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Characterization of Pectate Lyase from Alkalitolerant *Bacillus* sp. YA-14: Its Action Pattern and Active Center

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Pectate lyase from alkalitolerant *Bacillus* sp. YA-14 is an endo-type pectate lyase which acts randomly at the α-1,4-galacturonan linkage, and requires calcium or strontium ions for its activity. The enzyme is active on low methyl esterified pectin, but the activity toward a high methyl esterified substrate is reduced. The apparent Km's of the enzyme toward sodium polygalacturonic acid, polygalacturonic acid, and various pectins such as apple pectin, citrus pectin, and genu pectin are 0.826 mg/ml, 0.685 mg/ml, and 1.14 mg/ml, respectively. The enzyme activity is inhibited by SDS, urea, and sodium azide, but not by various reducing reagents, such as β-mercaptoethanol, Na-thiosulfate, Na-sulfate, cystein, and L-ascorbic acid. The enzyme is inactivated by N-bromosuccinimide, I_2, H_2O_2, PMSF, and iodoacetate. Judging from the results of their inhibition types, we speculate that tryptophan and serine residues are directly involved in enzyme activity, while tyrosine and methionine residues are indirectly involved in its activity.

Various pectolytic enzymes are used as processing aids in the production of fruit and vegetable juices (13). To date, there have been many reports on maceration and plant cell killing effects of the endopolygalacturonase and multiple forms of endopectolytic enzymes (2).

Pectic enzymes are classified into three main groups; de-esterifying enzymes (pectin esterase), and two groups of chain splitting enzymes (hydrolase and lyase). Pectate lyase (PL) cleaves internal glycosidic linkages in pectic substances by β-elimination. The β-eliminative attack of this enzyme results in formation of products with a double bond between C4 and C5 (2, 13). Some kinds of PL produced by different bacteria have been studied in detail (1, 5, 8, 9, 14, 17). Pectins, as substrates of the various pectolytic enzymes, have been characterized by defining structural domains of the carbohydrates. The most common domains include homogalacturonan (also known as pectate or polygalacturonate), rhamnogalacturonan I, rhamnogalacturonan II, oligosaccharide side chains and arabinogalactan (17).

In previous studies of the alkalitolerant *Bacillus* sp. YA-14, which was isolated from soil,it has been shown that this strain produces pectate lyase (19, 20). Recently this enzyme was purified to a higher degree and its general properties, amino acid composition, and N-terminal amino acid sequence were studied (4).

This paper describes the action pattern of pectate lyase from alkalitolerant *Bacillus* sp. YA-14 on polygalacturonates and the amino acids involved in the enzyme active center are determined.

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Key words: Pectate lyase, *alkalitolerant Bacillus* sp., active center

MATERIALS AND METHODS

Assay of Pectate Lyase Activity

PL activity was assayed on the basis of the enzymatic production of unsaturated products which had an absorption maximum at 232 nm. Reaction mixture contained 1.3 ml of approximately diluted enzyme, 0.5 ml of 0.2M Tris-HCl (pH 8.5), 0.2 ml of 3 mM $CaCl_2$, and to start the reaction, 1 ml of 1.0% (w/v) sodium polypectate. Absorbancy changes at 232 nm were measured in a Hitachi Model 200-20 spectrophotometer. One unit of PL activity was defined as the change in absorbancy per minute under the experimental conditions.

Paper Chromatography

A reaction mixture of enzyme with 1% sodium polygalacturonic acid was incubated at 30℃ and samples were taken at specific intervals of time and treated with WK 10 resin (Diaion) previously equlibrated with 50 mM

Tris-HCl buffer (pH 7.5). Filtering, followed by vacuum evaporation concentration, was then performed. Paper chromatography was performed on Whatman No. 1 filter paper, and developed by the ascending method with a solvent system of ethylacetate:water:acetic acid (5:3: 2.5, v/v). Dry chromatograms were dipped in a solution of 2% aniline and 2% trichloroacetic acid in ethylacetate, dried and then heated at 100℃ for 5 minutes (10).

