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Acidic condition-inducible polygalacturonase of *Aspergillus kawachii* [1]

Yoichiro Kojima, Tatsuji Sakamoto, Masao Kishida, Takuo Sakai, Haruhiko Kawasaki *

Department of Applied Biochemistry, College of Agriculture, Osaka Prefecture University, Gakuen-cho 1-1, Sakai, Osaka 599-8531, Japan

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Abstract

Aspergillus kawachii IFO 4308, which can grow in an extremely acidic condition (pH 2), produced some extracellular polygalacturonases (PGase). However, pH 2 and pH 5 culture filtrates showed different pH PGase activity profiles. Anion exchange chromatographies revealed that the PGase compositions of the two culture filtrates were different, and dominant enzymes (PGase-A1 and -A2 in the pH 2 culture and PGase-B in the pH 5 culture) were purified and characterized. The optimal pH was pH 4 for A1, pH 3 for A2, and pH 5 for B. PGase-A1 and -A2 were more stable at low pH than PGase-B. Molecular masses of PGase-A1, -A2, and -B were 43, 83, and 71 kDa, respectively. The N-terminal amino acid sequence of PGase-B was similar to those of other fungal PGases, but distinctly different from those of PGase-A1 and -A2. These results suggest that PGase-A1 and -A2 may be acidic condition-inducible enzymes and that a pH-regulated expression system is involved in the PGase production of *A. kawachii*.

Keywords: *Aspergillus kawachii*; Polygalacturonase; Acid-inducible enzyme; Response to acidity; pH-regulated gene expression

1. Introduction

In general, extreme environments influence organisms as stresses, to which they inevitably respond in order to survive in different ways depending on the nature of the stress and the kind of the organism. Bacteria are known to mount cellular responses to high temperature, hypertonicity and oxidative stimulation [1–3]. Responses of yeast to high temperature and hypertonicity have also been studied [4,5], and heat shock proteins (Hsps) play an important role in the cellular responses [6].

Homeostasis of pH has been observed in bacteria. *Escherichia coli* and *Salmonella typhimurium* have been shown to respond to and tolerate high acidity conditions by inducible transcription [7–10]. Three reasons why some bacteria can grow under acidic conditions have been proposed [10,11]: (1) the pH homeostasis system is induced by a change in proton-pumping or by a decrease in permeability, (2) some kinds of stress proteins (Hsp-like acid shock proteins) are induced and play an important role

* Corresponding author. Tel.: +81-722-54-9456; Fax: +81-722-54-9921; E-mail: hkawasak@biochem.osakafu-u.ac.jp

[1] Dedicated to Professor Hideaki Yamada in honor of his 70th birthday.

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in the resistance to and survival under acidic conditions, (3) the pH homeostasis system and the stress proteins function cooperatively. These studies suggest that the gene expression regulated by pH conditions occurs in bacteria, resulting in acid-tolerance. In fact, many genes regulated by pH have been found in bacteria [9], and also a transducer protein that transmits external pH signals to the inside of the cell has been known [12].

In fungi, however, cellular machineries that respond to acidic conditions have not yet been elucidated in detail. The pH-regulated expression of a penicillin biosynthetic gene in *Aspergillus nidulans* [13–15] is a rare precious observation. Some strains of the genus *Aspergillus* are known to be able to grow in an extremely acidic environment (pH 2) and produce peculiar acid-stable enzymes, including an α-amylase [16], a xylanase [17], and an acid-cellulase [18]. Our collaborators have previously demonstrated that *A. awamori* grown in a pH 2 medium produces acid-stable protopectin-solubilizing enzymes, called protopectinases [19]. These enzymes, however, have not been proved to be acidic condition-inducible. If pH-regulated gene expression systems are present in fungi, they are expected to play an important roll in fungal adaptation to extremely acidic circumstances.

In this study, we compare polygalacturonases (PGase) produced by *A. kawachii* in pH 2 and pH 5 culture conditions and discuss the possibility of a pH-regulated gene expression occurring in this organism.

2. Experimental

2.1. Strain, media, and culture conditions

A. kawachii IFO 4308 was used. A culture medium consisting of 1.5% soybean flour, 0.1% Polypeptone, and 0.1% $NaNO_3$ was adjusted to pH 2 by H_3PO_4 or pH 5 with 1 M KH_2PO_4–K_2HPO_4. *A. kawachii* was cultured with shaking at 37°C in a 500 ml flask containing 100 ml of the medium. A large scale culture was done in a 30 l jar fermentor containing 10 l of the medium with aerating at 6 l/min and stirring at 300 rpm.

