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Study of some physico-chemical characteristics of a *Saccharomyces cerevisiae* endopolygalacturonase: a possible use in beverage industry

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Zymograms of a crude protein extract from *S. cerevisiae* strain SCPP containing endopolygalacturonase were studied and compared to the purified enzyme by determining their physico-chemical properties. The results obtained with crude extract were similar to those of the purified enzyme. The endopolygalacturonase from both sources displayed a pH optimum between 3.0 and 4.0, and was active at temperatures between 4 and 50°C on a large panel of substrates. These characteristics make this *S. cerevisiae* endopolygalacturonase an attractive tool for the beverage industry. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 296–300.

Keywords: *Saccharomyces cerevisiae*; pectinases; polygalacturonase; *PGL1–1* gene; characterization

Introduction

Pectolytic enzymes are responsible for the degradation of pectic polymers, which constitute the main building block of the primary cell wall of plants [7,18]. Some of the pectins may pass into the juice during pressing, leading to an increase of viscosity and limiting clarification [21]. This difficulty can be overcome by adding a suitable pectinase [2]. The most commonly used commercial pectinases come from *A. niger* [20]; nevertheless this fungus secretes a variety of other enzymes some of which possess oxidizing activities that are not desirable in the production of wine and fruit juices, for example arabinofuranosidase, which can cause the formation of haze [21]. *Saccharomyces cerevisiae* strain SCPP, which produces all three types of pectolytic activities, including depolymerizing and de-esterifying enzymes (polygalacturonases (PG; EC: 3.2.1), pectin-lyases (PL; EC: 4.2.2) and pectin-esterases (PE; EC: 3.1.1) was isolated from Champagne wine during alcoholic fermentation by Gainvors *et al* [8]. In a previous study, we reported that the wild-type SCPP strain carried out simultaneously fermentation and pectin removal, leading to wine without pectins [9]. However, for non-fermented beverages, an isolated enzyme is required. For this reason, the *PGL1–1* gene encoding endoPG was recently cloned from the SCPP strain by Gognies *et al* [11]. In this study, an endoPG was produced from the activity of the *PGL1–1* gene isolated from the SCPP strain. Results obtained with this purified endoPG were compared with results from the crude extract obtained from the wild-type SCPP strain. Two major studies have been reported on endoPG enzyme activity from other *S. cerevisiae* strains [4,13]. So far, no one has described the relationship between the physico-chemical properties and these endoPG biotechnological applications. In this paper, we also discuss the endoPG characteristics coupled with industrial applications.

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Materials and methods

Microorganisms

The SCPP yeast, a wild-type strain secreting all three pectolytic activities, was isolated from fermenting Champagne wine and classified as a *Saccharomyces cerevisiae* [8]. Overproduction of endopolygalacturonase activity was obtained by transforming the *S. cerevisiae* ATCC 28583 strain using the plasmid pPGL1–1 [11]. This plasmid carried the *PGL1–1* gene encoding endoPG and was cloned from the SCPP strain according to Gognies *et al* [11]. As a reference, we used a X2180–1A (MAT a) strain (Yeast Genetic Stock Center, Bethesda Research Laboratories, Gaithersburg, MD, USA), a *S. cerevisiae* without any pectolytic activity [8].

Media

The yeast strains SCPP and X2180–1A were grown in a medium containing 0.67% yeast nitrogen base (Difco, MI and WI), 1% glucose and 50 mM potassium buffer pH 5.5. The transformed strain ATCC 28583 was grown in SD medium (0.67% yeast nitrogen base (Difco) without amino acid, 1% glucose, 50 mM potassium buffer pH 5.5) supplemented with tryptophan (50 μg ml^{-1}). Cultures were incubated at 30°C. Experiments were performed with 50 ml of medium in 500-ml Erlenmeyer flasks and agitated at 100 rpm (aerobic conditions) or in 50-ml capped conical tubes without stirring (anaerobic conditions).

