Food Chemistry 111 (2008) 824–829

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Biochemical and thermal characterization of crude exo-polygalacturonase produced by *Aspergillus sojae*

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ARTICLE INFO

Article history:
Received 19 February 2008
Received in revised form 13 March 2008
Accepted 24 April 2008

Keywords:
Polygalacturonase
Aspergillus sojae
Biochemical and thermal properties
Microbial enzymes
Enzyme characterization

ABSTRACT

Crude exo-polygalacturonase enzyme (produced by *Aspergillus sojae*), significant for industrial processes, was characterized with respect to its biochemical and thermal properties. The optimum pH and temperature for maximum crude exo-polygalacturonase activity were pH 5 and 55 °C, respectively. It retained 60–70% of its activity over a broad pH range and 80% of its initial activity at 65 °C for 1 h. The thermal stability study indicated an inactivation energy of E_d = 152 kJ mol^{-1}. The half lives at 75 and 85 °C were estimated as 3.6 and 1.02 h, respectively. Thermodynamic parameters, ΔH^*, ΔS^* and ΔG^*, were determined as a function of temperature. The kinetic constants K_m and V_{max}, using polygalacturonic acid as substrate, were determined as 0.424 g l^{-1} and 80 μmol min^{-1}, respectively. SDS-PAGE profiling revealed three major bands with molecular weights of 36, 53 and 68 kDa. This enzyme can be considered as a potential candidate in various applications of waste treatment, in food, paper and textile industries.

1. Introduction

Pectic substances, mainly degraded by pectolytic enzymes, contribute to the firmness and structure of plant cells (Naidu & Panda, 2003). These enzymes are of multiple nature and various forms due to the complex nature of their substrates. Endo-polygalacturonase (PGL, EC 3.2.1.15), exo-PGL (EC 3.2.1.67), pectate lyase (EC 4.2.2.2), pectin lyase (EC 4.2.2.10) and pectin methyl esterase (EC 3.1.1.11) form a consortium of enzymes which are necessary for the hydrolysis of pectin (Gadre, Driessche, Beeumen, & Bhat, 2003). Pectolytic enzymes of fungal origin attract the most attention since they offer tremendous potential to the industry. Some of their applications are in retting of flax and vegetable fibres, de-pectinisation and clarification of fruit juices, extraction of oils from vegetables and citrus peels, manufacturing of paper and pulp and pre-treatment of pectic waste water (Hoondal, Tiwari, Tiwari, Dahiya, & Beg, 2002; Jayani, Saxena, & Gupta, 2005; Moyo, Gashe, Collison, & Mpuchane, 2003; Saito, Takakuwa, & Oda, 2004). Almost all of the commercial preparations of pectinases are produced from fungal sources, mainly from *Aspergillus niger*. In fact microbial pectinases account for almost 25% of the global food enzyme sales (Jayani et al., 2005). It is also observed that applications of pectinases in various fields are increasing, demanding the discovery of new strains producing pectinases with novel properties. Therefore, it is highly important to determine these characteristics for an efficient application.

Hence, the objective of this study was to determine the biochemical and thermal properties of the crude exo-polygalacturonase (from here on for simplicity polygalacturonase only) mostly considered among pectinases. This enzyme was produced by *Aspergillus sojae* ATCC 20235, not recognized so far. In our previous work (Gogus, Tari, Unluturk, Oncu, & Tokatlı, 2006; Tari, Gogus, & Tokatlı, 2007; Ustok, Tari, & Gogus, 2007), we reported that the organism exhibits a major potential for the production of this enzyme in submerged and solid state fermentation. Therefore, this paper will complement our previous studies.

In fact, the search for polygalacturonases with new industrial potential requires the discovery of new microbial strains and an understanding of the structure–stability relationship of this enzyme. The knowledge gained, will improve the potential and its effective usage in such diverse and broad areas. Furthermore, it will help to establish additional information required to maintain the desired level of enzyme activity over a long period of time and improve its stability. These are important parameters taken into account in the selection and design of enzymes (Gummadi & Panda, 2003). In this study characterization of the crude enzyme was considered, since the crude enzyme application can offer advantages in certain circumstances. For example, the collaborative action of different pectolytic enzymes in a crude enzyme sample preparation can increase the overall yield of the extraction or clarification process in the fruit juice industry. In fact a brief comparison of the crude enzyme with the purified enzyme, using three-phase partitioning (Dogan & Tari, 2008), is given at the end of this paper. It has been reported that a crude enzyme is more stable than a

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doi:10.1016/j.foodchem.2008.04.056

Nomenclature

E_d	inactivation energy (kJ mol^{-1})	T	absolute temperature (K)
ΔG^*	Gibb's free energy change (kJ mol^{-1})	V_{max}	maximum reaction velocity (μmol min^{-1})
h	Plank's constant (J s)	K_m	Michaelis constant (g l^{-1})
ΔH^*	enthalpy change (kJ mol^{-1})		
k_d	first-order deactivation rate constant (min^{-1})	*Greek symbol*	
R	universal gas constant (J mol^{-1} K^{-1})	κ	Boltzmann's constant (J K^{-1})
ΔS^*	entropy change (J mol^{-1} K^{-1})		
$t_{1/2}$	half-life time of enzyme (min)		

purified enzyme (Naidu & Panda, 2003). This observation was in agreement with our own results, highlighted in this paper.