Determination of Action Pattern of Purified Pectate Lyase

The random splitting mechanism of pectate lyase was demonstrated by comparing the decrease in viscosity to the percentage of degradation of sodium polygalacturonic acid as a function of time. Reaction mixtures consisted of 1% polygalacturonic acid, 0.2 M Tris-HCl buffer (pH 8.5), 3 mM $CaCl_2$ and 15 units of the enzyme in a total volume of 90 ml. Immediately after addition of the enzyme 50 ml of the reaction mixture was placed in a capillary viscosimeter and flow times were periodically measured. The rate of reaction was determined by removing 0.5 ml of the mixture at specific time intervals from the remaining 40 ml, and measuring absorbancy at 232 nm. The reaction temperature was 30℃.

RESULTS

Action Pattern of Purified Pectate Lyase

The mode of attack, terminal or random, can be determined by simultaneously measuring viscosity changes and percentage of cleavage of the substrate. The endo-pectate lyase caused a 50% reduction in relative viscosity of pectic acid when only about 2.0% of the bonds were broken, whereas the exo-pectate lyase had degraded 22.5% of pectic acid bonds when a 50% reduction in viscosity had occured. As shown in Fig. 1, pectate lyase of alkalitolerant *Bacillus* sp. YA-14 caused a 50% reduction in relative viscosity when about 15% of the bonds were degraded. These results suggested that pectate lyase of this *Bacillus* species degraded pectic acid randomly rather than terminally. Also, in the preliminary experiments, it was confirmed that the shorter chain products were not unsaturated galacturonic acids, based on paper chromatography result (Fig. 2).

Substrate Specificity

The enzymic activities on polygalacturonate and pectin, with various degrees of esterification of the substrate, were examined at pH 8.5. As shown in Fig. 3 the reaction rate on partially esterified pectin was higher than on a highly esterified substrate (tomato pectin). The K_m and V_{max} constants for various pectins were estimated from Lineweaver-Burk plots. These results are shown in Table 1. These data show that the enzyme has a greater affinity for polygalacturonic acid (no sodium salt form) and the affinity for 50 to 79% esterified pectin (apple or citrus pectin) is relatively low.

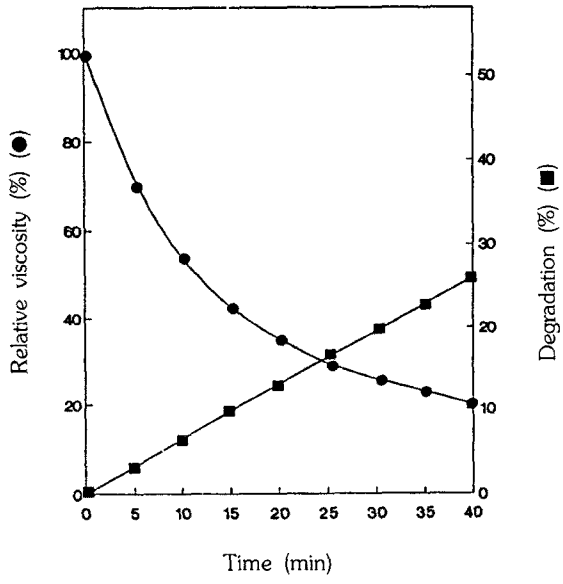

Fig. 1. Relationship of viscosity reduction due to degradation of sodium-polygalacturonic acid by pectate lyase.

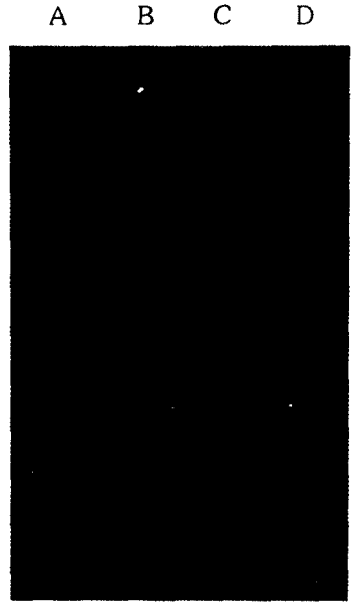

Fig. 2. Paper chromatogram of the reaction product of sodium polygalacturonic acid by pectate lyase.
A: α-D-galacturonic acid, B: polypectate digest for 24 hrs, C: polypectate digest for 8 hrs, D: control, reaction mixture

