Purification and characterization of acidic endo-polygalacturonase encoded by the PGL1-1 gene from Saccharomyces cerevisiae

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Received 18 September 1999; received in revised form 6 December 1999; accepted 10 December 1999

Abstract

The PGL1 gene of the yeast Saccharomyces cerevisiae has been shown to encode polygalacturonase. Cloning of the PGL1 open reading frame behind the ADH1 promoter allowed overexpression of polygalacturonase activity in S. cerevisiae. This enzyme was purified to apparent homogeneity from cultures of recombinant S. cerevisiae on synthetic medium using one-step purification by anionic exchange chromatography. The enzyme, named Pgl1P, had an apparent Mr of 42 kDa as shown by SDS-PAGE. Pgl1P was active from pH 3 to 5.5, with an optimum temperature at 25°C. This enzyme hydrolyzed polygalacturonic acid as an endo-polygalacturonase as demonstrated by independent methods. The purified protein was N-glycosylated. However, the activity remained in the N-deglycosylated form. The N-terminal amino acid sequence was also determined as D-S-C-T-L-T-G-S-S-L. ß 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Endo-polygalacturonase; PGL1 gene; Pectinase; Pectin; Saccharomyces cerevisiae; Purification; Characterization

1. Introduction

Pectic substances are glycosidic macromolecules with high molecular mass that are present in the cell walls of higher plants. The pectin molecule is a polymer of α-(1,4)-linked β-galacturonic acid units. Pectins are naturally degraded by pectinolytic enzymes which have been classified on the basis of their mechanism of attack of the galacturanon backbone.

The two classes of pectinases are esterases and depolymerases [1]. The first group is represented by pectin esterase (PE) and the second by polygalacturonase (PG) and pectin lyase (PL). PG (EC 3.2.1) is a depolymerizing pectinase that catalyzes the hydrolysis of α-(1,4) glycosidic bonds between two non-esterified galacturonic acid units [2]. This enzyme is produced by plants [3–5] and by microorganisms such as bacteria and fungi [6–8] but only a few yeast species show this ability [9–13]. In relation to their activity PG cleaving α-(1,4) glycosidic bonds of non-esterified galacturonic acid residues are classified as endo-polygalacturonase (endoPG; EC 3.2.1.15) and exo-polygalacturonase (exoPG; EC 3.2.1.67). The prefixes endo and exo denote random or terminal cleavage activity respectively. EndoPG has an important role in fruit softening and plant infection processes [14]. It is also widely used in the industrial processing of fruits and vegetables [15] and specifically in beverage clarification.

Although Saccharomyces cerevisiae is frequently used in the food industry, few studies have been undertaken concerning pectinase production [16,17]. We reported a wild-type S. cerevisiae strain, designated SCPP, already used in enology [18], secreting all forms of pectinases [13]. Recently, the open reading frame (ORF, accession number SCY JR153W) encoding polygalacturonase has been cloned [19–21]. Jia and Wheals [22] reported that when the PGL1 gene was introduced into a strain of Escherichia coli or S. cerevisiae, PG activity was not always detected. However, we have successfully cloned a gene using the SCPP strain encoding a biologically active polygalacturonase [21].

In this study, we describe the purification, characterization and N-terminal amino acid sequence of PG from SCPP, a wild-type S. cerevisiae strain.
2. Materials and methods

2.1. Culture conditions

*S. cerevisiae* (strain ATCC 28583, [pPGL1-1]) [21] was grown for 2 days at 30°C without stirring in SD medium (0.67% (w/v) yeast nitrogen base (Difco) without amino acids, 2% (w/v) glucose) supplemented with tryptophan (50 μl/ml) and 50 mM K₂HPO₄/KH₂PO₄ buffer. Cultures were inoculated at 5×10⁶ cells ml⁻¹.

2.2. Enzyme production

After cultivation, cells were removed by centrifugation at 5000×g for 5 min at 4°C and the culture medium was collected. The supernatant was then subjected to tangential ultrafiltration using a 30-kDa cut-off polysulfone membrane (Sartocon mini from Sartorius) in order to concentrate it 10-fold. The retentate was precipitated with three volumes of cold acetone according to Gainvors et al. [13]. The final protein pellet was used for polygalacturonase purification.

