Purification and Biochemical Characterization of Polygalacturonases Produced by *Aureobasidium pullulans*

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The extracellular polygalacturonases produced by *Aureobasidium pullulans* isolated from waters of the Danube river were partially purified and characterized. The pH optima of polygalacturonases produced in the first phases of cultivation (48 h) and after 10 d as well as their optima of temperature, thermal stabilities, molecular masses, isoelectric points, action pattern and ability to cleave polymeric and oligomeric substrates were compared. Polygalacturonases with a random action pattern (random cleavage of pectate forming a mixture of galactosiduronides with a lower degree of polymerization) [EC 3.2.1.15] were produced only in the first phases of growth, while exopolygalacturonases [EC 3.2.1.67] with a terminal action pattern (cleavage of pectate from the nonreducing end forming D-galactopyranuronic acid as a product) were found during the whole growth. The main enzyme form with a random action pattern was glycosylated and its active site had the arrangement described previously for the active site of polygalacturonase of phytopathogenic fungi.

Key words: *Aureobasidium pullulans*, Exopolygalacturonase, Polygalacturonase

Introduction

The yeast-like fungus *Aureobasidium pullulans* (De Bary) Arnaud is an ubiquitous saprophyte that occurs commonly in the phyllosphere of many crop plants and on various tropical fruits (Deshpande *et al.*, 1992 and references cited therein). Due to the production of melanin it is popularly known as black yeast. *A. pullulans* has been considered mainly as a plant pathogen causing softening of plant tissue.

Because of production of pullulan and many industrially usable enzymes *A. pullulans* has become an important organism in applied microbiology. *A. pullulans* also produces pectolytic enzymes (Biely and Sláviková, 1994 and references cited therein), which are widely used in the maceration of fruit pulps and for the clarification of juices and wines. The main pectolytic enzymes are polygalacturonases. Polygalacturonase and exopolygalacturonase [EC 3.2.1.15 and EC 3.2.1.67] cleave α-1,4-glycosidic linkages between linked deesterified galacturonic acid units in the linear part of pectin molecules (Rexová-Benková and Markovič, 1976).

The production of pectolytic enzymes has been widely reported and thoroughly studied in bacteria and filamentous fungi because they play an essential role in the phytopathogenesis. The pectinase production in yeasts has received less attention and a few species show this ability (Blanco *et al.*, 1999 and references cited therein). Only a few yeasts that, like filamentous fungi, have the ability to grow using pectic substances as the sole carbon source, suggest a more complex enzymic system in this type of yeasts (Federici, 1985; Stratilová *et al.*, 1998). *A. pullulans* belongs to this type of pectolytic yeasts. It is able to utilize as the single C-source in the cultivation medium pectin, pectate or oligogalacturonides (Biely *et al.*, 1996). Isoenzymes of polygalacturonase produced by *A. pullulans* were observed (Archer and Fielding, 1979) and the production of pectin methylesterase (Archer 1979) and exopolygalacturonase (Biely *et al.*, 1996) was described. Activities of pectate and pectin lyases were detected by Manachini *et al.* (1988).

The pectolytic enzymes produced by fungi and yeasts, mainly the extracellular ones, are mostly induced by the presence of pectin or its fragments in cultivation media (Federici, 1985; Stratilová *et al.*, 1998). But this is not generally valid and some yeasts produce polygalacturonase constitu-

tively (Schwan and Rose, 1994; Blanco *et al.*, 1997). According to Biely *et al.* (1996) D-galacturonic acid serves in *A. pullulans* as the natural inducer of pectolytic enzymes, or at least as a precursor of a natural inducer. On the other hand, glucose in the medium is very often described as a repressor of polygalacturonase production (Federici, 1985; Schwan and Rose, 1994; Blanco *et al.*, 1997). The production of pectolytic enzymes in *A. pullulans* was not a subject of a derepression type of control (Biely *et al.,* 1996).

The aim of this work was to characterize the polygalacturonases produced by *Aureobasidium pullulans* isolated from waters of the Danube river. The rules of their production and characteristic features of the main forms were compared with polygalacturonases produced by other microorganisms, especially with polygalacturonases of phytopathogenic fungi.