2.2. Assay of PGase activity and protopectinase activity

A PGase reaction was done at 37°C for 20 min in 100 mM sodium acetate buffer (pH 4 or 5) containing 0.1% polygalacturonate (Sigma) as a substrate, and the amount of reducing groups generated from polygalacturonate was measured by the method of Somogyi [20]. One unit of PGase activity was defined as the activity that generates reducing groups corresponding to 1 μmol of D-galacturonic acid for 1 h.

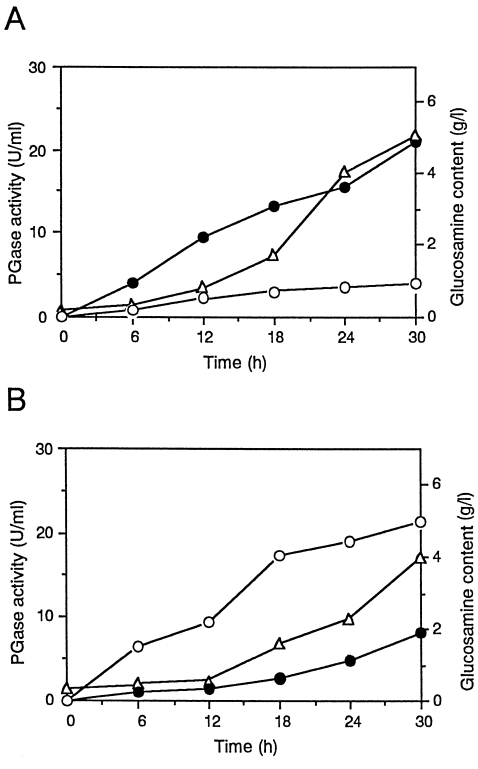

Fig. 1. Time courses of the cultures of *A. kawachii* IFO 4308 at pH 2 (A) and pH 5 (B). Fungal growth was indicated by glucosamine contents (△). The PGase activity in the culture filtrate was assayed at pH 4 (●) and pH 5 (○).

Protopectinase activity was assayed as described by Hours et al. [19]; the amount of pectin solubilized from solid lemon-protopectin was determined by the carbazole–H_2SO_4 method.

2.3. Measurement of fungal growth

Since the culture medium contained insoluble materials, the amount of glucosamine of fungal cell wall was measured as described by Aidoo et al. [21].

2.4. Column chromatographies

Culture filtrate (10 l) was concentrated to 1/10 volume by the Filtron ultrafiltration system and dialyzed against 20 mM sodium acetate buffer (pH 5). The dialyzate was applied on a DEAE-Toyopearl 650 M column (4.5 × 30 cm, Tosoh) equilibrated with 20 mM acetate buffer (pH 5) and eluted with a linear gradient of NaCl from 0 to 0.8 M. Mono-Q and Mono-S columns (both 1 × 30 cm, Pharmacia) were equilibrated with 20 mM acetate buffer pH 5 and pH 3, respectively, and the enzymes were eluted with a linear gradient of NaCl from 0 to 0.8 M. Gel-filtration was done using a Superdex 200 HR10/30 column (Pharmacia) equilibrated with 20 mM acetate buffer (pH 5) containing 150 mM NaCl.

Protein was measured either by the Lowry's method or by absorption at 280 nm.

2.5. SDS-polyacrylamide gel electrophoresis

SDS-PAGE was done as described by Laemmli [22] using a 10% gel. A kit of 10 kDa Protein Ladder (Gibco BRL) was used as molecular mass standards.

2.6. Determination of N-terminal amino acid sequences

Protein (100 pmol) was blotted on a PVDF membrane and analyzed by the Shimadzu protein sequencer PSQ-1 (Kyoto, Japan).