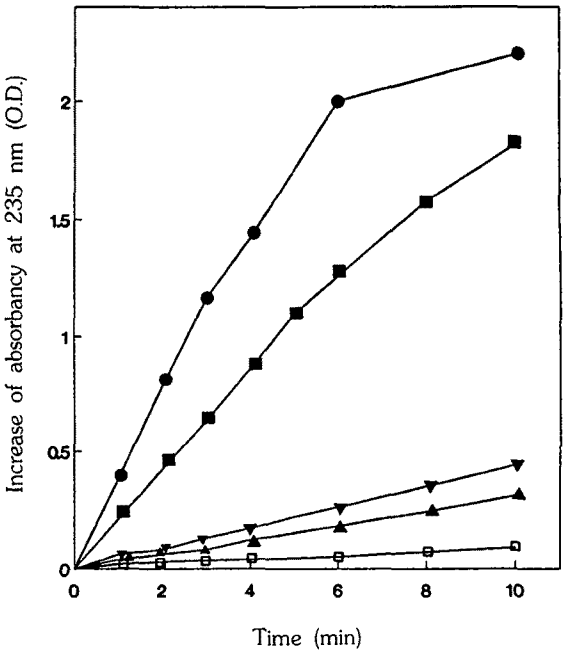

Fig. 3. Degradation of various polypectates and pectins by pectate lyase and the effect of degree of methyl esterification on enzyme activity.
Polygalacturonic acid (●), Sodium polygalacturonic acid (■), Apple pectin (▼), Citrus pectin (▲), Tomato, spray dried (□)

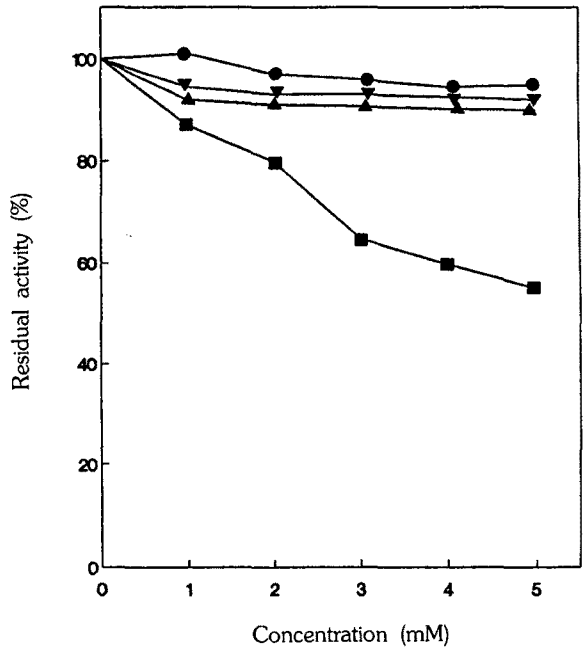

Fig. 4. Effect of iodoacetate, pCMB, NEM, and DTNB on the cystein residue modification of pectate lyase.
●; pCMB in 50 mM Sodium acetate (pH 6.0), ▼; DTNB in 20 mM ris-HCl (pH 7.5), ▲; NEM in 50 mM Tris-HCl (pH 7.5), ■; Iodoacetate in 50 mM sodium acetate (pH 4.5) in dark

Table 1. Kinetic parameters for the various substrate of the pectate lyase from *Bacillus* sp. YA-14.

Substrate (0.3%)	K_m (mg/ml)	V_{max} (ΔA_{235}/min/mg)
Sodium polygalacturonic acid	0.826	8.776
Polygalacturonic acid	0.685	14.351
Apple pectin	1.06	1.694
Citrus pectin	1.14	1.552
Genu pectin	1.21	1.753
Tomato, spray dried	5.0	0.821

Effects of Modification on Cystein

The most useful class of reagents for the modification of cysteine residues in proteins has been α-haloacetates and its corresponding amides. Because a rapid reaction was desired, iodine containing compounds, such as iodoacetate, were used. In addition, β-mercaptoethanol, N-ethylmaleimide (NEM), 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), and ρ-chloromercuribenzoate (pCMB) were used. Each reaction was performed at 30℃ for 15 minutes with various concentrations of reagents, and the effects were determined by measuring residual enzyme activity. The reaction with NEM, pCMB and DTNB did not induce activity reduction judging from the increasing concentration (Fig. 4). In the case of reaction with iodoacetate it was supposed that the reaction was affected by micro-environmental factors and its specificity to cysteine modification was not perfect. That is, histidine or methionine was simultaneously oxidized by this reagent. Therefore, its possible effect for causing the activity reduction was not seriously considered. As the increasing absorbance at 250 nm by the reaction with pCMB to cysteine was previously reported, the reaction with NEM was detected by a reduction of absorbance at 300 nm, its maximum absorbancy. This was confirmed by a change of absorbancy (7). Each reagent modified the cysteine residue but a significant activity change was not seen (Fig. 5). Therefore, cysteine was not involved in the enzyme active site.

