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ORIGINAL PAPER

Structural stability and unfolding transition of β-glucosidases: a comparative investigation on isozymes from a thermo-tolerant yeast

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Abstract The folding of proteins in the milieu of the cellular environment involves various interactions among the residues of the polypeptide chain and the microenvironment where it resides. These interactions are responsible for stabilizing the protein molecule, and disruption of the same provides information about the stability of the molecule. β-Glucosidase isozymes, despite having high homology in their primary and tertiary designs, show deviations in their properties such as unfolding, refolding, and stability. In a comparative study on two large cell-wall-bound isozymes, β-glucosidase I (BGLI) and β-glucosidase II (BGLII) from a thermo-tolerant yeast, *Pichia etchellsii,* we have investigated guanidine hydrochloride (GdnHCl)-induced, alkali-induced, and thermal-unfolding transitions using CD and fluorescence spectroscopy and high sensitivity differential scanning calorimetry. Using spectral parameters (MRE 222 nm) to monitor the conformational transitions of the GdnHCl-induced unfolding phenomenon, it was observed that the midpoints of unfolding, apparent C_m, occurred at 1.2 M ± 0.05 and 0.8 M ± 0.03 GdnHCl, respectively, for BGLI and BGLII. The alkali-induced unfolding process indicated that BGLI showed a mid-transition point at pH 11 ± 0.17, while for BGLII it was at pH 10 ± 0.40, further indicating BGLI to be more stable to alkali denaturation than BGLII. In the case of thermal unfolding, the midpoint of transition was observed at 63 ± 0.12°C for BGLI and at 58 ± 0.55°C for BGLII. Analysis by high sensitivity differential scanning calorimeter supported the unfolding data in which BGLI showed higher melting temperature, T_m, (56.07°C ± 0.34) than BGLII (54.02°C ± 0.36). Our results clearly indicate that BGLI is structurally more rigid and stable than BGLII.

Keywords Conformational changes · Circular dichroism · Fluorescence · Differential scanning colorimetry · Dynamic light scattering · Glycosylation

Abbreviations

BGLI	β-Glucosidase I
BGLII	β-Glucosidase II
C_m	Mid-point of transition
C_p	Excess heat capacity
DSC	Differential scanning calorimetry
GdnHCl	Guanidine hydrochloride
ΔH	Enthalpy of denaturation
MRE	Molar residue ellipticity
N and U	Native and unfolded states, respectively
T_m	Temperature of maximum heat capacity

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Introduction

β-Glucosidases constitute a major group among glycosyl-hydrolases. These enzymes hydrolyze O-linked β-glucosidic

bonds (β-D-glucoside glucohydrolase, EC 3.2.1.21) and occur in all domains of life (Archaea, Eubacteria, and Eukarya). Most of these enzymes occur as multiple forms and share a common hydrolase fold. β-Glucosidases have been placed in family 1 and family 3 of glycosylhydrolases (Henrissat and Davies 1997). Although a number of family 1 structures have been solved from different organisms (Wiesmann et al. 1997; Hakulinen et al. 2000; Tolley et al. 1993; Kaper et al. 2000; Czjzek et al. 2001), only a few structures are available for family 3 glycosylhydrolases, e.g., *Hordaeum vulgare* (Varghese et al. 1999) and *Kluyveromyces marxianus* (Yoshida et al. 2010). Hence, we know less about the structure-function relationship of family 3 glycosylhydrolases. In order to understand the structure-function relationship of the large isozymic proteins of family 3 glycosylhydrolases, systematic unfolding studies were conducted on β-glucosidase I and β-glucosidase II (BGLI and BGLII). Both are cell-wall-bound proteins and have been biochemically characterized (Wallecha and Mishra 2003). The two proteins were found to be similar in terms of amino acid composition, pH, temperature optima, and hydrolyzing ability. These enzymes were found to be large polypeptide chains of about 847 amino acids, which existed as dimers in the case of BGLI and tetramers in the case of BGLII. As they belong to the same family they are expected to share a common fold. The most detailed investigation yet of the conformational changes of proteins during folding/unfolding was performed with small globular proteins formed of 100–200 amino acid residues (Struksberg et al. 2007).