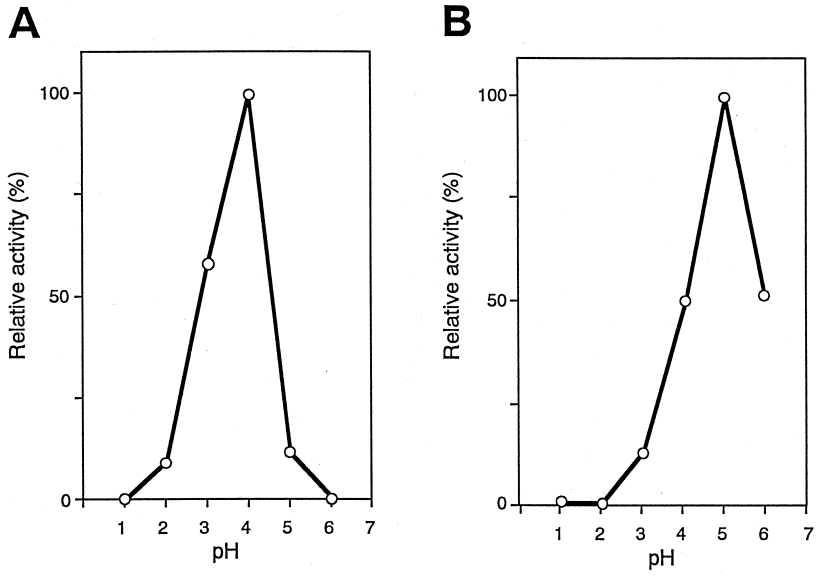

Fig. 2. pH-activity curves of the extracellular PGases produced at pH 2 and pH 5. *A. kawachii* IFO 4308 was cultured in the pH 2 and pH 5 media for 28 h and the PGase activities of the culture filtrates were assayed at indicated pHs. (A) The pH 2 culture filtrate; (B) the pH 5 culture filtrate.

3. Results and discussion

3.1. A. kawachii IFO 4308 selected as a PGase producer

About twenty strains of *Aspergillus*, including *A. awamori*, *A. cinnamoneus*, *A. luchuensis*, and *A. kawachii*, which were selected from a laboratory collection of fungi by a potential ability to grow in the pH 2 medium, were examined for protopectin-solubilizing activity produced in the culture filtrates. *A. kawachii* IFO 4308 grew well at pH 2 comparably with at pH 5 and produced the highest enzyme activity among the strains tested (data not shown). It was proven that the protopectin-solubilizing activity of this organism was mainly attributed to the action of extracellular PGases. Therefore, we focused on the PGases produced by *A. kawachii* IFO 4308 in the acidic culture conditions.

3.2. Comparison of PGases produced in pH 2 and pH 5 culture conditions

A. kawachii IFO 4308 was cultured in the pH 2 and pH 5 media, and the growth and the extracellular PGase activity were followed (Fig. 1). The pH of each medium was maintained through the cultivation. The growth at pH 2 was nearly comparable with that at pH 5. When the PGase production was followed by the activity assayed at pH 5, the pH 5 culture produced a considerable amount of PGase(s) along with the growth, while the pH 2 culture did not produced so much. However, when the enzyme activity was assayed at pH 4, it was shown that the pH 2 culture produced a large amount of PGase, whereas the activity of the pH 5 culture was reduced to less than half.

The different pH-property of the PGase activity of the two cultures was more definitely demonstrated by pH-activity curves (Fig. 2) estimated using the 28-h culture filtrates. The pH 5 culture filtrate gave the maximum activity at pH 5 and the half activity at pH 4, while the pH 2 culture filtrate showed the maximum at pH 4 and a little at pH 5. This suggested that the PGase composition was different between the pH 2 and pH 5 culture filtrates.

Both of the pH 2 and pH 5 culture filtrates retained the enzyme activities and the pH-activity profiles after dialysis against either pH 2, pH 4 or pH 6 buffer at 4°C overnight. This implied that the different PGase compositions were not caused by selective enzyme-inactivation owing to the different pH-instability of individual enzymes. In addition, since the intracellular PGase activity was only negligible in both cultures at pH 2 and pH 5, it was not possible that the different PGase compositions were caused by pH-dependent secretion of the enzyme. Therefore, this fungus might possibly produce different PGases depending upon the pH conditions.

