Mold Pectinase Modified with Dialdehyde Derivatives of Dextran and Cellulose

Mikihiko Kobayashi, Yogo Chiba, Kazumi Funane, Setsuko Ohya, and Yoji Kato*

National Food Research Institute, Tsukuba 305, Japan
*Faculty of Education, Hirosaki University, Hirosaki 036, Japan

Received October 17, 1995

Chemical modification of mold pectinase with dextran- and cellulose-dialdehydes was examined to improve the enzyme characteristics. The modified pectinase with dextran-dialdehyde retained about 50% of the original activity, and more than 80% of the total amino groups were modified. HPLC gel filtration analysis showed an increase in molecular weight of the reaction product. Reaction with cellulose-dialdehyde provided an immobilized form of pectinase. The immobilized pectinase was resistant to both acidic and alkaline pHs, and also acquired heat stability at 60°C. The optimum pH of the modified enzyme shifted from pH 4.5 to 5.0–5.5, and this enzyme had higher activity at neutral pH regions than the native enzyme. A rather low recovery of immobilized enzyme (14.5%) should be improved by the combination with various methods hitherto established.

Key words: beet pulp; cellulose-dialdehyde; chemical modification; dextran-dialdehyde; pectinase

Chemical modification causes the change in enzyme characteristics, mostly in the direction of negative and unfavorable states. Owing to these changes, essential amino acid residues for the catalytic activity were identified referring to the loss of enzyme activity. Modification of amino groups generally gave no such drastic decrease in enzyme activity, and occasionally led to the improvement of stability and reactivity. For example, modification of proteases by dextran-dialdehyde reduced the self-digestion of enzyme and increased the storage stability. Heat stability of an α-amylase from Bacillus amyloliquefaciens increased by 10°C, after the modification with dextran-dialdehyde.

In preceding studies, we examined enzymic saccharification of beet pulp, and showed a mold pectinase was effective to obtain reducing sugars. Exhaustive saccharification of beet pulp allows 1.3-fold increase in the amount of usable carbon, such as mono- and oligosaccharides from sugar beet for ethanol fermentation.

Therefore, improvement of enzymic characteristics of pectinase might be important for an increase in ethanol production. Moreover, the fermentation process using a bioreactor system would require the immobilized pectinase. For the basis of these application steps, modifications of mold pectinase with a water-soluble matrix of dextran-dialdehyde and a water-insoluble matrix of cellulose-dialdehyde were examined.

Materials and Methods

Materials. Sugar beets, Beta vulgaris L. var. monohyaccae and fodder beets, B. vulgaris L. var. MGM that were harvested in the autumn of 1992 were provided by the Agricultural Experimental Station of Hokkaido, Sapporo. Mold pectinase (Fluka 76290; 0.02 units/mg) and citrus pectin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Clinical dextran and cellulose powder (300 mesh) were the products of Meito Sangyo Co. and Advancee Toyo Co., respectively.

Modification of pectinase with dialdehyde polymers. Mold pectinase (10 mg/mL), dextran-dialdehyde (10 mg/mL), and TEMED (N,N,N′,N′-tetramethylethylenediamine) buffer (pH 6.8, 7.8, 8.8, and 9.8) were mixed and incubated at 30°C. The reaction mixture (50 μL) was removed at suitable times, and diluted with 40 μL acetate buffer (pH 5.2, 950 μL). For preparative purposes, pectinase (200 μL), dextran-dialdehyde (500 μL), and TEMED buffer (pH 8.5, 500 μL) were incubated at 30°C for 4 h. In the case of cellulose-dialdehyde, the reaction mixtures were prepared as above and centrifuged. The resulting precipitate was suspended in the acetate buffer.

Assay of pectinase activity. The reaction mixture containing enzyme solution (25 μL) and 1% citrus pectin (35 μL) dissolved in acetate buffer (pH 5.2) was incubated at 30°C for 20 min. The reducing sugar produced was measured by the Nelson Somogyi method as described previously. The amino acid content was measured by the OPA (o-phthalaldehyde) method.

Saccharification of various polysaccharides. Two-hundred μL of native pectinase (1 mg/mL) and modified pectinase with cellulose-dialdehyde (0.005 units/5 mg) were incubated with various polysaccharides (1% solution, 200 μL) in 40 μL acetate buffer (pH 5.2) at 30°C for 24 h. The reducing sugar was measured as described above.