Effect of Modifiers on Methionine

The specific or selective modification of methionine in proteins and peptides is somewhat difficult to achive under relatively mild conditions. The majority of the modification reactions used to study methionine involve either oxidation by chloramine T, H_2O_2 and sodium periodate, or alkylation by iodoacetate at the thioether sulfur. After the enzyme was incubated with each reagent at 30℃ for 15 minutes, its residual activity was measured (Fig. 6). Hydrogen peroxide and sodium periodate redu-

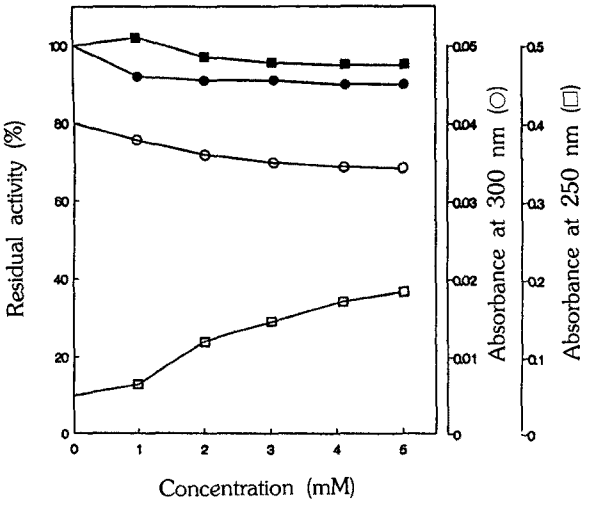

Fig. 5. Detection of the reaction of the modification of pectate lyase by pCMB (■) and NEM (●).
□; Absorbancy at 250 nm caused by the modification with pCMB, ○; Absorbancy at 300 nm caused by the modification with NEM

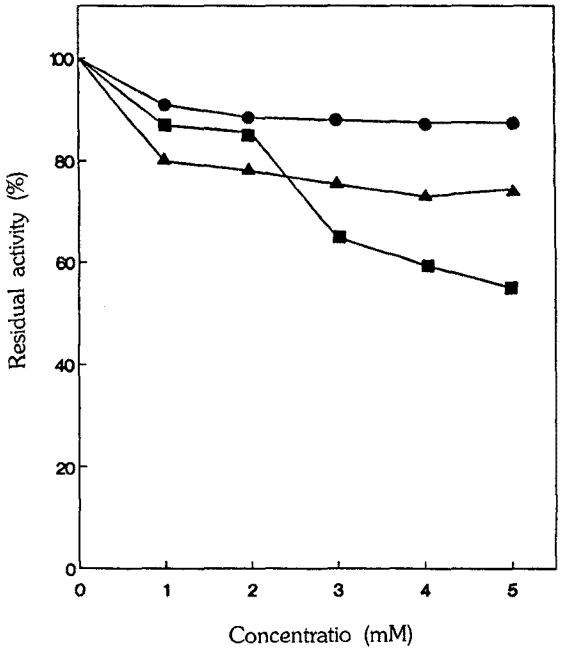

Fig. 6. Effect of H_2O_2, sodium periodate, and iodoacetate on the methionine residue modification of pectate lyase.
●; sodium periodate in 0.1 M sodium acetate (pH 5.0), ▲; H_2O_2 in 50 mM sodium acetate (pH 4.5), ■; Iodoacetate in 50 mM sodium acetate (pH 4.5) in dark