The three-dimensional structure of a protein, which is a prerequisite for its proper functioning, is maintained by different types of interactions between the amino acids and the solvent molecules. Perturbations of these interactions by various agents such as temperature, pH, or denaturants can provide vital information about structural stability and structural flexibility. This behavior towards structural perturbants differs significantly for different proteins. Even isozymic enzymes produced by the same organism that share a common fold show deviations in structural stability and flexibility.

Considering the limited number of studies done on large family 3 glycosylhydrolases, investigations focusing on the interplay of structural organization, conformational changes, and enzymatic activity assume great significance. β-Glucosidases from thermo-tolerant yeast provide a good model for studying conformational stability of large isozymic proteins especially as they play a role in a variety of fundamental biological processes, such as growth and development, chemical defense, host-parasite interactions, and cell signaling, and in biotechnological applications, such as synthesis of nonionic surfactants, oligosaccharides, and other glycoconjugates (Bhatia et al. 2002).

Materials and methods

Chemicals, reagents, and buffers

Guanidine hydrochloride was purchased from Aldrich (USA), and *p*-nitrophenyl-β-D-glucoside (*p*NPG) was procured either from Aldrich or Sisco Research Laboratories (India). Para-nitrophenol (*p*NP) was purchased from SRL (India). All buffers were made from analytical grade reagents and prepared in Milli-Q water and filtered through 0.45 μm filters (Millipore, USA). The stock concentration of GdnHCl was determined by refractometer (Atago, 3T, Japan), while that of protein solutions was measured by Bradford assay. The Bradford reagent was purchased from Aldrich (USA). The buffers used were 10 mM sodium phosphate, pH 7, for BGLI and 25 mM sodium phosphate, pH 7, for BGLII for spectroscopic measurements.

Enzyme purification

BGLI and BGLII were isolated from the cell wall of *Pichia etchellsii* JFG-2201 (Deutsche Sammlung Von Mikroorganismen, Germany) by modification of the method described previously (Wallecha and Mishra 2003). The cells grown in 10 l phosphate–succinate medium were resuspended in 50 mM sodium phosphate buffer, pH 7, to make a 15% cell suspension (wcw/v). This was disrupted in a dynomill in discontinuous batch operations at 4°C. The cell debris was removed by centrifugation (Sigma 3K30) at 12,000 rpm for 30 min at 4°C. The cell-free extract was applied to an XK26/70 column (Pharmacia, Sweden) packed with strong anion exchanger Q-Sepharose FF (Amersham Biosciences, USA) previously equilibrated with 50 mM sodium phosphate buffer, pH 7. Elution of β-glucosidases was carried out with a step gradient of 130 mM KCl. Fractions of 7 ml size were collected at a flow rate of 2 ml/min. Those showing activity were analyzed on 10% SDS-PAGE and pooled. The pooled fractions were concentrated by ammonium sulphate and applied to an XK26/100 column (Pharmacia) packed with Sephadex G 200 to a bed volume of 0.5 l, previously equilibrated with 50 mM sodium phosphate buffer, pH 7, containing 100 mM NaCl. Fractions of 5 ml were collected at 0.2 ml/min and analyzed in 10% SDS-PAGE. Positive fractions were pooled, dialyzed against 20 mM sodium phosphate buffer, pH 6.8, and applied to an XK16/30 column (Pharmacia) packed with hydroxyapatite (CHT-ceramic, Bio-Rad, USA), equilibrated with 20 mM sodium phosphate buffer, pH 7. Elution of BGLI and BGLII was carried out by step gradients of 125 and 250 mM sodium phosphate buffer, pH 7, respectively, at a flow rate of 1 ml/min. Fractions showing BGLI were pooled separately from those containing BGLII. All the purification steps were carried out on AktaExplorer (GE

Health Care, USA) at 4°C except hydroxyapatite chromatography, which was done at 20°C.

