2O. Sterile seeds were aseptically transferred to agar plates, germinated, and grown under an alternating temp cycle of 12 hr at 24° and 12 hr at 18°. Enzymes were extracted by adding 1 vol of cold sodium citrate buffer-sodium chloride solution, pH 5·5 (0·05 M sodium citrate, 1·7 M NaCl) to the seedlings and homogenizing the mixture in a blender. The resulting fluid was allowed to stand overnight at 4°. After standing, the fluid was centrifuged (15000 *g* for 1 hr) to remove insoluble plant material. The supernatant fluid was dialyzed against cold glass distilled H_2O, sodium citrate buffer, and polyethylene glycol to remove NaCl and to concentrate the extract. Enzyme activity was measured by a viscosity method [15], by following the increase in reducing units [16], or by measure of the absorbance at 235 nm [8]. Size 300 Cannon Fenske viscometers were used. Substrates were pectic acid (1% soln of Sodium Polypectate No. 6024, Sunkist Growers, Inc., Ontario, Cal.) pectin (1% soln of Pectin, L.M. No. 3446, Sunkist Growers) and methylcellulose (0·5% soln U.S.P.) Substrates were suspended in 0·05 M, pH 5·5 sodium citrate buffer. Merthiolate was added to a final concn of 0·002% to prevent bacterial growth. For paper chromatography 1 ml soybean extract and 5 ml substrate soln were mixed and incubated for 40 hr at 30°. Insoluble material was removed by centrifugation (6000 *g* for 15 min) before spotting. The solvent was *n*-BuOH–HOAc–H_2O (5:2:3). Chromatograms were developed overnight by the ascending method on Whatman No. 4 paper at room temp; sugars were detected with aniline phthalate.

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