Cold-Active Polygalacturonase from Psychrophilic-Basidiomycetous Yeast
Cystofilobasidium capitatum Strain PPY-1

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Received October 21, 2004; Accepted November 16, 2004

We purified and characterized a cold-active polygalacturonase (PG) from the extracellular fraction of Cystofilobasidium capitatum strain PPY-1. The purified PG from strain PPY-1 has a molecular mass of about 44 kDa, and exhibited high activity at 0 °C, although its optimum temperature was 45 °C. Although the $K_m$ value for polygalacturonate as a substrate at 45 °C was found to be 11.2 mg/ml, it decreased gradually with decreasing temperature, and it was 0.66 mg/ml at 0 °C. Moreover, its cleavage pattern was of the endo-type. These findings might indicate that PG from strain PPY-1 is a novel type of cold-active endo-PG that is able to degrade pectin compounds at low temperatures.

Key words: Cystofilobasidium capitatum; polygalacturonase; cold-active enzyme

Cold-active enzymes are attractive for the processing of foods, since there is an industrial trend to treat food-stuffs under mild conditions in order to avoid spoilage and changes in taste and nutritional values. Therefore, interest in cold-active enzymes has been increasing in recent years, and various candidates have been identified for application in several food industries.

Pectin is one of the main constituents of the primary cell walls and the middle lamellae of higher plant cells with cellulose and xyloglucan. In the food industry, pectin compounds are a source of problems that arise during the extraction, filtration, concentration, and clarification of fruit juice. Therefore, pectin-depolymerizing enzymes e.g., polygalacturonase (PG), are widely used for the treatment of pectin compounds in the fruit and vegetable processing industries.

In previous studies, we isolated several Pectinolytic and Psychrophilic Yeast (PPY) strains from forest soil from Abashiri, Hokkaido, Japan. Among these strains, Cystofilobasidium capitatum strain PPY-1 grew on pectic compounds at temperatures below 5 °C, and the extracellular fraction of strain PPY-1 exhibited high PG activity at 5 °C. These findings might indicate that strain PPY-1 produces a cold-active PG able to degrade pectic compounds at low temperatures. In this study, we attempted to purify and characterize this cold-active PG from strain PPY-1.

First we attempted to purify PG from the extracellular fraction of C. capitatum strain PPY-1. Yeast cells grown on MI medium containing 1% (w/v) pectin (Sigma Chemicals, St. Louis, MO) were separated by centrifugation at 12,000 g for 10 min at 4 °C. Supernatant derived from the culture medium was applied to a DE52 (Whatman International, Maidstone, U.K.), and eluted with 50 mM sodium phosphate buffer (pH 7.5). The non-adsorbed fraction, which exhibited PG activity, was collected. The PG-active fraction was applied to a Butyl Toyopearl 650S (Tosoh, Kanagawa, Japan) column (φ 33 by 270 mm), and eluted with a linear gradient of 50 mM sodium phosphate buffer (pH 7.5) containing 1,500 to 0 mM (NH$_4$)$_2$SO$_4$. PG activity appeared at fractions with about 900 mM (NH$_4$)$_2$SO$_4$. The fraction containing PG showed a single band on SDS–PAGE. PG activity was assayed by measurement of the increase in reducing groups.

The PG was purified about 6.7-fold, with a yield of 41.9% total activity, and gave a single band, which corresponded to a molecular mass of about 44 kDa, on SDS–PAGE (Fig. 1). The specific activity of the purified PG was calculated to be 458 U/mg at 45 °C. The purified PG exhibited high residual activity at 0 °C, which was one-fifth of the activity against that at 45 °C, although the optimum temperature was 45 °C (Fig. 2A). This suggests that PG has sufficient ability to catalyze pectin degradation under cold conditions, e.g., at 5 °C. The optimum pH for purified PG was 4.4, and the enzyme was stable at pH 2.5 to 7.0 (Fig. 2B). On the other hand, no effect on activity was detected with 1 mM chloride salts of Fe$^{3+}$, Ni$^{2+}$, Zn$^{2+}$, Ca$^{2+}$, Co$^{2+}$, Ba$^{2+}$, Mn$^{2+}$, Mg$^{2+}$, or Cu$^{2+}$, and PG was not inhibited by 5 mM EDTA. The $K_m$ of the purified PG for polygalacturonate was found to be 11.2 mg/ml at 45 °C, but the $K_m$ value decreased gradually with decreasing temperature, and it was 0.66 mg/ml at 0 °C. The N-terminal amino acid sequence of the purified PG was WSATISSLNNDVAAKKCTS, which exhibits 52% identity with that of PG from Saccharomyces cerevisiae.
Next we determined the substrate specificity and cleavage pattern of the purified PG. First, the influence of substrate methylation on the enzymatic activity was examined by evaluating the activity of the enzyme toward pectins with increasing degrees of methyl esterification (DE). Among the pectic substrates, maximum activity was found toward polygalacturonate (DE0%), and PG hydrolyzed up to DE60% pectin, although it did not hydrolyze DE90% pectin (data not shown). Di- and tri-galacturonates were not hydrolyzed by PG (data not shown). The mode of action of the enzyme (exo-PG or endo-PG) was determined by viscosimetric assay. Viscosity changes of polygalacturonate were measured with an Ostwald-type viscometer at 5°C with 1% polygalacturonate in 0.1 mM acetate buffer, pH 4.4. PG reduced the viscosity of a 1% polygalacturonate (DE0%) solution (Fig. 3A). When PG caused a 50% decrease in the viscosity of a polygalacturonate solution, the extent of hydrolysis was less than 10% (Fig. 3A). This indicates that the purified PG has an endo-action. On the other hand, analysis of the PG reaction products was performed by thin-layer chromatography (TLC). TLC analysis indicated PG produced di- and tri-galacturonates from polygalacturonate in the initial stages of hydrolysis, which accumulated throughout the incubation period, although a little mono-galacturonate was found among the reaction products (Fig. 3B).

In conclusion, it has been reported that several yeast species produce PG, but to our knowledge no cold-active PG from psychrophilic yeast has been reported. So far, some PPY strains, which can grow on pectin at 5°C, have been isolated and characterized by our group and by Birgisson et al. In this study, we purified and characterized PG from C. capitatum strain PPY-1, the most dominant PPY species. The enzymatic property of most interest in this study was the temperature dependency of PG. Although the optimal temperature was 45°C, PG exhibited high residual activity and a low $K_m$ value at 0°C. This suggests that PG from strain PPY-1 is a cold-active enzyme. Moreover, its N-terminal amino acid sequence did not show high homology with those of any other PG, although it shows 52% identity with that of S. cerevisiae PG. On the other hand, the cleavage pattern of the PG was of the endo-type. This suggests that PG degraded the substrate with a certain degree of multiple attack, since most of the reaction products derived from polygalacturonate are tri- and di-galacturonates in the initial stages of the incubation period. This degradation pattern might be similar to the mixed endo/exo mode of PG cleavage described by Cook et al. In this mechanism, the enzyme might catalyze the hydrolysis of several bonds before it dissociates and forms a new active complex.
with another polymer chain, resulting in the liberation of oligogalacturonates.\textsuperscript{16)

Based these facts, it appears that PG from strain PPY-1 is a novel type of cold-active endo-PG, and it might be possible that the PG will be applicable in the food industry.

Acknowledgments

We are indebted to Professor Toshihiro Watanabe of Tokyo University of Agriculture for his help in the amino acid sequence determination.

References