Enzyme assays

β-Glucosidase activity was measured with *p*NPG and cellobiose by following the release of colored *p*NP at 420 nm or glucose, measured by glucose-oxidase peroxidase method (Wallecha and Mishra 2003). One unit of enzyme activity corresponds to release of 1 μmol of *p*NP or glucose at 50°C per min. The activity assay is not affected by the presence of the denaturant. We monitored the breakdown of substrate by the formation of the product *p*NP (yellow in color) under alkaline conditions.

Circular dichroism (CD) spectroscopy

Far-UV CD spectra of the native and denatured samples were recorded on a Jasco J-810 (JASCO) spectropolarimeter (Japan) under constant nitrogen flow. The spectra were recorded at room temperature between 200 and 250 nm in a 1 mm quartz cuvette, averaged over three scans, and buffer subtracted. The protein concentration chosen was 0.5 μM for BGLI and 0.25 μM for BGLII in the buffers described above. However for thermal-unfolding experiments, equal concentrations of the proteins, 6 μM, were used. Molar residue ellipticity (MRE; deg cm^2 $dmol^{-1}$) was calculated by using the following formula:

$$[\Theta] = \Theta/\text{n.c.l}$$

where Θ is measured ellipticity (millidegree), n is the number of amino acid residues in the protein, c is the molar concentration of protein, and l is the path length (mm). The calculated MRE values were plotted against different concentrations of GdnHCl or different alkaline pH values and temperatures to obtain denaturation curves. The percentage of the secondary structures was determined with the k_2d secondary structure analysis program from Dichroweb online secondary structure analysis software (Andrade et al. 1993).

Fluorescence spectroscopy

Intrinsic fluorescence

Steady-state intrinsic fluorescence spectra of the native and denatured samples (GdnHCl and pH denatured) were recorded on LS 55 luminescence spectrofluorometer (Perkin-Elmer, USA). The excitation wavelength of 295 nm was used with a scan speed of 100 nm/min, and the emission spectra were recorded between 310 and 390 nm with excitation and emission slit of 8 nm. A protein concentration of 0.5 μM was used for BGLI in 10 mM sodium phosphate buffer, pH 7, and 0.25 μM for BGLII in 25 mM sodium phosphate buffer, pH 7, in a 1 mm quartz cuvette. Unfolding transitions were monitored by determining the change in relative fluorescence intensity and shift of the emission maximum under different denaturing conditions.

Differential scanning calorimetry

DSC scans of the change in excess heat capacity as a function of temperature were measured using Micro-Cal VP-DSC (MicroCal Europe, Milton Keynes, UK; cell volume 0.5 ml). DSC measurements were carried out by taking BGLI and BGLII at a concentration of 5 μM in 10 mM sodium phosphate buffer, pH 7. The buffer and sample were gently degassed prior to measurements. Scanning was carried out from 25 to 80°C and at a scan rate of 1°C/min. Data were corrected for the buffer baseline (buffer scans performed under identical conditions) and analyzed using standard Micro-Cal Origin version 7. The reversibility was also investigated by cooling the samples from 80 to 25°C under same conditions.

Dynamic light scattering

Dynamic laser light scattering was employed to determine the size distribution of proteins for folded and unfolded states. We used a Melvaron NanoZep dynamic light scattering system from Melvaron Instruments (UK) with a 10 mm path length, 1 ml quartz cuvette. Samples with protein concentration 0.1 mg/ml were centrifuged before measurements in the DLS at 25°C. Data were analyzed with the software provided with the instrument. Using this software, autocorrelation functions were calculated and a regularization fit was performed in order to obtain the size.

Glycosylation of BGLI and BGLII

Glycosylation of BGLI and BGLII was determined by the phenol-sulphuric acid method (Masuko et al. 2005) using phenol 5% (v/v) as reagent A and sulphuric acid as reagent B.

Purified BGLI (25 μg) and BGLII (25 μg) were placed in 200 μl of 25 mM sodium phosphate buffer, pH 7. To the protein solution, 1 ml of concentrated sulphuric acid was added and rapidly vortexed for 2 min. Immediately afterward, 200 μl of reagent A was added to the mixture and the solution was vortexed briefly. The test tubes were incubated at 90°C for 5 min. The samples were cooled to room temperature, and absorbance was recorded at 490 nm. A standard curve with glucose was made (1–10 μg) in the same way.