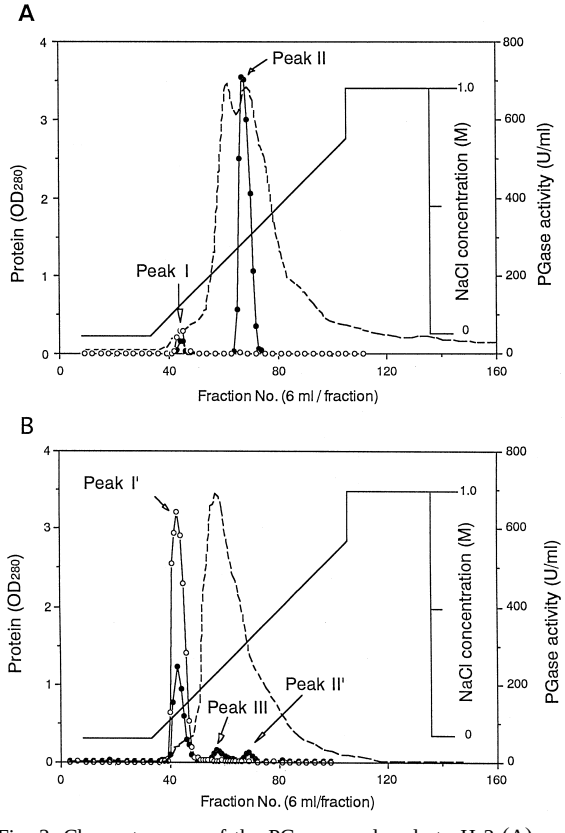

Fig. 3. Chromatograms of the PGases produced at pH 2 (A) and pH 5 (B) on a DEAE-Toyopearl 650 M column. The PGase activity was assayed at pH 4 (●) and pH 5 (○). Broken line indicates the absorbance at 280 nm.

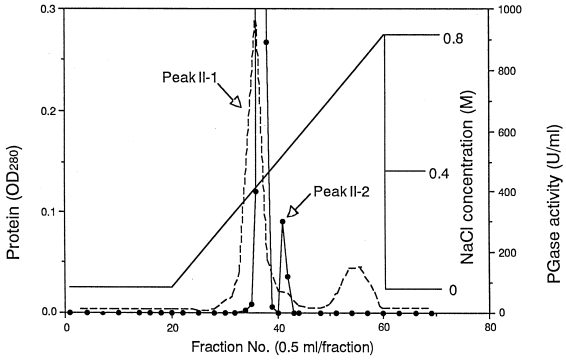

Fig. 4. Chromatogram of the peak II PGases on a Mono-S column. The PGase activity (●) was assayed at pH 4, and broken line indicates the absorbance at 280 nm.

Different PGase compositions in the two filtrates were clearly demonstrated by DEAE-Toyopearl 650 M column chromatography (Fig. 3). Two peaks of PGase activity (I and II) were observed in the chromatogram of the pH 2 filtrate and three peaks (I′, II′, and III) in the pH 5 filtrate. The eluted positions of the peaks I and II agreed with those of the peaks I′ and II′, respectively, under the same chromatographic conditions. The enzymes of the peaks I and I′ were more active at pH 5 than at pH 4, while the peaks II and II′ were active at pH 4 but inert at pH 5. The peak II was much larger than the peak I and occupied 80% of the pH 4-PGase

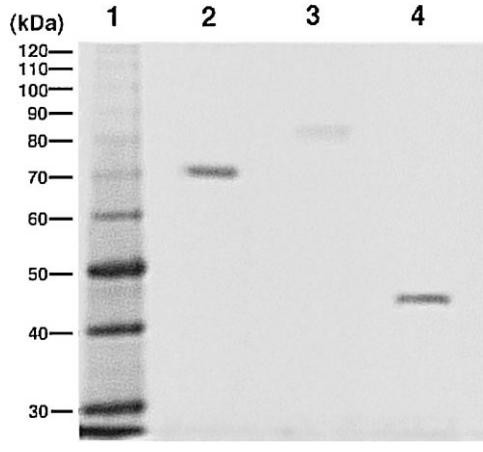

Fig. 5. SDS-PAGE of purified PGase-A1, -A2, and -B. The proteins were developed in a 10% gel and visualized with Coomassie blue staining. Lane 1, marker proteins; lane 2, PGase-A1; lane 3, PGase-A2; lane 4, PGase-B.

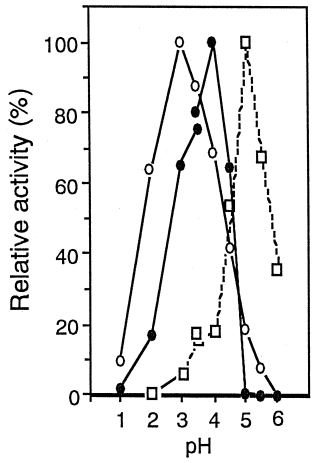

Fig. 6. The pH-activity profiles of PGase-A1 (●), -A2 (○), and -B (□). Sodium acetate–HCl buffer (pH 1 ~ 3.5) and sodium acetate–acetic acid buffer (pH 3.5 ~ 6) were used.

activity in the pH 2 filtrate. In the pH 5 filtrate, the peak I′ was dominant and occupied not less than 70% of the total pH 5-activity. The peak III that was very small was observed only in the pH 5 filtrate.