2. Materials and methods

2.1. Microorganism and development of inoculum

Aspergillus sojae ATCC 20235 was purchased from Promochem Inc., an international distributor of ATCC (American Type of Culture Collection) in Europe. The propagation was done on yeast malt extract (YME) agar and incubated at 30 °C until sporulation. Stock cultures were prepared in 20% glycerol water and stored at −80 °C.

The spore suspensions used as inoculum were obtained on molasses agar slants, described in Tari et al. (2007).

All the analytical grade materials and the culture media ingredients were obtained from Sigma Aldrich.

2.2. Production of polygalacturonase

The production of polygalacturonase was done in shaken flasks (50 ml in 250 ml Erlenmeyer) containing glucose (25 g l^{-1}), peptone (2.5 g l^{-1}), disodium phosphate (3.2 g l^{-1}), monosodium phosphate (3.3 g l^{-1}) and maltrin (120 g l^{-1}). The fermentation was conducted at 350 rpm at 30 °C for 96 h. After this time, each flask was assayed for polygalacturonase enzyme activity. Enzyme activity was determined on supernatant obtained after the centrifugation of the broth at 6000 rpm for 15 min.

2.3. Enzyme assay

Polygalacturonase activity was defined and assayed according to the procedure given by Panda, Naidu, and Sinha (1999), using polygalacturonic acid as substrate at pH 6.6 and 26 °C.

2.4. Protein determination

The total protein contents of samples were determined according to the method reported by Lowry, Rosebrough, Farr, and Randall (1951); the protein standard used was bovine serum albumin.

2.5. SDS-PAGE profiling

SDS-PAGE was performed according to the procedure described by Laemmli (1970) using 10% resolving and 5% stacking gel. Protein bands were visualized using Coomassie brilliant blue R250 and methanol–water (containing 10% acetic acid) as staining and destaining agents, respectively. Fermentas SM0661 was used as the molecular weight marker, with the broad range of 10–200 kDa.

2.6. Effect of pH on activity and stability

The effect of pH on the activity of polygalacturonase was determined by assaying the enzyme activity at different pH values ranging from 3.0 to 7.0, using 0.1 M concentrations of the following buffer systems: citrate (pH 3.0), acetate (pH 4.0, 5.0) and phosphate (pH 6.0, 7.0). The relative activities were based on the ratio of the activity obtained at a certain pH to the maximum activity obtained at that range and expressed as a percentage. The pH stability of *A. sojae* polygalacturonase was investigated in the pH range 3.0–8.0, using 0.1 M citrate (pH 3.0), acetate (pH 4.0, 5.0) and phosphate (pH 6.0, 7.0, 8.0) buffer systems. Therefore, 2 ml of the crude enzyme were mixed with 2 ml of the buffer solutions indicated above and incubated at 30 °C for 2 h. After this period, aliquots of the mixtures were taken to measure the residual polygalacturonase activity (%) with respect to control, under standard assay conditions.

2.7. Effect of temperature on activity and stability

In order to determine the effect of temperature on the activity of polygalacturonase, the standard polygalacturonase assay procedure at different temperatures, ranging from 25 to 75 °C (25, 37, 45, 55, 65 and 75 °C), was performed. Prior to the addition of the enzyme, the substrate (0.24% (w/v) polygalacturonic acid) was pre-incubated at the respective temperature for 10 min. The relative activities as percentages, were expressed as the ratio of the polygalacturonase activity at a certain temperature to the maximum activity at the given temperature range. The thermostability of the crude polygalacturonase was investigated by measuring the residual activity after incubating the enzyme at various temperatures ranging from 25 to 65 °C (25, 37, 45, 55 and 65 °C) for 30 and 60 min.

2.8. Kinetics of thermal inactivation and estimation of the inactivation energy

In order to study the thermal inactivation kinetics of polygalacturonase, the crude enzyme was incubated at different temperatures (75, 80, 82.5 and 85 °C) in the absence of the substrate. At periodic intervals, aliquots were withdrawn and cooled in an ice bath prior to assay as described above. The residual activity was expressed as percent of the initial activity. The inactivation rate constants (k_d) were calculated from slopes of a semilogarithmic plot of residual activity versus time and apparent half lives were estimated using Eq. (1). The time where the residual activity reaches 50% is known as the half-life

$$t_{1/2} = \frac{\ln 2}{k_d}. \quad (1)$$

The temperature dependence of k_d was analyzed using the Arrhenius plot (Shuler & Kargi, 2002). The inactivation energy was calculated from the Arrhenius equation as

$$\ln(k_d) = \ln(k_0) - \left(\frac{E}{R}\right)\frac{1}{T}. \quad (2)$$

The values of E and k_0 were estimated from the slope and intercept of the plot of $\ln(k_d)$ versus $1/T$, respectively.