Materials and Methods

The strain *Aureobasidium pullulans* used in this work was isolated from the waters of the Danube river (Sláviková and Vadkertiová, 1997) and is also deposited in the Culture Collection of Yeasts at the Institute of Chemistry, Slovak Academy of Sciences, under the number CCY 27-1-111. In comparison with other strains deposited in this collection, CCY 27-1-111 differed by low melanin production under physiological conditions.

The strain was cultured on Czapek's Dox media (Difco, Michigan) with purified pectin as the only carbon source. Commercially available citrus pectin (GENU Pectin, Denmark, with d.e. 45%) was purified by washing with acidified 60% ethanol (5 ml concentrated HCl/100 ml of 60% ethanol), followed by washing with 60% and 96% neutral ethanol as previously described (Kohn and Furda, 1967) and used at 13 g/l. Thus purified pectin contained 87% uronic acid in dry matter.

The cells were precultured aerobically in test tubes with liquid mineral salt medium (all components from Merck) containing 2% w/v glucose (Difco) (Šajbidor *et al.*, 1989) until the stationary phase was achieved.

The yeasts were grown aerobically in the liquid pectin medium on a shaker (100 rpm) at 28 °C. The cells were separated in time intervals (48 h and 10 d) by centrifugation. The concentration of cells in the medium was determined by calculation of cells in the Bürker cell utilizing a Fluoval 2 microscope (Zeiss, Jena, Germany).

The extracellular protein mixture was obtained by the precipitation of centrifuged cultivation media with ammonium sulphate (saturation 90%) and ethanol (1:4) followed by desalting on a Sephadex G-25 Medium column (Pharmacia, Sweden).

Ion-exchange chromatography was performed on a DEAE-Sephadex A-50 column utilizing a sample application with 0.05 M phosphate buffer, pH 8.0, stepwise elution with 0.10 M phosphate buffer, pH 8.0 and finally 1.0 M acetate buffer, pH 4.0, containing 1.0 M NaCl (column size: 30×100 mm; fraction size: 6 ml per 30 min). Fractions with polygalacturonase activity were desalted on a Sephadex G-25 Medium column and freeze-dried. For further characterization the first fractions with polygalacturonase activity (4–10) from ion-exchange chromatography were used.

The total polygalacturonase and exopolygalacturonase activity was assayed at 30 °C (except the determination of temperature optima where temperatures from 20 °C to 70 °C were utilized) in time intervals by measuring the increase of reducing groups (Somogyi, 1952) in the reaction mixture containing one aliquot of sodium pectate (5 g/l) in 0.1 M acetate buffer, pH 4.6, as a substrate (except the determination of pH optima where the substrates with pH values in the range of 3.6–5.6 were used) and one aliquot of protein solution with proper concentration. The total activity of the polygalacturonase/exopolygalacturonase system was expressed in mmol of reducing groups liberated within a time unit per mg of extracellular protein precipitate and determined by means of standard graph for D-galactopyranuronic acid. Pectate as a substrate was prepared from purified pectin by total alkaline deesterification (Kohn and Furda, 1967). The activity of exopolygalacturonase was evaluated in the same manner utilizing di-(D-galactosiduronic) acid as the substrate of difference. This was obtained by enzymatic hydrolysis of pectate followed by gel permeation chromatography on a Sephadex G-25 Fine and by desalting on a Sephadex G-10 column (Heinrichová, 1983).

Pectate and highly esterified pectin (degree of esterification 92%) were used at 5 g/l in 0.1 M phosphate buffer (pH 5.7–8.0) or in 0.1 M acetate buffer (pH 5.0–5.6) as substrates for pectate and pectin lyases. These activities were determined by

recording the absorbance at 235 nm during incubation with substrates.

The thermal stability of polygalacturonases was evaluated after 2 h incubation of enzyme solutions at 20 °C–70 °C and a following enzyme assay at 30 °C.

The viscosity measurements were provided during degradation of polymeric substrate by extracellular enzymes in a Ubbelohde viscosimeter. At the same time intervals the measurement of liberated reducing groups was performed (with the molecular mass of pectin used for counting of the degree of degradation). The action pattern of produced mixtures of polygalacturonase was demonstrated by the correlation of viscosity decrease of polymeric substrate with the degree of its degradation.