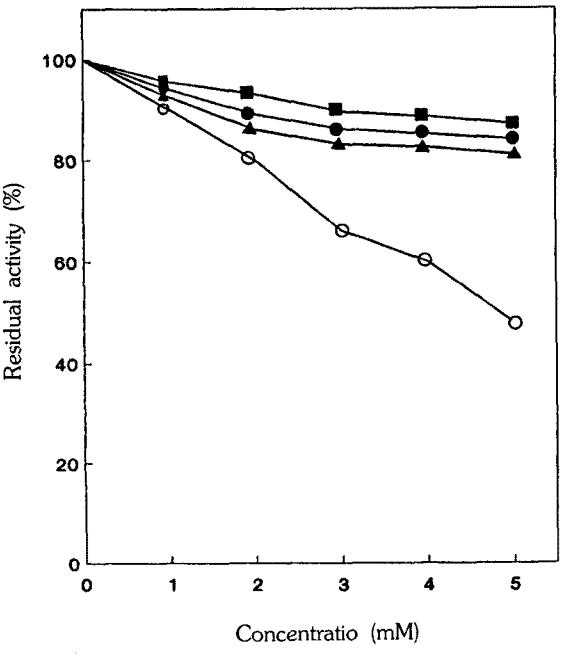

Fig. 7. Effect of DEP, hydroxylamine, PMSF, and $HgCl_2$ on pectate lyase activity.
■; $HgCl_2$, ●; DEP in 50 mM sodium acetate (pH 6.0) at 10℃, ▲; Hydroxylamine in 50 mM Tris-HCl (pH 7.5), ○; PMSF

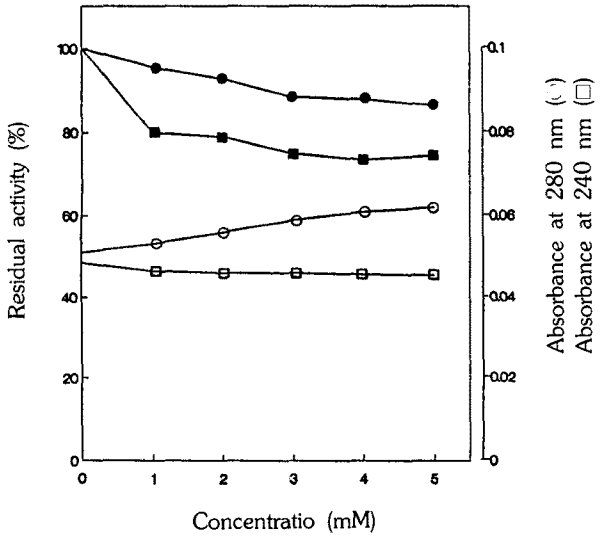

Fig. 8. Detection of the reaction of the modification of pectate lyase by H_2O_2 (■) and DEP (●).
○; Absorbancy at 240 nm caused by modification with DEP, □; Absorbancy at 280 nm caused by modification with H_2O_2 in 50 mM sodium acetate (pH 4.5)

ced the enzyme to 80 to 91% of residual activity at 1 mM concentration, but the effect of iodoacetate was as significant as increasing the concentration. Because hydrogen peroxide was oxidized by the tryptophan residue under alkalic condition the reaction was performed under acidic condition. This was confirmed by measuring

whether there was a change in absorbancy at 280 nm resulting from modification of tryotophan (Fig. 8). As a result the reaction with hydrogen peroxide was relatively specific to methionine.

Effect of Histidine Modification

Modification of histidine by alkylation with iodoacetate or ρ-bromophenacyl bromide is a common process. Diethylpyrocabonate (DEPC) has become useful for studies involving the specific modification of histidine because its modification of histidine causes an increase in absorbance at 240 nm. As shown in Fig. 7, 95% of enzyme activity was maintained after treatment with 1 mM of DEPC and 88% of activity was maintained after treatmemt with to 5 mM of DEP. Also, as shown in Fig. 8, no change in absorbance at 240 nm was noted. Although DEP significantly modified histidyl residue, enzyme activity was preserved. Therefore, the possibility of involvement of histidine in the active center was thought to be remote.

Effect of Modifiers on Hydroxylamine

It has been reported that hydroxylamine modifies the carboxyl group of aspartic acid and glutamic acid. However, as shown Fig. 7, hydroxylamine, in stepwise concentrations, did not effect enzyme activity. These results suggest the possibility that aspartate or glutamate are involved in the active center is remote.

Effect of Serine Modification

PMSF(Phenylmethylsulfurfluoride), which is a specific inhibitor to serine, strongly affects enzyme activity, as shown in Fig. 7. Enzyme activity was reduced by 1 mM PMSF, and only 55% of enzyme activity remained after a one hour reaction with PMSF (data not shown). As the composition of serine in the enzyme was relatively high the effect of gradual reduction in enzyme activity, as a function of increasing the concentration and incubation time with PMSF, seemed to be caused by a stepwise modification of serine. Therefore, it was determined that serine was involved in the enzyme active center.

