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Characterization of Acid-Induced Partially Folded Conformation Resembling a Molten Globule State of Polygalacturonase from a Filamentous Fungus *Tetracoccosporium* sp.

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Abstract Acid-induced unfolding of a *Tetracoccosporium* sp. polygalacturonase enzyme (PG) was studied by a comprehensive series of biophysical and biochemical techniques. At pH 1.0, PG acquires partially folded state, which reveals characteristics of molten globule (MG) state, i.e., reduction of defined tertiary structure with minimal changes in the secondary structure. In this study PG unfolds exposing its hydrophobic surface to a greater extent than the native form at acidic pH with more tryptophan residues exposed to the solvent. Collectively, our data imply the presence of MG state of PG at low pH, suggesting the phenomenon of hydrophobic collapse model for folding and integration into cell membrane.

Keywords Aggregation · Molten globule · Polygalacturonase · Refolding · *Tetracoccosporium* sp.

Introduction

Proteins may unfold through intermediate(s) retaining high content of secondary structure but little or no tertiary structure known as molten globule (MG) [1, 2]. The MG state may have some features of the native fold. However, this state differs from native state by the absence of close packing throughout the molecule and by a substantial increase in

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fluctuation in side chains as well as larger parts of the molecule [3–5]. This folding intermediate state [6] has been demonstrated to be both as an equilibrium state under mild denaturing conditions [3] and as a kinetic intermediate of protein folding [4, 7]. Protein structures react with the surrounding environment, consisting of many factors including pH, and certain proteins at low pH behave as MG [8]. They have compact dimension, abundant secondary structure, and largely disordered tertiary structure [1, 9].

Pectolytic enzymes are important additives in beverages, food, and feed industries. Studies on the physiochemical properties, pH, and thermal stability of these enzymes can unravel more functionally efficient ones, greatly affecting aforementioned industries. Since applications of polygalacturonases (PGs) in various fields are broadening, it is important to discover new strains producing enzymes with novel properties and understand the nature and properties of these enzymes for their efficient and effective usage. In most industrial applications, fungal polygalacturonases prove to be very useful owing to the higher enzyme activity and their optimum activity at a lower pH range, which is suited to most fruit- and vegetable-processing applications [10, 11]. PGs, enzymes that act on the α-1,4-D linkages of polygalacturonic acids in pectic polysaccharides, are generally active at the acidic pH (4–6) [12]. PGs are single polypeptide chains with a unique parallel β-helix motif [13, 14].

A natively isolated PG from a filamentous fungus, *Tetracoccosporium* sp., demonstrated to be functional and stable under acidic conditions. This polygalacturonase not only was active and stable under acidic conditions but also exhibited a broad range of thermostability at temperatures up to 80 °C, as well as tolerance to metal ions and surface-active agents. A similar pH range for the activity of the enzyme was reported in *Sporotrichum thermophile* [15], *Streptomyces* sp. QG-11-3 [16], *Verticillum albo-atrum* [15], *Aspergillus niger* [17], *Lentinus edodes* [18], and Pectinol A1 and Röhapect D5S from Röhm [19]. An active PG in low pH can revolutionized the pectin processing in many relevant biotechnological processes. In the present study, we have followed the changes in the enzyme structure in the pH range of 1–9. The fluorescence and circular dichroism (CD) results demonstrated that the enzyme resembles a molten globule like structure at pH 1.

Materials and Methods

Chemicals

Polygalacturonic acid was obtained from Fluka (Switzerland). α-D-galacturonic acid, 3,5-dinitrosalicylic acid (DNS), and 1-anilino naphthalene-8-sulfonate (ANS) were purchased from Sigma (St. Louis, MO. USA). Q-Sepharose, Mono-Q-Sepharose and Superdex 75 were provided by Pharmacia (Uppsala, Sweden). All other chemicals were obtained from Merck (Darmstadt, Germany). The polygalcturonase was purified from *Tetracoccosporium* sp. as stated in Aminzadeh et al. [20].