Unfolding transitions

Unfolding reactions of BGLI and BGLII were carried out by incubation of the proteins in the presence of different

concentrations of GdnHCl (0–6 M) or in different alkaline buffers (7–12) with protein concentrations of 0.5 μM for BGLI and 0.25 μM for BGLII in 10 mM sodium phosphate buffer, pH 7, and 25 mM sodium phosphate buffer, pH 7, respectively. The unfolding was allowed to occur for 3 h at room temperature. Unfolding transitions were followed by monitoring the changes in enzymatic activity, CD ellipticity, intensity and λ_{max} of fluorescence emission of both the proteins. In case of thermal unfolding, 6 μM protein samples were taken in 10 mM sodium phosphate buffer, pH 7 and the temperature was raised from 25–75°C at the rate of 5°C/min.

Results

Folded states

The far-UV CD spectra are used to determine the secondary structure content of proteins. The chromophore in this region is the peptide backbone itself and shows intense circular dichroism bands when organized into regular and rigid conformations. The far-UV CD spectra of BGLI and BGLII at physiological pH are characterized by a double minima at 220 and 208 nm, which is a signature of the α/β class of proteins (Fig. 1a, b). The signal is greater in magnitude at 208 nm and indicates that the enzyme has a higher β-sheet or random coil content. The secondary structures when analyzed by k_2d program (Andrade et al. 1993) showed BGLI to have an α-helical content of 16% and β-sheet content of 33%, while BGLII had 14% α-helix and 33% β-sheet.

β-Glucosidase I is a multitryptophan and multityrosine containing protein (Tyr = 27, Tryp = 11) with the amino acids distributed throughout the protein sequence. Although little sequence information is available for BGLII, it is also expected to be a tryptophan- and tyrosine-rich protein as both the proteins have similar amino acid composition (Wallecha and Mishra 2003). The tryptophan fluorescence is very sensitive to environmental factors such as polarity, and this makes it a valuable tool to study the folding and unfolding properties of proteins. The spectral parameters of tryptophan fluorescence such as position,