HPLC analysis. Gel filtration of the modified pectinase was done by high pressure liquid chromatography (HPLC), on a JASCO PU-980 chromatograph with a column of Tosoh G3000 SW and patterns were monitored by an RI detector (JASCO RI-830) and UV detector (JASCO UV-807) at 280 nm. Hydrolysis patterns of beet pulp were analyzed by an Aminex HPX-87C column (Bio-Rad Co.) at 60°C. Peaks were monitored by an RI-830 detector.

Results

Modification with dextran-dialdehyde

Dextran-dialdehyde was prepared by the periodate oxidation of clinical dextran (MW 63,000) with NaIO₄. The course of the reaction of mold pectinase with the dextran-dialdehyde is shown in Fig. 1, where the incubation was done at pH 6.8–9.8. Modification of amino groups of pectinase by dialdehyde groups of oxidized dextran proceeded faster at higher pHs of the reaction mixture. According to the modification, enzyme activity was decreased and seemed to reach a constant at about 60% (Fig. 1a). The free-amino groups of pectinase were measured by the fluorescent OPA method, and almost complete modification was attained within 120 min at pH 9.8 (Fig. 1b). Although the amount of free-amino groups in the reaction mixture at pH 7.8 was lower than that at pH 6.8, the reason was not clear.
Fig. 1. Effects of pH on the Reaction of Pectinase with Dextran-dialdehyde.

Pectinase (1% solution, 100 µl) was mixed with 1% dextran-dialdehyde (100 µl) and 0.1 M TEMED buffer, pH 6.8 (●), 7.8 (■), 8.8 (▲), and 9.8 (■), and incubated at 30°C. A 50 µl sample was removed and diluted with 40 mM acetic buffer, pH 5.2 (450 µl). (a) Enzyme activity. (b) Amino group content measured by the OPA method.

Fig. 2. Effects of Temperature on the Reaction of Pectinase with Dextran-dialdehyde.

Pectinase (1% solution, 100 µl) was mixed with 1% dextran-dialdehyde (200 µl) and 0.1 M TEMED buffer, pH 8.5 (●), and incubated at 30°C (●), 40°C (■), and 50°C (▲). (a) Assay of residual enzyme activity (a) and amino group contents (b) were done as described in the legend for Fig. 1.

Inactivation of pectinase was stimulated by higher concentrations of dextran-dialdehyde. This was illustrated by the results shown in Fig. 2. Use of 0.5% dextran-dialdehyde gave faster modification of pectinase than the case with 0.25% concentration shown in Fig. 1. Moreover, Fig. 2 showed that a higher temperature for incubation caused rapid modification of the enzyme. Loss of pectinase activity remained constant at the level of 50%. Thus, the exhaustive modification of amino groups in the current pectinase caused no critical damage of the enzyme activity.

Modification with cellulose-dialdehyde

Since the peridate oxidation was applicable to the water-insoluble polysaccharides, cellulose powder was oxidized to prepare the cellulose-dialdehyde, which served as the water-insoluble matrix. Reaction of pectinase with cellulose-dialdehyde proceeded in a similar way to those of dextran-dialdehyde. However, the pectinase attached to cellulose-dialdehyde was converted to the water-insoluble form (Fig. 3), and free and bound pectinases could be separated by centrifugation of the reaction mixture. Reactivity of pectinase for the increasing concentrations of cellulose-dialdehyde was indicated by the decrease and increase of enzyme activities in the supernatant and precipitate fractions, respectively (Fig. 3a). A large decrease of amino groups in the supernatant fraction also supported the conversion of pectinase into the water-insoluble form (Fig. 3b). The pectinase bound to the cellulose-dialdehyde was equivalent to the immobilized enzyme.

In this experiment, 1 mg of pectinase (1.93 units) was incubated with 0–50 mg of cellulose-dialdehyde at pH 7.6 and 25°C for 1 h. The maximum amount of pectinase immobilized to the matrix was about 0.28 units, which corresponded to 14.5% of pectinase activity in the reaction being attached to the matrix.

Characteristics of modified pectinase

Changes in molecular forms of pectinase attached to the dextran-dialdehyde were measured by HPLC gel filtration.
Fig. 5. Stability of Pectinase Modified with Cellulose-dialdehyde.
Native pectinase (0.004 units) (○, ○) and modified pectinase with cellulose-dialdehyde (0.001 units/1 mg) (●, ▽) were incubated at 60°C with 40 mM acetate buffer, pH 5.2 (○, ●) or triethanolamine HCl buffer, pH 8.5 (○, ▽). Residual activity was measured as described in the text. The alkaline-treated samples were neutralized with 1 N HCl before the activity assay.