Enzyme Catalytic Assay and Protein Determination

The PG has been purified by two-step chromatography (fast protein liquid chromatography (FPLC)), namely DEAE-Sepharose and Mono-Q sepharose [20]. Its activity was assayed by quantifying reducing groups expressed as galacturonic acid units, which had been liberated during the incubation of 200 μl of 1% (*w*/*v*) polygalacturonic acid in 20 mM sodium acetate buffer, pH 4.3 (optimum pH), with 200 μl of suitably diluted enzyme (0.047 mg ml^{-1}) at 25 °C for 3 min by DNS method [21]. One unit of PG was defined as the amount of enzyme

required to release 1 μmol of galacturonic acid (as a standard) from the polygalacturonic acid per minute. The protein content was determined by Bradford method [22].

Acid Titration of Enzyme and Refolding Process

Acid-induced unfolding of PG was carried out in 10 mM solution of the mix buffer that contains glycine, acetate, and Tris at various pH values. In order to assess the reversibility of acid-induced unfolding, PG at pH 1.0 was diluted 30-fold by 20 mM acetate buffer, pH 4.3. Refolding experiments were conducted under steady-state conditions.

Fluorescence Measurements

Fluorescence measurements were performed at 25 °C using a Perkin-Elmer luminescence spectrometer LS 50B. Intrinsic spectra were recorded between 300 and 400 nm with excitation wavelength of 280 nm. Both excitation and emission slit widths set at 5 nm. The fluorescence spectra were measured at a protein concentration of 50 μg ml^{-1}. All the fluorescence measurements were carried out using a 10-mm path cell in 20 mM mix buffer (glycine/HCl buffer, pH 2.2–3.4; acetate buffer, pH 3.6–5.4; phosphate buffer, pH 5.8–8.0; glycine/NaOH buffer, pH 8.4–10). Before making measurements, the solutions were incubated for 30 min at 25 °C.

CD Studies

CD spectra were recorded on a JASCO (Tokyo, Japan) J-715 spectropolarimeter equipped with a thermostatically controlled cell holder. The instrument was calibrated with (+)-10-camphorsulfonic acid prior to obtaining the spectra [23]. Samples were dialyzed against 20 mM borate buffer, and far-UV CD spectra were measured at a protein concentration of 0.15 mg ml^{-1}, using a 1-mm path length quartz cell. The path length for near-UV CD spectra was 10 mm with the same concentration of protein. Results are expressed as molar ellipticity, [Θ] (deg $cm^2 dmol^{-1}$), based on a mean amino acid residue weight (MRW) of 115. The molar ellipticity [Θ] was calculated from the formula:

$$[\Theta]_\lambda = (\theta \times 100\mathrm{MRW})/(cl)$$

where c is the protein concentration in mg ml^{-1}, l is the light path length in centimeters, and θ is the measured ellipticity in degrees at wavelength λ. Determination of the secondary structure was performed using the software "SSE-338", which deconvolutes far-UV CD spectra [24, 25].

ANS Binding Experiments

To study the ANS binding [26–28], an enzyme solution with concentration of 50 μg ml^{-1} in 20 mM mix buffer at different pH values was incubated with 30 μM ANS at 25 °C for 10 min, and the spectra were recorded in the region 400–600 nm. The excitation was set at 360 nm, and the excitation and emission slit widths were 5 and 7 nm, respectively.

Acrylamide Quenching Measurement

To determine the solvent exposure of tryptophan residues, fluorescence quenching measurements by acrylamide were acquired [29]. In these experiments, aliquots of 0.5 M

quencher stock solution were added to a protein stock solution (50 μg ml^{-1}) to achieve the desired range of quencher concentration (0.05–0.5 M). Titration was carried out by excitation at 280 with 5 nm slit widths for both excitation and emission.

The quenching data were analyzed in terms of the Stern–Volmer constant, K_{SV}, which was calculated from the ratio of the unquenched and the quenched fluorescence intensities, F_0/F, and the molar concentration of the quencher [Q], using the following equation (Eftink [38]);

$$(F_0/F) - 1 = K_{SV}[Q]$$

The intrinsic protein fluorescence F was corrected for the acrylamide inner filter effect f, using an extinction coefficient ε for acrylamide at 280 nm of 4.3 $M^{-1}cm^{-1}$as follows;

$$f = 10^{-\varepsilon[Q]/2}$$

Aggregation Measurements

The partly folded enzyme at a concentration of 0.1 mg ml^{-1} in mix buffer, pH 1 was placed in the cuvette of Perkin-Elmer luminescence spectrometer LS 50B. The excitation and emission monochromators were set at 350 nm with band passes of 1.5 nm, and the extent of light scattering was monitored [30].