Thin-layer chromatography of degradation products of pectate and oligogalacturonides was performed on commercially available Silufol plates (Kavalier, Czech Republic) in a mixture of *n*-butanol/formic acid/water (2:3:1) (Koller and Neukom, 1964). After detection with aniline phthalate the products were identified on the basis of their retention factor as a function of their degree of polymerization.

Molecular mass evaluation was performed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) as well as by gel permeation chromatography on a Superose 12™, HR 10/30, column (FPLC device, Pharmacia, Sweden; fraction size: 0.5 ml/min; mobil phase: 0.05 M phosphate buffer, pH 7.0, containing 0.15 M NaCl). Molecular masses were approximately evaluated from a calibration curve obtained with a molecular weight marker kit (Sigma) with the molecular weight range 12.5–700 kDa.

For deglycosylation, a *N*-glycosidase F Deglycosylation Kit (Roche Diagnostics, Germany) was used. The cleavage of polygalacturonase was performed as recommended for *N*-glycosidase F in 0.1 M phosphate buffer, pH 7.2, in 0.1% SDS, 1% mercaptoethanol, 0.025 M EDTA, and 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). Enzymes denatured by a boiling water bath (10 min) in the presence of 5% mercaptoethanol and 1% SDS were used. The cleavage was performed for 24 h at 37 °C. The change of relative molecular masses of polygalacturonases was detected by SDS-PAGE.

SDS-PAGE for molecular weight analysis of native and deglycosylated polygalacturonase was performed on a Mini-Protean 3 Electrophoresis System (Bio-Rad Laboratories, California) under reducing conditions (with βmercaptoethanol). The silver-staining method was used for band visualization (Wray *et al.*, 1981). As the markers of molecular size the calibration proteins in the range 17–95 kDa (Serva) were utilized.

The cultivation of strain was repeated three times and the presented results show the overview of individual experiments obtained with each protein precipitate of medium.

Results and Discussion

A. pullulans isolated from waters of the Danube river (CCY 27-1-111) was able to grow on medium containing pectin as the only carbon source. The natural acidic pH of cultivation was not very suitable for growth of this microorganism, but *A. pullulans* was able to change it on the final pH about 8.5, the pH value supporting growth. A similar change of the pH value was observed for the strain LV 10 (Manachini *et al.*, 1988) or the fungus *Aspergillus niger* (Stratilová *et al.*, 1996).

The evaluation of pH optima of the mixture of polygalacturonases produced by the strain showed differences between enzymes produced in the first phases of growth (48 h) and the later ones (10 d) (Fig. 1). Further differences were shown by the determination of temperature optima of produced enzymes (Fig. 2A) as well as by the evaluation of their thermal stabilities (Fig. 2B). The extracellular polygalacturonases produced by the strain were compared on the basis of their distribution of molecular masses (Fig. 2C) and action pattern on polymeric substrate (Fig. 2D), too. Because of
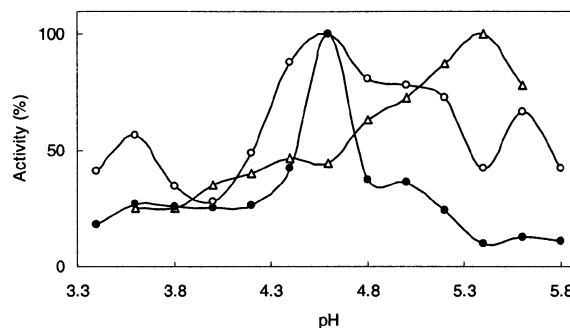