Effect of Arginine Modification

The modification of arginyl residues was possible with three different reagents; phenylglyoxal, 2,3-butanedione and 1,2-cyclohexanedione. It has been reported that the modification effects of phenylglyoxal are confirmed by increasing absorbance at 475 nm region. This result is shown in Fig. 9. Additionally, the absorbance change at 475 nm caused by modification with 2,3-butanedione (11, 12), was also determined. Though modification was substantial with phenylglyoxal, enzyme activity inhibition rates were reduced by phenylglyoxal or 2,3-butanedione. Therefore, it was confirmed that there was no important role for arginine in enzyme activity.

Effect of Modification on Tryptophan and Tyrosine

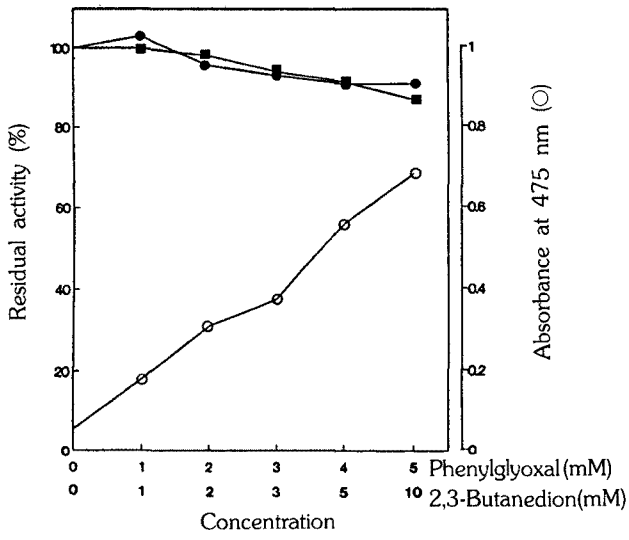

Fig. 9. Effect of phenylglyoxal and 2,3-butanedion on the arginine residue modification of pectate lyase.
●; Phenylglyoxal in 50 mM Tris-HCl (pH 8.0), ■; 2,3-Butanedion in 50 mM sodium borate (pH 7.5), ○; Absorbancy at 475nm caused by modification with 2,3-butanedione

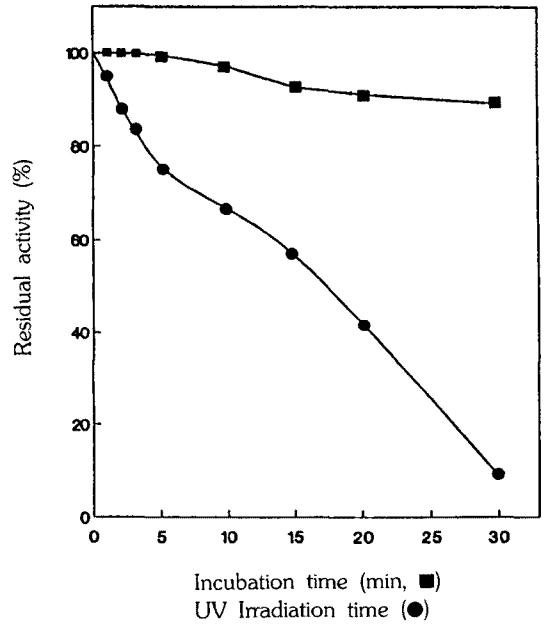

Fig. 10. Effect of UV irradiation time in the presence of 2,3-butanedion on pectate lyase activity.
■; control, ●; After $UV_{366\,nm}$ irradiation in 10 mM 2,3-butanedion

2,3-butanedione, N-bromosuccinimide(NBS), hydrogen peroxide and iodine were used to examine the effect of tryptophan on enzyme activity. Fliss and Viswanatha have reported that 2,3-butanedione is a photo-sensitizing agent in the presence of oxygen (3). In our experiments