Molecular Sieve Chromatography by FPLC

Gel filtration measurements were carried out using a Superdex 75 column (30×1 cm) on a FPLC system. The columns were pre-equilibrated with 150 mM sodium acetate buffer pH 4.3 and glycine HCl buffer, pH 1. Two milliliters of 0.5 mg/ml native and pH-treated protein solution were applied to the columns. The flow rate was 0.2 ml min^{-1} at 25°C. The eluted fractions were read at 280 nm.

Results presented in this paper are the mean from at least three repeated experiments in a typical run in order to confirm reproducibility.

Results

pH-Induced Changes in Fluorescence

The PG had little degradation at low pH (data not shown), and it was decided to probe its conformational changes by intrinsic fluorescence studies at different pH values.

The intrinsic fluorescence is an excellent parameter to monitor both the polarity of tryptophan environment in the protein and the protein conformation [31, 32]. The fluorescence emission spectra of PG at various pH values are presented in Fig. 1a. The native enzyme (pH 4.3) had an emission maximum at 336 nm. The relative fluorescence intensity of the enzyme was reduced with a decrease in pH (Fig. 1a). This observation suggests that there is a change in the degree of tryptophan residues exposure to the solvent at acidic pH. The results revealed that the intrinsic spectra at pH range of 3–8 has the same λ_{max} and at pHs 1,2,9, and 10 are accompanied by red shift. Similar red-shift fluorescence has also been reported earlier for glucose/xylose isomerase [33]. Goto et al. [34] have proposed that upon acid titration, intramolecular charge repulsions are the driving force for

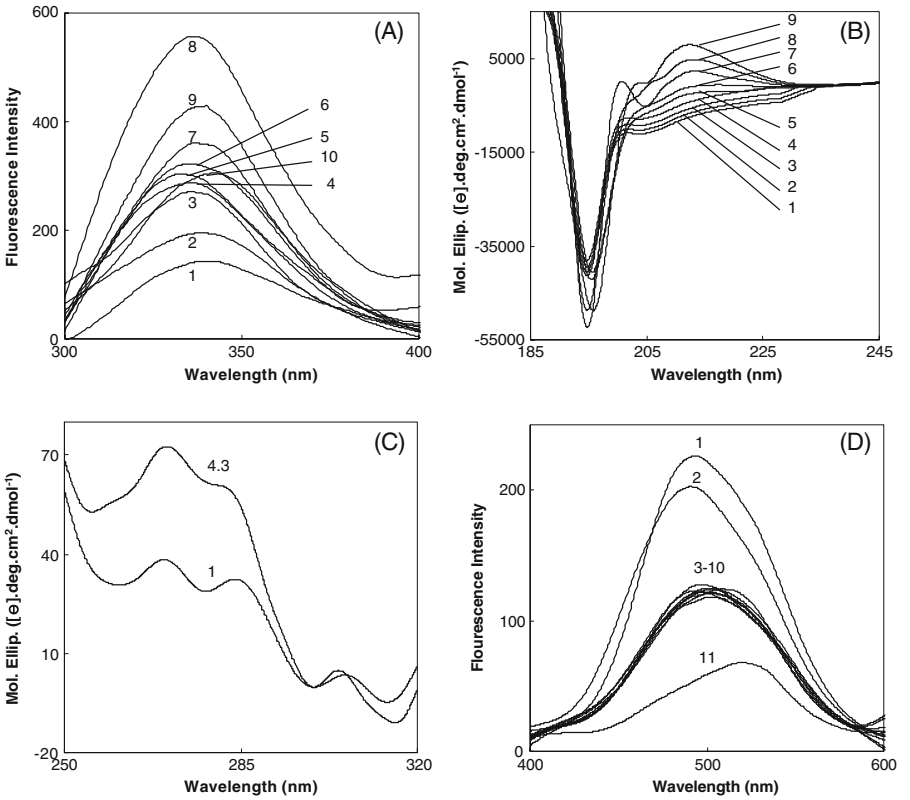

Fig. 1 **a** Fluorescence emission spectra of PG at pH 1.0–10 (*1–10*), **b** far-UV CD spectra of PG at pH 1.0–9.0 (*1–9*), **c** near-UV CD spectra of PG at pH 1 and pH 4.3, **d** fluorescence spectra of 30 μM ANS in the presence of PG at pH 1.0–11 (*1–11*)