Fig. 6. Optimum pH of Pectinase Modified with Cellulose-dialdehyde.
Native (○) and modified pectinase (●) were incubated at 30°C with 40 mM Tris acetate buffer in the presence of the substrate, citrus pectin. Residual activity was measured as described in the legend for Fig. 5.

analysis (Fig. 4). Panels U1 and R1 were the control at zero time measured by UV at 280 nm and R1 detector, respectively. Clinical dextran shown in R1 gave a much lower response than the dextran-dialdehyde in the reaction mixture. A small but definite change was detected by UV pattern, where the major peak of $R_t$ (retention time) 16.4 min decreased in its height, and heavy tailing toward the high-MW region (smaller $R_t$) was observed, indicating a complex formation of pectinase and dextran-dialdehyde. R1 analysis for dextran-dialdehyde found no significant change though the height of whole peak decreased slightly.

SDS-PAGE analysis also gave no sharp band of enzyme-dextran complex, but the protein band at the position of original enzyme became thinner compared with the control (data not shown).

Incubation of pectinase at pH 3.0 and 50°C caused a significant loss of activity, i.e., 78% inactivation for 20 min, while the modified pectinases with dextran and cellulose-dialdehydes retained most of the activity. Another example of the heat stability of modified pectinase is shown in Fig. 5. Enzymes were incubated at pH 5.2 or 8.5 and 60°C. Native pectinase was inactivated faster at lower pH. In contrast, pectinase attached to cellulose-dialdehyde retained higher activities at both pHs for much longer incubation periods.

Table Saccharification of Various Polysaccharides by Modified and Native Pectinases

<table>
<thead>
<tr>
<th>Polysaccharides</th>
<th>Modified pectinase</th>
<th>Native pectinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beet pulp</td>
<td>244 (29)</td>
<td>142 (17)</td>
</tr>
<tr>
<td>Apple pectin</td>
<td>833 (100)</td>
<td>814 (100)</td>
</tr>
<tr>
<td>Citrus pectin</td>
<td>816 (98)</td>
<td>804 (99)</td>
</tr>
<tr>
<td>CM-Cellulose</td>
<td>264 (32)</td>
<td>150 (18)</td>
</tr>
<tr>
<td>Guar gum</td>
<td>273 (33)</td>
<td>156 (19)</td>
</tr>
<tr>
<td>Xylan</td>
<td>385 (46)</td>
<td>258 (32)</td>
</tr>
</tbody>
</table>

* Amount of reducing sugar (represented as μg of glucose) released from 2.0 mg of each polysaccharide by the incubation at 30°C for 24 h is indicated. Values in the parentheses are relative (%) to the substrate apple pectin.

Fig. 7. Double Reciprocal Plots for Native and Modified Pectinases.
Native pectinase (0.004 units (○) and modified pectinase with cellulose-dialdehyde (0.001 units/1 mg) (●) were incubated with 0.250 μg of substrate, citrus pectin 50 μl of reaction mixture at 30°C for 30 min.

Native pectinase had a sharp optimum pH at 4.5. At pH 3.5 and 5.5, this enzyme had less than 20% of activity. Modification of pectinase with cellulose-dialdehyde shifted the optimum pH by 0.5–1.0 pH units toward the neutral region and greatly improved the reactivity at pH 5.0–6.5 (Fig. 6). This desensitization of pectinase at neutral pH would stimulate the synergistic use of this enzyme with the other enzymes such as cellulase and xylanases.

Action of modified pectinase

Cellulose-dialdehyde-modified pectinase was used for the hydrolysis of various polysaccharides including beet pulp. Comparison of hydrolysis (%) with modified enzyme for those with native pectinase showed some improvements in the modified enzyme (Table). Rather low susceptibility to beet pulp may indicate the higher contents of cellulose and semi-cellulose polymers in the beet pulp. Moreover, production of reducing sugars from the polysaccharides other than pectins and beet pulp indicated the presence of cellulase and xylanase activities in the mold pectinase preparation as described previously. HPLC analysis of the hydrolysis products from beet pulps obtained from the sugar beet (monohomare) and fodder beet (MGM) gave almost the same profiles.

Kinetic evaluation of the modified pectinase was done by the measurement of $K_m$ (Fig. 7). Double reciprocal plots for citrus pectin with the native pectin gave a $K_m$ of 3.8 mg/ml. The modified pectinase with cellulose-dialdehyde had a $K_m$ of 6.7 mg/ml. Therefore, the modified enzyme showed about 1.8-fold lower affinity for the substrate, citrus pectin. $V_{max}$ for the native and modified enzymes were 5.18
and 1.03 μmol min⁻¹ μg⁻¹, respectively. Decrease in the substrate affinity would be ascribed to the modification of a large number of amino groups in the enzyme molecule.