Fig. 1. pH optima of polygalacturonases produced by strain 27-1-111 in the first (○–○) and later (△–△) phases of cultivation; (●–●) polygalacturonase produced in the first phases of growth partially purified on a DEAE-Sephadex A-50 column.
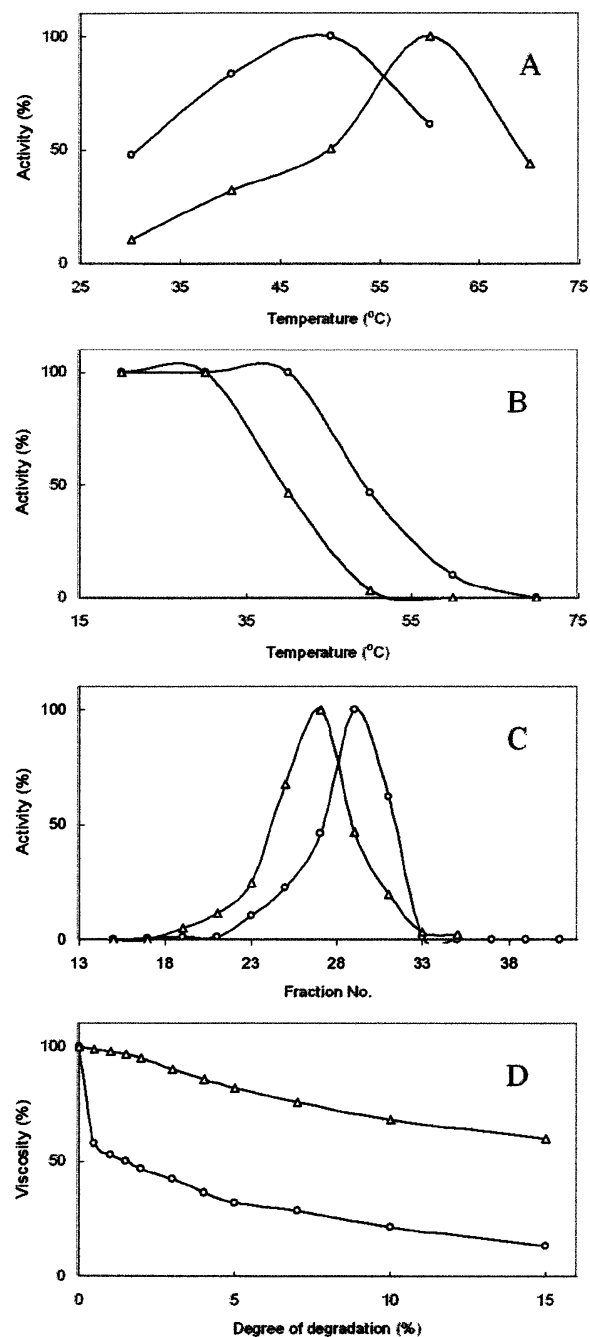
Fig. 2. The optima of temperature (A), the thermal stability (B), the molecular mass determination utilizing gel-permeation chromatography on Superose 12 (FPLC device) (C) and the correlation of viscosity decrease of polymeric substrate during reaction (D) by polygalacturonases produced by strain 27-1-111 in the first (○–○) and later (△–△) phases of cultivation.

multiple pH optima of the protein mixture (Fig. 1; 48 h cultivation) partially purified polygalacturonase on a DEAE-Sephadex column (fractions 4– 10) with a pH optimum at 4.6 (Fig. 1) was used in these experiments.

The results (10 d cultivation) show only the presence of enzymes with a terminal action pattern on polymeric substrate and with the ability to cleave di-(D-galactosiduronic) acid. This was evaluated by correlation of viscosity decrease and increase of degree of substrate degradation during reaction (Fig. 2D) as well as by the initial rate on di-(D-galactosiduronic) acid as a substrate and by thin-layer chromatography of degradation products, where only D-galactopyranuronic acid was observed. The initial rates of enzymes on pectate and di-(D-galactosiduronic) acid were higher for the polymer (pectate – 1.511 μmol/min · mg, dimer – 0.097 μmol/ min · mg). As the enzymes catalyzed the cleavage of pectate with more than fifteen times higher speed as the cleavage of the dimer, they can be supposed to be typical exopolygalacturonases [EC 3.2.1.67]. The main form had the pH optimum 5.4 (Fig. 1), the optimum of temperature was 60 °C (Fig. 2A), it was stable only at temperatures lower than 30 °C (Fig. 2B) and the molecular weight was about 54 kDa (Fig. 2C). A higher optimum of temperature and the value of molecular mass are typical features of exopolygalacturonases, when compared generally with polygalacturonases. These enzymes are not able to seriously destroy the pectin structure of plants and so they are not supposed to be one of the factors of phytopathogenity of microorganism. Exopolygalacturonases in the medium were not the result of cell lysis, because about 99% of cells were still living.