partial unfolding of the protein molecule The red shift is a good indicator of exposure of tryptophan residues to the polar solvent. In addition, fluorescence intensity of the enzyme decreased at pH 9 and 10 as compared to pH 8. The unfolding of protein leads to a decrease in fluorescence intensity.

Circular Dichroism Studies of Native and Molten Globule States

Far-UV CD

MG is usually characterized by an intensive far-UV CD spectrum [1]. Thus, the effect of pH on PG was followed at pH 1 to 9 through inspection of far UV CD spectra (Fig. 1.b). The molar ellipticity values did not significantly change at pH 1, and the spectrum is close to that of the native state. A negative peak at 195 nm ($n \rightarrow \pi^*$) was observed, implicating substantial random coil formation and to lower extent some secondary structural deformities at pH 1 and 4.3. Analysis of secondary structure at pH 1 and 4.3 demonstrated that the enzyme consists of about 40% random coil (Table 1). Similarly, the deformation of secondary structures were evident at pH 8 and 9 (Fig. 1b).

Table 1 Statistical test of secondary structure contents of PG estimated from far-UV CD spectra at 200 to 250 nm regions.

Secondary structure	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random (%)
PG at pH 1	5.6±1	24.3±2.4	29.1±2.4	41.0±1
PG at pH 4.3	5.5±1	28.3±2.5	25.4±2.4	40.9±1

Near UV CD

The CD signals at pH 1 (Fig. 1c) are significantly lower than that at pH 4.3, suggesting that the addition of acid will induce a looser and more flexible environment nearby aromatic residues [35].

ANS Fluorescence Studies

In order to identify the induction of any intermediates, extrinsic fluorescence studies were performed in presence of ANS, a fluorescent hydrophobic dye widely used to detect the intermediate states of proteins, as it has higher affinity for partially folded or unfolded states with exposed hydrophobic patches [36, 37]. An increase in the relative fluorescence intensity of ANS was observed by lowering the pH from 10 to 1 (Fig. 1d). The results indicated the partial unfolding of the protein molecule at pH 1 and 2 and, therefore, exposing the hydrophobic groups to which ANS can bind. Moreover, PG at low pH has enhanced solvent accessible clusters of hydrophobic regions, which were initially buried in the interior of native PG at pH 4.3. At pH 1 as compared to pH 4.3, the emission maximum of the ANS-bound protein is shifted by about 10 nm. This blue shift of ANS fluorescence is also due to the increased hydrophobicity of the environment around ANS.

Fluorescence Quenching by Acrylamide

The exposure of the fluorophore residues in the native state was compared to that in the acid-denatured state by fluorescence quenching experiment, using uncharged molecules of acrylamide as described by Eftink and Ghiron [38]. The corresponding Stern–Volmer constant (K_{SV}) values at pH 4.3 and 1 were found to be 29 and 61 M^{-1}, respectively (Fig. 2). This was indicative that the tryptophan residues (aromatic amino acid) at pH 1 are more exposed compared to the native folded conformation at pH 4.3. In addition, fluorescence quenching experiments as Stern–Volmer constant (K_{SV}) showed that the flexibility of PG at pH 1 is higher than pH 4.3 (i.e., the flexibility of the MG state of PG is more than the native state).

Aggregation Measurements, Refolding Studies, and Effect of pH on the State of Association

The formation of aggregates was followed by light scattering methods [39]. The light scattering profile of PG (0.1 mg ml^{-1}) was shown as a function of time at pH 1 (Fig. 3a). Protein intermediates including MG state expose hydrophobic surfaces and become accessible to the hydrophobic dyes such as ANS (Fig. 1d) with a tendency to aggregate (Fig. 3a).