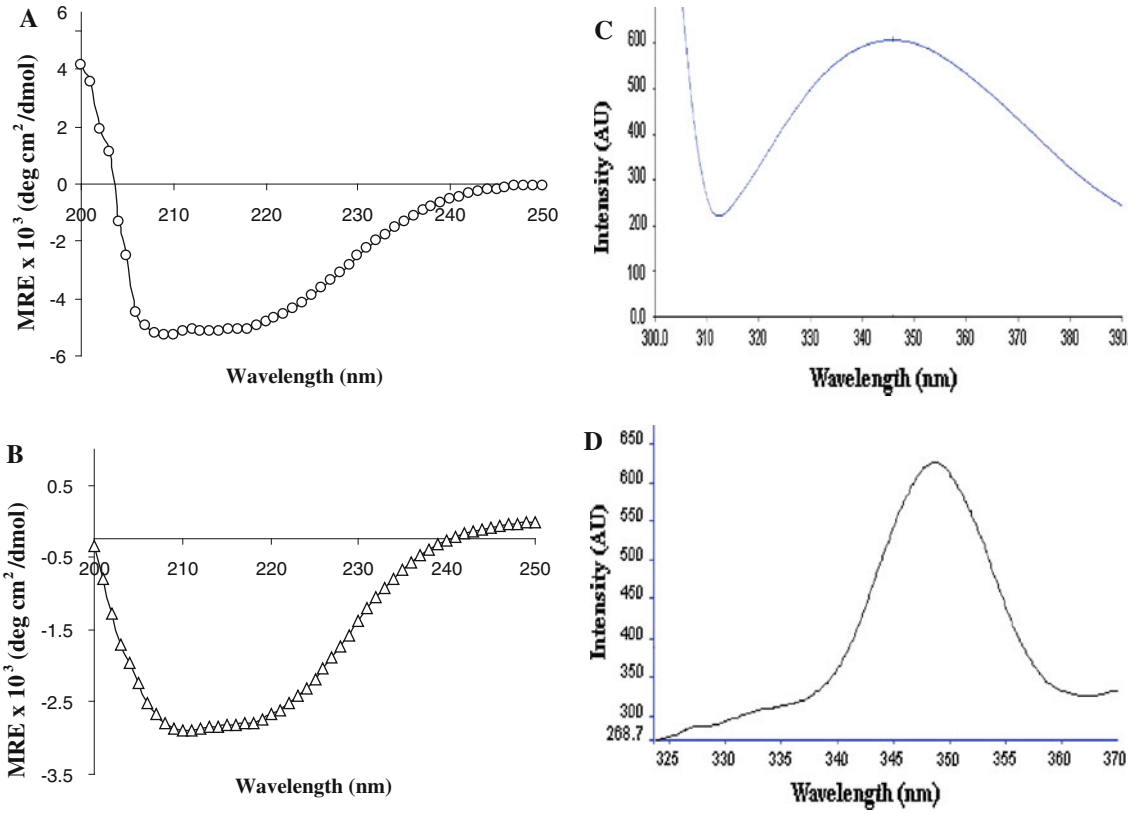

Fig. 1 Far-UV CD spectra of native BGLI (**a**) and BGLII (**b**) in 10 mM sodium phosphate buffer, pH 7, and 25 mM sodium phosphate buffer, pH 7, respectively. The protein concentrations were 0.5 and 0.25 μM, respectively. CD spectra were measured at 25°C in a quartz cuvette of 1 cm path length. Intrinsic tryptophan fluorescence spectra of BGLI (**c**) and BGLII (**d**). The protein concentrations were 0.5 μM in 10 mM sodium phosphate buffer, pH 7, for BGLI and 0.25 μM in 25 mM sodium phosphate buffer, pH 7, for BGLII. The emission spectra were recorded between 300 and 400 nm with the excitation wavelength of 295 nm at 25°C

shape, and intensity are dependent on the electron and dynamic properties of the chromophore environment. Therefore steady-state tryptophan fluorescence has been extensively used to obtain information on the structural and dynamic properties of the protein (Hayashi and Nakamura 1981). The tryptophan fluorescence maxima suffers a red shift when chromophores are more exposed to solvent, and the quantum yield of fluorescence decreases when the chromophores interact with quenching agents either in solvent, i.e., intermolecular quenching, or in the protein itself, i.e., intramolecular quenching (Ptitsyn 1992; Halfman and Nishida 1971; Cairoli et al. 1994). The emission maxima and the quantum yield of the tryptophan fluorescence are characteristic of the native three-dimensional structure of the proteins. Intrinsic fluorescence spectra of BGLI at pH 7 show emission maxima of 345 nm, while BGLII showed 348 nm; these maxima characterize the native state of the proteins (Fig. 1c, d).

The native and unique structure is held only over a particular range of temperatures, pHs, and salinities and is an intrinsic property of the protein molecule (Anfinsen 1973). Outside this range, the molecule starts losing its secondary and tertiary structures.

The unfolded states

Guanidine hydrochloride–induced unfolding

Guanidinium salts are the most interesting and commonly used protein perturbants because of the strong denaturing activity associated with the guanidinium ion (Arakawaf and Timasheff 1984). The secondary structural perturbations of BGLI and BGLII with GdnHCl when monitored at 222 nm showed that both the enzymes retained all of their spectral parameters up to denaturant concentrations of 0.5 M, after which there was a rapid loss until complete melting occurred at 2.0 M for BGLI and 1.5 M for BGLII (Fig. 2). Since β-glucosidase activity is a direct measurement of integrity of the tertiary structure of the protein, the effect of different concentrations of GdnHCl on activities was also monitored and is shown in Fig. 3. Up to 0.5 M concentration of GdnHCl, BGLI maintained the active site geometry and retained 81% activity, while BGLII retained about 98% of its enzymatic activity. BGLI lost 50% of its activity at 0.75 M, while there was a 50% loss in the β-glucosidase activity for BGLII at 1 M concentration of the denaturant. Almost complete loss of the active site geometry (95%) occurred at about 2 M for BGLI with complete denaturation at 4 M, while BGLII lost all of its activity at 1.5 M of denaturant. The transition to the inactive states was more rapid in the case of BGLII (0.75–1.5 M) than for BGLI (0.45–4 M). On further increase in GdnHCl concentration, the enzymes assumed