The enzyme obtained from the cultivation medium after 48 h with the pH optimum 4.6 (Fig. 1), optimum of temperature 50 °C (Fig. 2A), stability by 40 °C (Fig. 2B), isoelectric point at 9.4, molecular mass 31 kDa (Fig. 2C, 3A), glycosylated [a creation of a new protein band with a smaller molecular weight (about 28 kDa) after deglycosylation utilizing *N*-glycosidase F cleavage (Fig. 3B)], with high affinity to pectate (K_M 7.92×10^{-5} mol/l), not able to cleave di-(D-galactosiduronic) acid and with random action pattern on pectate [rapid decrease of viscosity corresponding to low degree of degradation of pectate (Fig. 2D) and a wide distribution of oligosaccharides from degradation of pectate as polymeric substrate (Fig. 4A)] was characterized
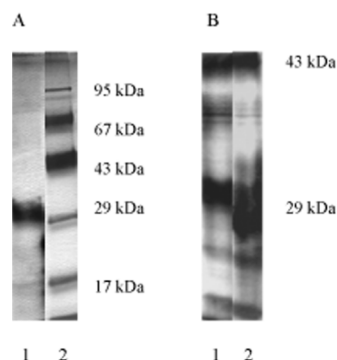
Fig. 3. SDS-PAGE electrophoresis of (A) purified native polygalacturonase produced by CCY 27-1-111 (lane 1) and standards (lane 2; M_r of standards: 95 kDa, 67 kDa, 43 kDa, 29 kDa, 17kDa), and (B) native enzyme (lane 1) and deglycosylated enzyme (lane 2). Silver staining for visualization of protein bands was used.
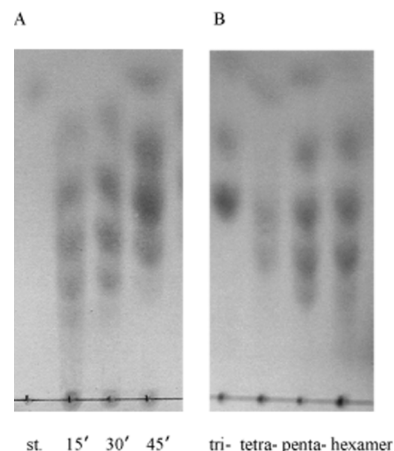

Fig. 4. TLC chromatography of products of degradation of substrates by polygalacturonases produced by CCY 27-1-111 in the first phases of growth. (A) incubation for 15, 30 and 45 min with pectate; di-(D-galactosiduronic) acid was used as a standard (st.); (B) incubation for 15 min with oligogalacturonides (trimer, tetramer, pentamer and hexamer).

as polygalacturonase [EC 3.2.1.15] (or endopolygalacturonase).

This enzyme cleaved oligogalactosiduronides by the same manner as was described for phytopathogenic fungi (for instance *A. niger*; Rexová-Benková and Markovič, 1976) with four subsites and a typical cleavage of tetra-(D-galactosiduronic) acid on trisaccharide and D-galactopyranuronic acid (Fig. 4B). The active site of yeast endopolygalacturonase (*Saccharomyces fragilis*) was described to contain seven subsites (McClendon, 1979).

In opposite to Manachini *et al.* (1988) the presence of pectate or pectin lyases was not observed in the extracellular protein precipitate.

Polygalacturonase with a random action pattern on pectic substances is considered to be one of the main enzymes making the colonization and destruction of plant cells by microorganism possible even if plants have developed barrier against invasion processes (Bellincampi *et al.*, 2004). *Aureobasidium pullulans* from waters of the Danube river (CCY 27-1-111) produced this enzyme only in the first phases of its growth. From this reason, the function by the colonization of the plant material rather than by destruction of plant is supposed in physiological conditions. This enzyme showed characteristic features of enzymes produced by phytopathogenic fungi rather as of enzymes produced by yeasts, however, it seemed that they differ in the pH stability.

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