3.3. Purification of PGases

The dominant PGases, the peak II in the pH 2 culture and the peak I′ in the pH 5 culture, were further purified as follows. The peak II enzyme(s) was subjected to Mono-Q column chromatography, by which the enzyme(s) was eluted as a single peak and purified about ten-

Table 1
Properties of PGase-A1, -A2 and -B

Property	PGase-A1	PGase-A2	PGase-B
Molecular weight			
by SDS-PAGE	43,000	83,000	71,000
by gel filtration	43,000	70,000	53,000
Optimum pH[a]	4	3	5
Optimum temperature[b]	50°C	60°C	60°C
pH-stability[c]	2 ~ 6	3 ~ 6	2 ~ 6
Thermo-stability[d]	~ 50°C	~ 50°C	~ 50°C

[a] The activity was assayed at 37°C.
[b] PGase-A1 and -A2 were assayed at pH 4 and PGase-B was at pH 5.
[c] Enzymes were incubated at various pH at 37°C for 18 h.
[d] Enzymes were incubated at various temperature at pH 5 for 1 h.

Fig. 7. Comparison of N-terminal amino acid sequences of some *Aspergillus* PGases. Identical amino acid residues are indicated by reversed letters. X means an indefinite residue. PG-I and PG-II are PGases of *A. niger* [23,24] and PG is the enzyme of *A. oryzae* [25].

fold. The active fraction was subsequently subjected to Mono-S column chromatography, by which the enzymes were separated into a main peak (II-1) and a minor peak (II-2) (Fig. 4). The enzyme of each peak was further purified by Superdex 200 gel-filtration chromatography twice, resulting in finally about a thousand-fold purification from the culture filtrate.

The peak I′ enzyme was purified by Mono-Q, Mono-S, and twice Superdex 200 column chromatographies about 1200-fold from the culture filtrate with about 50% yield. This enzyme was always eluted as a single peak through all chromatographies.

Three purified enzymes gave single bands on SDS-PAGE (Fig. 5). Incidentally, we may remark that the peak I enzyme purified from the pH 2 culture gave the same migration on SDS-PAGE as that of the peak I′ enzyme. The peaks II-1 and II-2 enzymes were designated as PGase-A1 and PGase-A2, respectively, and the peak I′ enzyme as PGase-B.

3.4. Properties of PGase-A1, -A2, and -B

The molecular masses of PGase-A1, -A2, and -B were estimated to be 43, 83, and 71 kDa, respectively, by SDS-PAGE (Fig. 5) and to be 43, 70, and 53 kDa, respectively, by Superdex 200 gel filtration. The optimal pH for the PGase reaction was pH 4 for PGase-A1, pH 3 for PGase-A2, and pH 5 for PGase-B (Fig. 6). These enzymes were stable in acidic conditions between pH 3 and pH 6, and PGase-A1 and -B maintained more than 80% of their initial activities even after 18-h incubation at pH 2 at 37°C. PGase-A1 was more stable than PGase-B and -A2. The properties of the PGases are summarized in Table 1.

The N-terminal amino acid sequences of three enzymes were entirely different each other (Fig. 7), and the sequence of PGase-B was similar to those of PGases produced by *A. niger* and *A. oryzae* [23–25].

The facts that PGase-A1, -A2, and -B were distinctly different in the molecular properties and that PGase-A1 and -B were dominantly produced at pH 2 and pH 5, respectively, suggest that *A. kawachii* has a specific PGase production system that is regulated by the culture pH. We are now trying to isolate the genes encoding PGase-A1 and -B in order to determine whether PGase-A1 and -B are produced from the same or different genes. If the two PGases are produced from the same gene, their different properties might be caused by some pH-dependent processing system, which might change a normal PGase to an acidophilic PGase. If these PGases are from different genes, this will be the case of pH-regulated gene expression. Studies of these genes will help to elucidate the cellular response to acidic or alkaline circumstances.

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