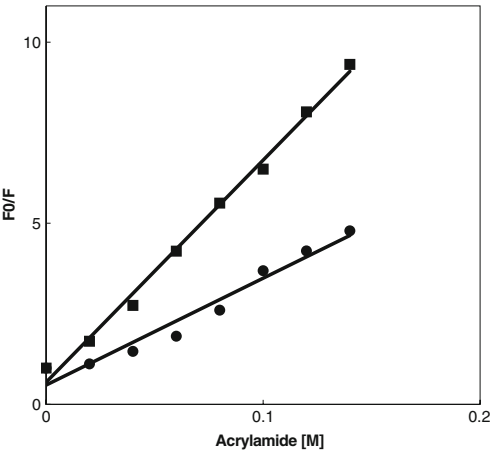

Fig. 2 Stern–Volmer plot of fluorescence quenching by acrylamide. Fluorescence quenching at 25°C for PG at pH 4.3 (*filled circles*) and pH 1 (*filled squares*). For more details, please see "Materials and Methods" section

In our experiments, the refolding of the enzyme depends on the length of incubation time at pH 1 (Fig. 3b). Within a short time of incubation, 0 to 25 min, a refolding yield greater than 50% was measured. Over longer periods, a decrease in the refolding yield was observed probably due to irreversible aggregation process of the partially folded protein molecules (Fig. 3a).

The effect of pH on the size and the state of association of PG was followed by gel filtration on FPLC (Fig. 3c). PG at pH 1 has a lower retention time than pH 4.3. This might be due to either partially aggregated proteins linked together or a looser conformation. A shoulder peak for PG at pH 1 (shown by arrow in Fig. 3c) is evident, suggesting that the protein is partially aggregated, and the native state retains at the same fraction as the PG at pH 4.3 elutes from the column.

Discussion

Proteins fold either with (hydrophobic collapse model) or without an intermediate (framework model) [32, 35–38, 40]. The molten globule state is a common equilibrium intermediate between native and unfolded states for many proteins in both directions of folding and unfolding transitions [7, 40–48]. Processes in which molten globule states are believed to participate include translocation of proteins across membranes, protein–protein interactions, and protein degradation [49]. Low pH is among other protein denaturing factors that occur in some organelles and causes MG to form [50]. Here, a large set of biophysical methods was used to monitor the folding state and conformational changes of a polygalacturonase isolated from culture broth of *Tetracoccosporium* sp. in acidic pH.

The PG had little degradation at low pH (data not shown) with a reduction in relative fluorescence intensity suggesting an unfolded state similar to other enzymes reported in previous studies [35, 51–55]. The folding process leading to the compact state is postulated to be driven primarily by anions binding to the positively charged groups and minimizing the intramolecular charge repulsion, in turn favoring intrinsic hydrophobic interaction. Therefore, upon acid titration, intramolecular charge repulsions are the driving force for partial unfolding of the protein molecule [45, 56]. The combined results of far and near UV CD spectra and fluorescence experiments together with the retention of the secondary