Preparation of proteins

Preparation of crude extract from the SCPP strain: A crude extract from the SCPP strain contain-

ing the endoPG enzyme was obtained after chilled acetone precipitation according to Gainvors *et al* [8]. Proteins contained in the retentate were precipitated by addition of chilled acetone; the extract was centrifuged for 5 min at 4000 × *g* at 4°C, and the pellet was washed twice with chilled ethanol (100%). The protein pellet was resuspended in 50 mM acetate buffer at pH 4.0.

Preparation of purified enzyme from the transformed ATCC 28583 strain: For purified enzyme preparations, the supernatants obtained by centrifuging the culture medium of the transformed ATCC 28583 strain at 5000 × *g* for 5 min at 4°C were subjected to tangential ultrafiltration on a Sartocon mini (Sartorius, Palaiseau, France) with a 30 000 Da cut-off membrane. They were concentrated 10-fold. The retentate was precipitated with chilled acetone as described above. The final protein pellet was redissolved in 10 mM Tris-HCl buffer at pH 7.0 and applied to a UNO™Q1 ion exchange chromatography column. Fractions containing PG activity were pooled and transferred to a dialysis cartridge (10 000 Da cut off; Centrisart 1, Sartorius) and concentrated 5-fold.

Zymogram procedure

In order to study the PG activity secreted by the transformed ATCC 28583 strain and the SCPP wild-type strain, we used the method of Cruickshank and Wade [5]. Protein-containing samples were analysed by gel electrophoresis under non-denaturating conditions on 10% acrylamide and 0.1% polygalacturonic acid (Sigma, St Quentin Fallavier, France) gels. The proteins from both preparations were standardized for cell concentration. Electrophoretic separation was performed overnight at 4°C at constant voltage (150 V). After 1 h incubation in 0.1 M malic acid, gels were incubated for 7 h in a 50 mM acetate buffer pH 4.0. After staining the gels with 0.02% Ruthenium Red and destaining them with several changes of distilled water, PG activity was revealed as clear zones corresponding to degradative zones. The zymogram procedure was optimized by determining the pH optimum, the temperature dependence and the stability of the endoPG as follows.

The pH optimum was determined by separating the proteins by gel electrophoresis as described above and incubating different runs in 50 mM buffers differing by their pH values for 7 h at 30°C. Reactions were visualized with Ruthenium Red. The temperature dependence study was carried out as described for the pH study, except that different temperatures were applied to similar electrophoretic runs using the optimal pH defined previously.

The stability of the protein extracts was tested using the optimal pH and temperature defined previously, except that before electrophoresis, protein extracts were incubated for 5 min at temperatures from 4°C to 100°C.

Detection of pectinolytic activity on solid medium

Pectinolytic activity was revealed through the coloration of the medium in Petri dishes with a solution of Ruthenium Red at 0.1% for 5 min, followed by a washing step with sterile distilled water [15].

EndoPG molecular weight determination

For the crude extract, the protein sample was separated on SDS-PAGE containing 0.1% polygalacturonic acid (Sigma) according to Laemmli [14]. Renaturation of the endoPG was obtained after a 2-h wash with 2.5% Triton X-100 in 50 mM acetate buffer pH 4.0 under agitation according to Heussen and Dowdle [12]. The gel was then incubated in 50 mM acetate buffer pH 4.0 for 7 h at 30°C and the PG activity was visualized. Molecular weight markers were visualized with Coomassie Blue G250 [19]. For the purified enzyme, electrophoresis was performed according to Laemmli [14] followed by silver staining according to Merril *et al* [16].