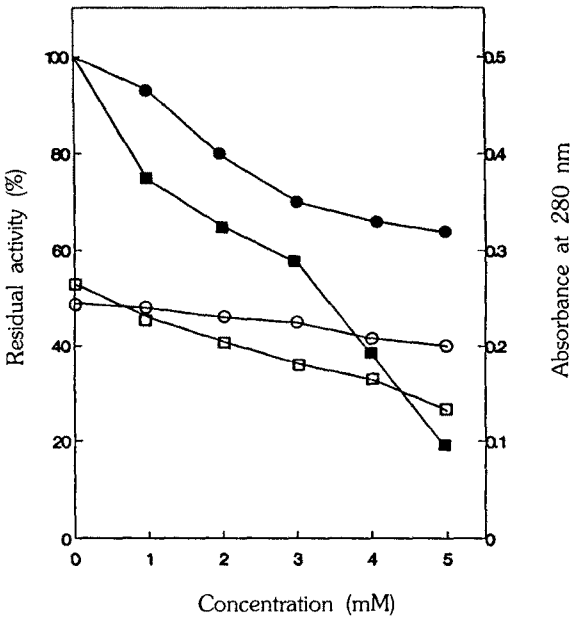

Fig. 11. Change in absorbancy at 280 nm due to I_2 modification of pectate lyase.
▼; I_2 in 25 mM Glycine-NaOH (pH 10.0), ■; Absorbancy at 280 nm by I_2 in high pH, ▲; I_2 in 25 mM sodium acetate (pH 4.0), ●; Absorbancy at 280 nm by I_2 in low pH

illumination was provided by a UV light of 366 nm wavelength positioned 15 cm above the surface of the enzyme solution. As shown in Fig. 10, the enzyme was gradually inactivated by photo-oxidation time, compared with a control without UV light. As this reduction of enzyme activity could not be caused only by destruction of tryptophan it ought to be confirmed by another reagent, such as iodine. Both alkali and acidic conditions were used to iodinate the amino acid. The degree of inactivation of tryptophan or tyrosine was examined by measuring absorbance at 280 nm, and enzyme activity. The reaction in acidic conditions showed a lower absorbancy and reduced enzyme activity than in alkali conditions. We speculated that tryptophan was involved in enzyme activity (Fig. 11). This was confirmed by the reaction with NBS. This reagent is a known specific modifier of tryptophan. Enzyme activity was significantly reduced as the reagent concentration was increasd, as shown in Fig. 12. Enzyme activity was completely inhibited by 3 mM NBS. Also, as seen in Fig. 12, oxidation of tryptophan by 5 mM H_2O_2 in alkali conditions caused a 45% reduction of enzyme activity.

Inhibition Type of Chemical Modifiers

Inhibition types of some chemical modifiers, such as N-bromosuccinimide, iodine, hydrogen peroxide, PMSF, and iodoacetate, were examined by Lineweaver-Burk