2.3. Enzyme assays and protein determination

The PGase activity was measured by different methods. First, this activity was detected on solid medium through the coloration of Petri dishes with a solution of ruthenium red (plate method [13]). Second, the PG activity was also assayed by the zymogram technique, according to the method of Cruickshank and Wade [23] modified by Gainvors et al. [13]. The pH optimum study was achieved by separating purified enzyme by gel electrophoresis as described above and by incubating different runs in 50 mM buffers with different pH values for 7 h at 30°C. Reactions were stopped after 1 h incubation in 0.1 M malic acid and visualized with ruthenium red.

The third assay measured reducing groups released by PG activity either directly on the culture broth or indirectly through the PG activity of the secreted proteins in the media as previously described [13,24,25].

The endo or exo mode of action of PG was also determined by measuring the decrease in relative viscosity in a Brookfield Model LDV II+ viscosimeter at 20°C and 6 rpm velocity. The reaction mixture contained PG enzyme in 500 ml of 5% (w/v) apple pectin (polygalacturonic acid, Sigma) solution in a 50 mM sodium acetate buffer, pH 3.5.

The method described by Gainvors and Belarbi [26] was also used in order to classify the purified PG.

Protein was determined according to the method of Lowry with bovine serum albumin as standard [27]. In the case of column chromatography, protein from each fraction was measured by the absorbance at 280 nm.

2.4. Chromatography

All operations were carried out at 4°C. Liquid chromatography was performed on a Bio-Rad FPLC system (Bio-Logic HR system). Ion exchange chromatography was carried out on a 1.3-ml UNO®Q1 column (3.5 cm×7 mm) equilibrated with Tris-HCl (10 mM, pH 7) and eluted at a flow rate of 0.5 ml min⁻¹ with a linear gradient of NaCl (0–0.5 M) prepared in the same buffer. Fractions containing PG activity were pooled and transferred to dialysis tubing (10-kDa cut-off; Centrisart 1 from Sartorius) and concentrated five-fold.

2.5. Removal of sugar chains of the endoPG

Removal of N-linked carbohydrate was carried out using N-endoglycosidase H and proceeded according to the instructions of the manufacturer (Boehringer Mannheim, France).

2.6. Analytical electrophoresis

The purity and molecular masses of endoPG were determined by SDS-PAGE on a slab gel prepared with 12% (resolving gel) and 5% (stacking gel) acrylamide by the method of Laemmli [28]. After electrophoresis, gels were subjected to silver staining according to Merril et al. [29].

2.7. N-terminal determination

N-terminal sequencing of purified PG was conducted by the protein microsequencing facility of the IBCG, CNRS Bordeaux on a Procise Sequencer (Applied Biosystems).

3. Results and discussion

3.1. Protein purification and physical characterization of PG

The supernatant from a 2-day-old culture with a maximum of activity of Pgl1P was subjected to ultrafiltration. The purification of the enzyme present in the retentate was achieved by a one-step separation using anionic exchange chromatography. The ultrafiltered fraction presented three peaks (data not shown). Using the plate method, we determined that only the fraction corresponding to peak 1 exhibited PG activity. The SDS-PAGE of this peak showed a single band with an apparent M₉ of 42 kDa (Fig. 1, lane 1). This observation agreed with the results reported for PG from *Cryptococcus albidus* var. *albinus* by Federici [12] and recently by Martel et al. [30] for PG from the fungus *Sclerotinia sclerotiorum*. 
The purification data are summarized in Table 1. The specific activity of the purified PG, named Pgl1P, was 219-fold greater than in the crude extract.

Usually, PG activity from other sources exhibited an activity between pH 4 and 5 [31]. However, the activity of Pgl1P showed activity over a broad pH range from 3 to 5 (Fig. 2). We observed that the optimal activity was obtained at pH 4, and Pgl1P expressed 80% of its activity at pH 3 (Fig. 2). To our knowledge, this is the first report of an enzyme with this activity at such a low pH. This discovery is interesting for the beverage industry because of the acidic pH of juices. The temperature optimum for PG activity was 25°C (data not shown). These results were similar to those obtained with endoPG from *Aspergillus japonicus* [32] and *Lactobacillus plantarum* [33].