Discussion

Based on the nucleotide sequence of two pectinase components, PG I and PG II, from *Aspergillus niger*, the primary structures of pectinases were deduced.¹⁰ PG I and PG II contained 17 and 20 Lys residues in the total 368 and 362 amino acid residues of the whole sequence, respectively. We showed the mold pectinase used in this experiment had an identical amino acid sequence to that of PG I.¹¹ Therefore, the OPA measurements of the modified pectinase (Figs. 1 and 2) indicated that modified enzyme retained more than half of its activity upon the almost complete modification of Lys groups in the enzyme molecule. For this mold pectinase preparation, however, the amino group modification with 10 mM TNBS (trinitrobenzene sulfonate) gave 90% loss of enzyme activity during the incubation at 30 °C for 30 min. Modification of carboxyl group with 10 mM EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide). His residues with 0.5 mM DEP (diethyl pyrocatechone), and Trp residues with 80 μM NBS (N-bromosuccinimide) more potently inactivated the pectinase.¹¹ Compared with these results of chemical modification, the current modification with dialdehyde-polymers would proceed in rather mild binding to Lys residues. Although the addition of substrate pectin to the reaction mixture to protect the enzyme from inactivation was tested, about 15% higher activity was recovered at pH 7.6 but no protective effects were observed at pH 9.8. In the latter case, pectinase for control lost 30% of activity, possibly because of the alkaline inactivation (data not shown).

The immobilized pectinase to cellulose-dialdehyde showed a large increase in pH stability at pH 3 and heat stability at 60 °C (Fig. 5). Since the modified pectinase was stable at pHs around 5.0, these improvements made this enzyme useful at higher pH together with Meicelase and the enzyme isolated from strain C as described in our previous paper.⁸ Compared with the native pectinase, modified enzyme showed somewhat higher saccharification of various polysaccharides as shown in the Table. It was interesting to note that the susceptibility to beet pulp, CM-cellulose, and guar gum increased to almost the same extent though the reason was not clear. Although the Km of the modified enzyme increased 1.8-fold, this was in the ranges obtained from the trypsin-dextran-dialdehyde (2-fold),²¹ serine proteinase-dextran-dialdehyde (1.3–1.9-fold).⁴³ The modified pectinase became less sensitive to the pH optimum and the maximum activity was obtained at pH 5.0–5.5 instead of pH 4.5 for native enzyme. Moreover, much higher activity was detected at neutral to alkaline pH (Fig. 6).

The mold pectinase used in this study has a MW 58,000, which was measured by SDS-PAGE.¹¹ Clinical dextran has a MW 63,000. Molecular forms of modified pectinase were analyzed by HPLC gel filtration (Fig. 4) and SDS-PAGE. The former results indicated time-dependent changes in the elution pattern but no definite MW of the products were measured. In most cases of the dextran-modified enzymes, assignment of a clear peak or band of modified product could not be done.²⁻⁶ One of the reason for these ambiguities would be the heterogeneous MW distribution of dextran, and the other would be conformational changes of enzyme structure, which caused by the binding of sugar chains to the enzyme at more than a single site. It is also conceivable that inter- and intra-molecular cross-linkings between enzymes and/or sugar chains would be produced.

For the bioreactor system of ethanol production, enzymes for saccharification of beet pulp would be preferably provided in an immobilized forms. In this respect, these results showed that pectinase modified with cellulose-dialdehyde was converted into more resistant forms for pH and heat stability than the native enzyme. Moreover, this method with cellulose-dialdehyde provided the immobilized pectinase by a convenient and simple reaction. Preparation of the immobilization matrix, cellulose-dialdehyde, was also simple and contained no particularly hazardous reagents such as cyanogen bromide or organic solvents. Although the recovery of immobilized pectinase (14.5%) was not sufficient for practical use, further improvement might be provided from studies on the combination of this method and various methods hitherto established. In the case of z-amylase, co-immobilization of amylose-cellulose-dialdehyde complex with polyacrylamide gave a sufficient yield of immobilized enzyme (unpublished result). Therefore, this method would be applicable to the pectinase immobilization with higher recovery than these results.

Acknowledgment. This work was supported by the Department of Ministry of Agriculture, Forestry, and Fisheries of Japan through the National Project of Bio-Renaissance.

References

6) K. Takatsu and M. Kobayashi, Abstracts of Papers, the Annual Meeting of the Japan Society for Biotechnology, and Agrochemistry, Tokyo, April, 1994, p. 34.