Isoelectric point

Concentrated proteins from the crude extract were desalted on a Centrisart cartridge (5000 Da cut-off) (Sartorius) and separated by isoelectrofocusing on Servalyt Precotes containing 5% ampholytes (pHi 3.0–10.0, Serva, Pharmacia, Orsay, France). Electrophoretic separation was achieved at 4°C as follows: 2 h at 2000 V, 25 mA and 3 W, followed by 2 h at 2000 V, 15 mA and 3 W using an LKB instrument (Pharmacia, Orsay, France). PG activity was visualized with an overlay containing 0.5% polygalacturonic acid and 0.8% agarose in 50 mM potassium buffer pH 4 [3]. Incu-

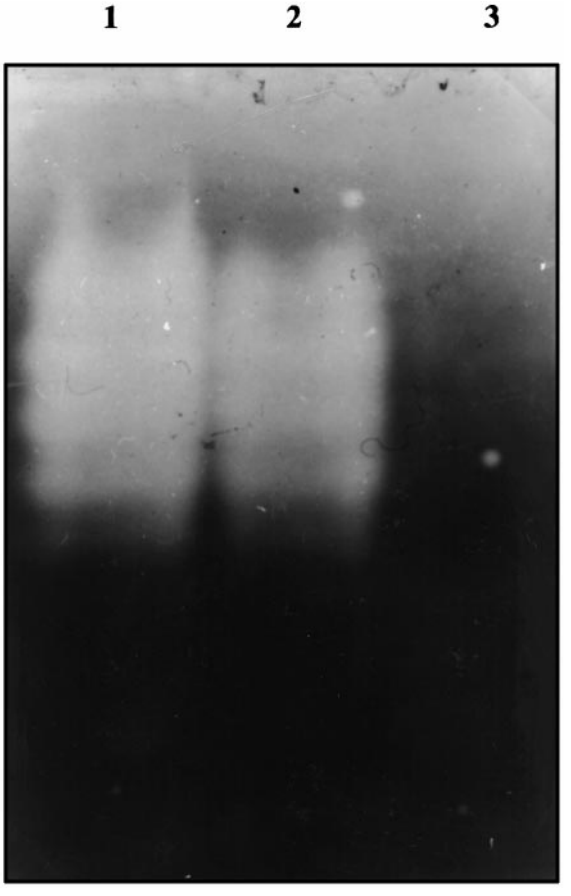

Figure 1 Detection of extracellular polygalacturonase activity by zymogram. Wells 1 and 2: 50 μl of 150× concentrated culture medium from SCPP grown on 1% glucose under anaerobic and aerobic conditions, respectively. Well 3: 50 μl of 150× concentrated culture medium from X2180 grown on 1% glucose under anaerobic conditions.