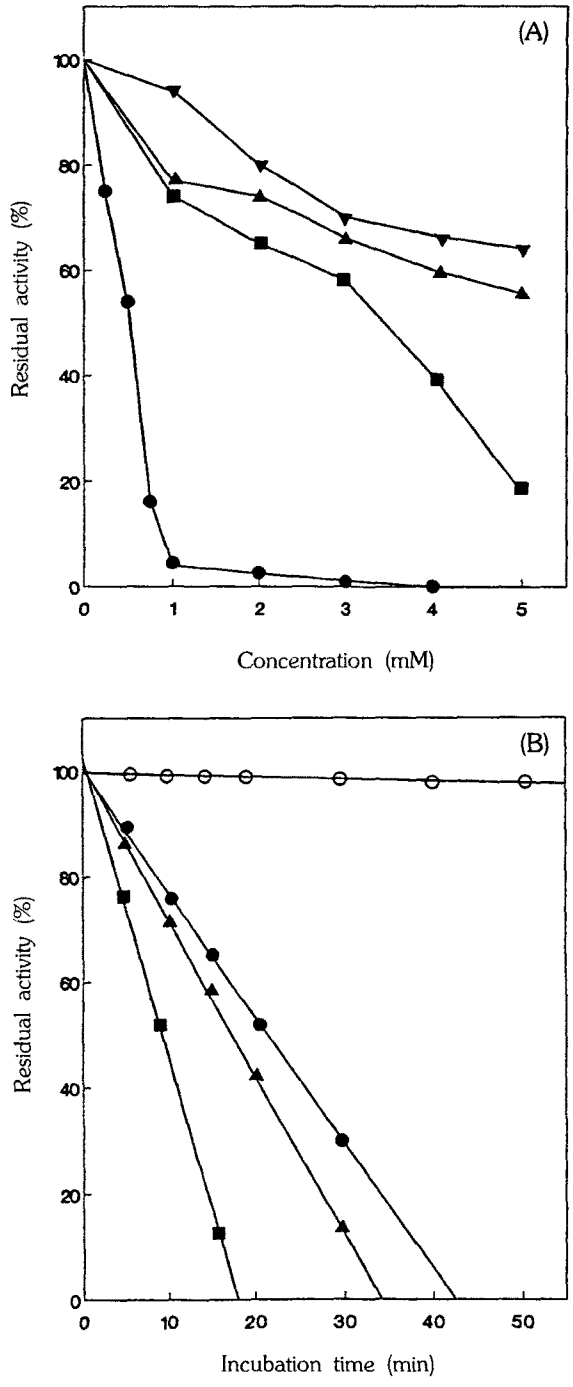

Fig. 12. (A) Effect of I_2, H_2O_2, NBS on the tryptophan residue modification of pectate lyase.
I_2 in 25 mM Glycine-NaOH (pH 10.0) (▼), I_2 in 25 mM sodium phosphate (pH 4.0) (■), H_2O_2 in 50 mM glycine-NaOH (pH 11.0) (▲) NBS in 0.1 M sodium acetate (pH 4.5) (●)
(B) Effect of NBS inhibition on pectate lyase as a function of concentration and time.
Control (○), NBS 0.25 mM (●), NBS 0.5 mM (▲), NBS 1.0 mM (■)

plots at various inhibitor concentrations. NBS, iodine, PMSF and hydrogen peroxide in alkali conditions exhibited a competitive inhibition type, but iodoacetate inhibited the enzyme activity as a noncompetitive type (data not shown). We speculated that tryptophan and serine are directly involved in the enzyme active center, as long as methionine and tyrosine are only indirectly involved.

DISCUSSION

If a polygalacturonate chain is attacked from the reducing end by a terminal mechanism, a double bond should be absent in the longer residual chain after unsaturated trimer units are removed from the molecule during the early reaction. If the chain is attacked from the non-reducing end, the longer residual chain should have an unsaturated unit at its non-reducing end. In order to determine whether pectate lyase acts on the reducing end or the non-reducing end, experiments were performed according to the methods of Nagel and Vaughn *et al.*.

The data illustrated in Fig. 3 and Table 1 show that lyase had a higher affinity for polygalacturonic acid than it had for 50 to 80% esterified pectin, and that the progress of cleavage by the enzyme was considerably inhibited by methyl ester groups in the substrate. In addition, this enzyme attacked the substrate in an endo-type manner (Fig. 1 and Fig. 2). Therefore, this enzyme was different from the bacterial exopolygalacturonate lyase which was demonstrated by Castelein and Pilnik to have the highest affinity for polygalacturonic acid (15). This alkali-tolerant *Bacillus* sp. YA-14 lyase should be named as an endo-low methoxy pectate lyase.

Various chemical reagents were used for the modification of enzyme activity. These included cystein, methionine, histidine, aspartic acid or glutamic acid, serine, arginine, tryptophan, and tyrosine. In these amino acid residues modifing reagents, the enzyme activity inhibition effect caused by the reaction of 2,3-butanedion in UV light, N-bromosuccinimide, I_2 in acidic condition, PMSF, iodoacetate, and H_2O_2 in alkali conditions was significant. Additionally, the inhibition type, as judged from Lineweaver-Burk plots, confirmed the role of these chemical modification reagents. N-bromosuccinimide, I_2, H_2O_2, and PMSF were competitive inhibitors, but iodoacetate was a non-competitive inhibitor. The modification mechanism of amino acids in protein by these chemical reagents was reported by Lundblad and Noyes (7). Therefore, we suggest that tryptophan and serine residues were directly involved in enzyme activity, while tyrosine and methionine residues were indirectly involved. On the basis of this results, we hope to identify the active site of pectate lyase, using molecular biological tools, comparing its amino acids in the active site to possible active sites in the pectate lyase DNA sequence. It will be possible to make a useful industrial pectate lyase having an enhanced stability of enzyme activity by modifing the active site.

Acknowledgement

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