3.2. Glycosylation determination

Glycosylation has been described for the endoPG from *Kluyveromyces marxianus* [34], *Aspergillus* [35] and *S. sclerotiorum* [30]. The treatment of Pgl1P with an endo-L-N-acetyl glucosaminidase H induced a decrease of the Mr from 42 to 37 kDa as shown by SDS-PAGE (Fig. 1, lane 2). This observation confirms that Pgl1P is also a glycoprotein with N-linked carbohydrate moiety (Fig. 1). The Mr value was comparable to that calculated from the deduced sequence of Pgl1P [21]. Furthermore, the study of this sequence presented two potential N-glycosylation sites [21]. The function of the carbohydrate moiety is not well established, although N-glycosylation was found to be essential for the secretion of an endoPG and a pectate lyase from *Fusarium oxysporum* [36,37]. Using the plate method, we observed endoPG activity of the deglycosylated protein (Fig. 3). In contrast, deglycosylation of polygalacturonase from *Aspergillus* sp. leads to complete enzyme inactivation [35].

3.3. N-terminal sequencing

Pgl1P is secreted into the culture medium by *S. cerevisiae* strain [13]. According to the rule of von Heijne [38], the putative optimal site for cleavage of the signal peptide of the Pgl1P gene is at Ala24 [21]. In this study, when purified PG was subjected to amino acid sequencing, a single amino acid peak was observed after each cycle, confirming the purity of this enzyme. The analysis of the N-terminal sequence exhibited a 10-residue sequence, D-S-C-T-L-T-G-S-S-L, matching the sequence of the structural PGL1 gene and indicating that the cleavage site for the signal peptide was at Arg24. This signal peptide did not present any significant homology to other sequences from data banks. Therefore, the sequence of the first 24 residues represented the signal peptide and the remaining sequence consisting of 337 amino acids was the mature protein.

**Table 1**

Summary of purification of endo-polygalacturonase

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U mg⁻¹)</th>
<th>Recovery (%)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme</td>
<td>1000</td>
<td>95</td>
<td>306</td>
<td>3.2</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>100</td>
<td>16.4</td>
<td>255</td>
<td>15.5</td>
<td>83</td>
<td>10.4</td>
</tr>
<tr>
<td>Anion exchange column</td>
<td>0.75</td>
<td>6.65 × 10⁻⁴</td>
<td>16.9</td>
<td>2533.8</td>
<td>5.5</td>
<td>219.4</td>
</tr>
</tbody>
</table>

*One unit was defined as the amount of enzyme resulting in a 1% reduction viscosity in 20 min.*
3.4. Polygalacturonase characteristics

The nature of the enzyme (exoPG or endoPG) was determined by three different methods. The viscosity of a 5% (w/v) polygalacturonic acid solution decreased by 63% within 30 min of incubation with the purified enzyme (Fig. 4). Baron and Thibault [39] pointed out that exoPG activity induces only a low decrease of viscosity, confirming that our PG exhibited an endoPG activity.

The assay of the reducing groups released by PG activity was done using the method previously described [24,25]. Results showed that no reducing groups were detected when polygalacturonic acid was used as substrate for Pgl1P after a long incubation time, while under the same conditions a commercial exoPG from Sigma released numerous galacturonic acid residues (data not shown). The method previously developed by Gainvors and Belarbi [26] pointed out that the galacturonic acid released by exoPG allowed a faster growth of the strain in pectin medium supplemented by glycerol. Our results reported a small growth but the activity detected by the method of McKay [40] was very important. These results clearly show that the polygalacturonase produced by the recombinant strain is an endoPG.

In conclusion, the results of the three described methods confirmed that the enzyme is an endoPG. This endoPG showed, in addition to its acidic pH activity, a maintained efficacy even after the deglycosylation step. For this reason, it remains stable facing endoglycosidases which can be present in most fruit juices. By these characteristics, endoPG from \textit{S. cerevisiae} could be widely used in clarification in the beverage industry. Moreover, this enzyme was easily recovered from the fermentation culture with a high yield of 5.5%. Therefore, recombinant \textit{S. cerevisiae} strains could be a potential source of endoPG.

Acknowledgements

The authors wish to thank Dr Aït Barka for his help during the preparation of the manuscript and Dr. Guillem for allowing us access to his facilities.

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