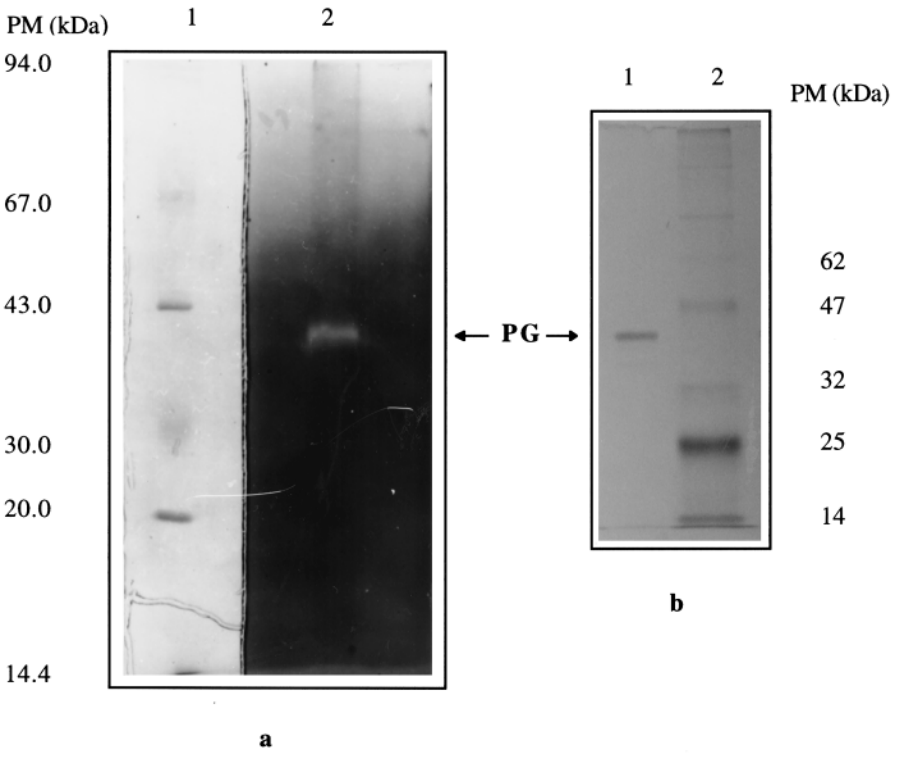

Figure 2 Molecular weight determination of *Saccharomyces cerevisiae* polygalacturonase. (a) Detection of polygalacturonase activity by zymogram under denaturating conditions. Well 1: Molecular weight markers. Well 2: 50 μl of 150× concentrated culture medium from SCPP grown on 1% glucose. (b) SDS-PAGE and silver staining of well 1, molecular weight markers; well 2, 10 μl of purified *S. cerevisiae* endoPG.

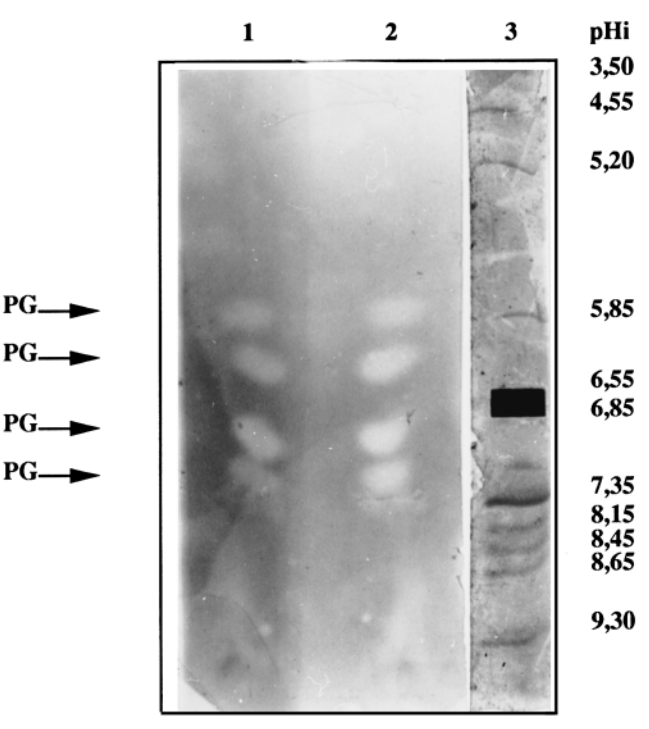

Figure 3 Isoelectrofocusing analysis of PG activity determined by zymogram under denaturating conditions. Wells 1 and 2: 10 μl of 150× concentrated culture medium from SCPP grown on 1% glucose. Well 3: pHi markers.

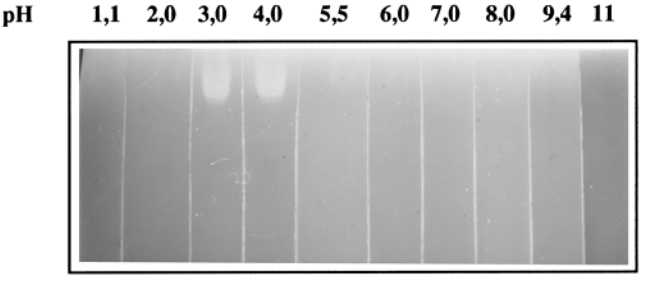

Figure 4 pH dependence of PG activity determined by zymogram. Each well was loaded with 50 μl of 150× concentrated culture medium from SCPP grown on 1% glucose.