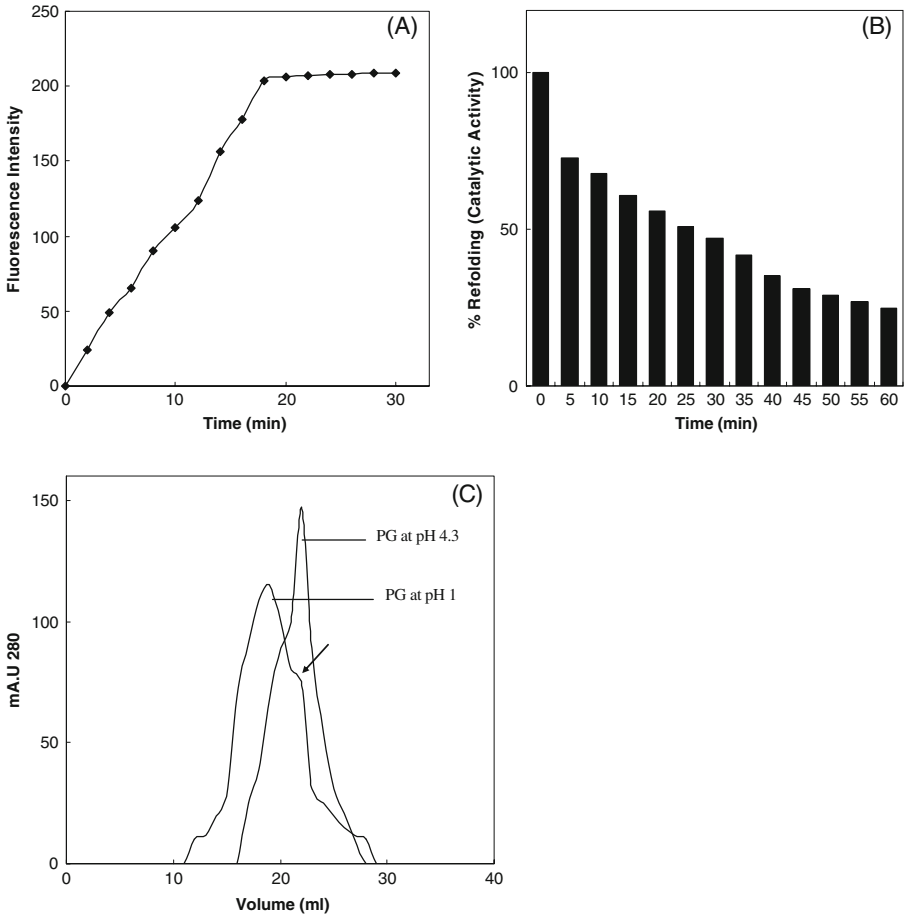

Fig. 3 **a** Aggregation of the molten globule state of PG at pH 1. The enzyme concentration was 0.1 mg/ml. The extent of aggregation was measured by monitoring the scattering of 355 nm. **b** Reactivation (*% Refolding*) of PG as a function of time. Refolding of PG was monitored by its activity after incubation at pH 1. The first column (0 min) refers to 100% enzyme activity in the case if the PG was not incubated at all in pH 1. Final protein concentration is 0.1 mg ml^{-1}. **c** The effect of pH on the elution profile of PG by size exclusion chromatography on FPLC. For more details, please see "Materials and Methods" section

structure [57–60] and loss of the tertiary structure can be suggestive of the existence of MG state at low pH [2].

Another criterion for determination of the molten globule state is the availability of more hydrophobic patches relative to the native state. It is known that globular protein polypeptide chains include both hydrophilic and hydrophobic side chains. Moreover, the secondary structure of proteins forms clusters of hydrophobic side chains that, in turn, form the hydrophobic core of proteins [61]. In order to study the MG state of proteins, ANS binding is being usually used [9, 28, 35, 46, 51, 62–65]. ANS binds to hydrophobic regions of proteins, resulting to an increase in fluorescence intensity [66]. Thus, to evaluate the Molten Globule formation of PG, an ANS binding assay was performed, and data demonstrated that upon pH reduction in the presence of ANS, an increase in fluorescent intensity can be obtained. Furthermore, the results of quenching experiments allowed us to

assess the relative solvent exposure of fluorophores. The more exposed a fluorophore is, the more effective collisional quencher will be leading to a reduction of the fluorescence intensity displayed by the molecule [67]. An increase in molar intensity of about threefold was observed in quenching experiments where the pH reduced from 4.3 to 1. This increase at low pH was indicative of exposure of previously hidden amino acid residues in PG, making the protein molecule structurally more flexible.

The aggregate formation has been recognized mainly as the result of nonspecific interactions between hydrophobic regions of polypeptide chains (unfolded protein molecules or folding intermediates) [68]. Meanwhile, protein renaturation is a competition process between folding and aggregation. Therefore, a suppression of the aggregation reaction can lead to the enhancement of refolding yield. In pH 1, the MG state of PG had a tendency to aggregate similar to cytochrome C [69], but as the incubation in low pH proceeded, PG started to refold up to 25 min.

The implication of the results presented in this paper may prove to be useful for elucidating the MG state of PG at low pH. Based on our results, PG upon acid titration does not fully unfold but directly transforms to the MG state. This state is proven to be functional and, therefore, can enhance the biotechnological processes in food industries at lower pH.

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