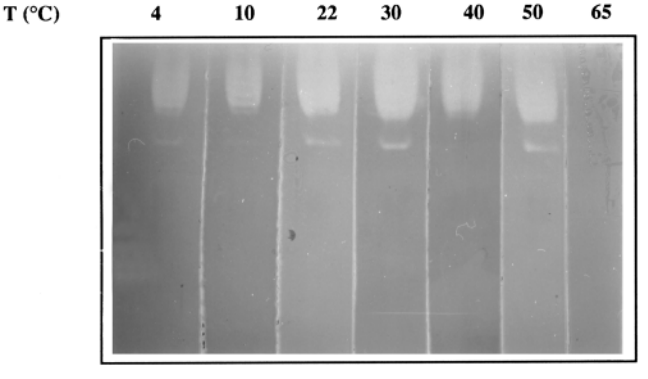

Figure 5 Temperature effect on extracellular PG activity determined by zymogram. Each well was loaded with 50 μl of 150× concentrated culture medium from SCPP grown on 1% glucose.

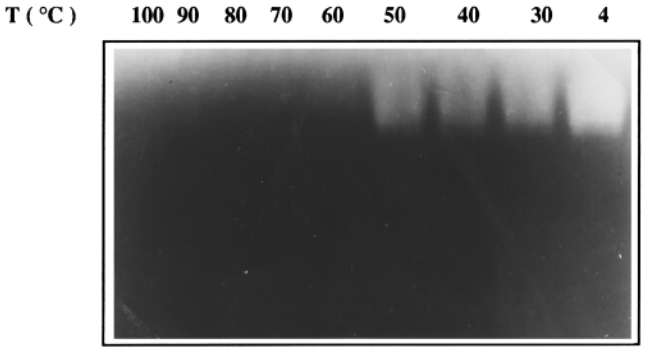

Figure 6 Temperature effect on stability of the polygalacturonase activity. Each well was loaded with 50 μl of 150× concentrated culture medium from SCPP grown on 1% glucose.

bations were at 30°C overnight. The isoelectric focusing gel was subsequently stained and washed as described for the zymogram. pHi markers were visualized with Coomassie Blue G250 [19].

Results and discussion

Relationship between culture conditions and PG production

Under optimal conditions, the zymogram showed that the production of polygalacturonase was higher under anaerobic than aerobic conditions for the wild-type SCPP strain, with maximal activity after 3 days (Figure 1). However, in a previous study, we showed that under the same conditions, the transformed strain exhibited maximum activity only after 1 day [11].

Physico-chemical properties of S. cerevisiae endoPG

Molecular weight determination: Crude extract from the wild-type SCPP strain, as well as purified enzyme from the transformed ATCC 28583 strain exhibited a band corresponding to endoPG at 42 kDa as shown in Figure 2a and b. In agreement with this result, Federici [6] and Yoshitake *et al* [22] obtained PG with a similar molecular weight from the yeasts *Cryptococcus albidus* var *albidus* and *Kluyveromyces wickerhamii*.

Isoelectric point: The isoelectric points of the PG isoforms secreted by the wild-type SCPP were determined according to Bertheau *et al* [3]. Four clear bands appeared with respective pIs of 5.7, 6.25, 6.7 and 7.20 corresponding to four PG activities (Figure 3). To our knowledge, this is the first time that PG isoforms are reported in *S. cerevisiae*.

Effect of pH on PG activity: We first determined the optimal pH by measuring the quantity of liberated reducing groups according to Milner and Avigad [17]. Polygalacturonic acid was hydrolyzed at a pH close to 11.0. Axelos *et al* [1] showed that under high temperatures pectins were more sensitive to hydrolytic and de-esterification reactions at pH 7.0 than at pH values between 3.0 and 4.5. Thus, the chemical hydrolysis of polygalacturonic acid could hide the endoPG activity at a pH higher than 4.5. We therefore turned our attention to the zymogram technique to characterize the optimal pH without the interferences caused by pectin hydrolysis. The pH optimum of the PG enzyme secreted by the wild-type SCPP strain as well by the purified enzyme was between 3.0 and 4.0 as shown in Figure 4. In the literature, the pH optimum is usually reported as between 4.0 and 5.5 for PG. At pH 3.0, for both strains, PG still exhibited 80% of its maximum activity, whereas there was only 10% activity at pH 5.5. This is the first report of such a characteristic, suggesting that this enzyme can be preferentially used in treatment of fruit juices which are usually acid.

Effect of temperature on PG activity: The *S. cerevisiae* endoPG was active at temperatures between 4 and 50°C with a maximum activity between 20 and 30°C (Figure 5). An optimum at 20°C for a PG extracted from *S. cerevisiae* CECT1389 strain was reported by Blanco *et al* [4].

Thermal stability of PG: After treatment for 5 min at different temperatures, PG was stable up to 50°C but was

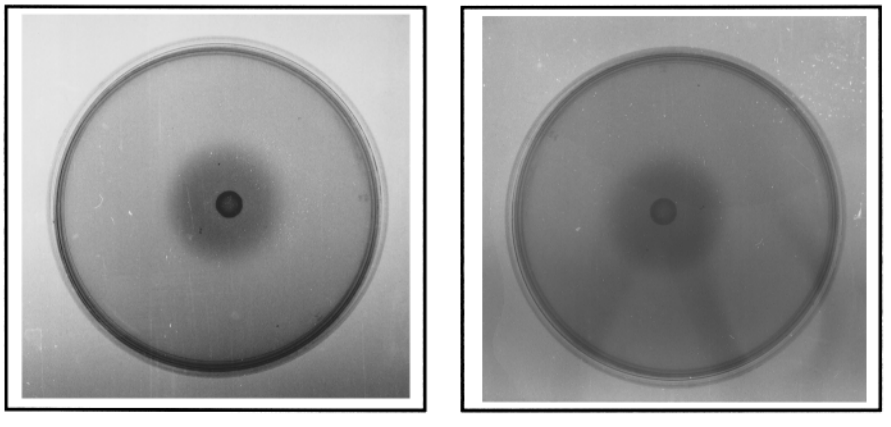

Figure 7 Relation between endoPG activity and degree of methylation of pectins. The transformant strain ATCC 28583 [pPGL1–1] was tested for endoPG activity by the plate method. Two microlitres of purified enzyme were applied on: (1) polygalacturonic acid medium; (2) apple pectin medium.

inactive beyond 60°C (Figure 6). The PG enzyme produced by the yeast strain *Kluyveromyces wickerhamii* exhibited the same characteristics [22].

Relationship between endoPG activity and substrate methylation: The ability of endoPG to degrade pectins with different degrees of methylation (DM) was determined for the purified enzyme. In Figure 7, a halo of depolymerization is visible indicating that this enzyme was active on polygalacturonic acid (DM <7%) or on apple pectins (DM =77%) as well.

Conclusion

The high commercial potential of a *S. cerevisiae* strain producing a polygalacturonase requires characterization of this enzyme. To avoid the necessity for purification of this enzyme, the zymogram technique was used to determine its physico-chemical properties. Despite the fact that the zymogram is not a quantitative method, it is possible to use it as a comparative means to study endoPG activity. Results obtained showed a similarity between the crude extract and the purified endoPG indicating that this technique is appropriate for the characterization of polygalacturonases. The enzyme activity was maintained at temperatures between 4 and 50°C, and the enzyme activity has an acidic pH optimum similar to the pH of numerous fruit juices. This enzyme can also be used during fermentations performed by cryotolerant *S. cerevisiae* [10]. Furthermore, *S. cerevisiae* endoPG allowed the degradation of pectins with different degrees of esterification and from diverse sources. The commercial mixture of pectolytic activity originating from *Aspergillus niger* contains other enzymes exhibiting unwanted activities. *S. cerevisiae* cells, however, secrete few proteins and especially not undesirable ones for wine and fruit juice production. For this reason, we can propose the recombinant *S. cerevisiae* strain as an alternative source of endoPG in the food industry.

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