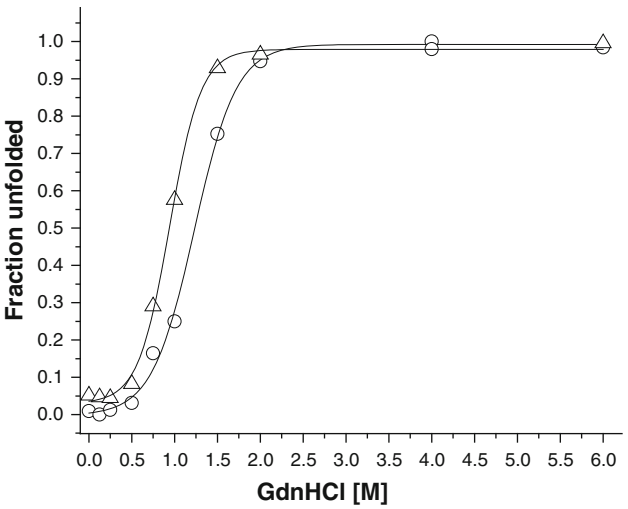

Fig. 2 GdnHCl-induced unfolding transitions as measured for BGLI (*circles*) and BGLII (*triangles*) after two-state fitting monitored by circular dichroism. BGLI and BGLII were titrated with different concentrations of denaturant (0.125, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 4.0, and 6.0 M) and incubated at 25°C. MRE values are plotted against different concentrations of denaturant. The *line* represents the theoretical two-state fitting

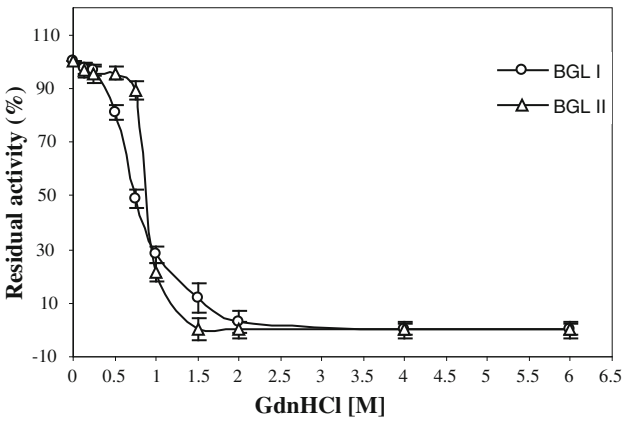

Fig. 3 Percent residual enzyme activity as a function of GdnHCl concentration for BGLI (*circles*) and BGLII (*triangles*). Enzymatic activity was measured at 50°C on *p*NPG substrate. BGLI lost activity at 2 M concentration of the denaturant, while BGLII was inactivated at 1.5 M

random coil conformation at 6 M of the denaturant, as observed by complete loss of CD signal and remained perfectly dissolved without any tendency toward aggregation, determined by absorbance at 360 nm. The MRE values at 222 nm when plotted against GdnHCl showed cooperativity of the unfolding process (Fig. 2). Apparent C_m, which is the midpoint of the unfolding transition, was calculated from the plots of MRE versus concentration of denaturants. The midpoint of transition (apparent C_{mNU})

for BGLI was observed at 1.20 ± 0.05 M, while for BGLII it was at 0.83 ± 0.03 M, indicating BGLI to be more stable to denaturant.

Tryptophan fluorescence

Intrinsic fluorescence The intrinsic tryptophan fluorescence spectra of BGLI and BGLII showed a biphasic intensity and wavelength response to increasing concentrations of GdnHCl. Thus it was possible to follow unfolding for BGLI and BGLII by both intensity and wavelength shifts. Intrinsic tryptophan fluorescence spectra of BGLI and BGLII as a function of GdnHCl are shown in Fig. 4a and b. In the native state, BGLI exhibited an emission maximum of 345 nm, while for BGLII it was 348 nm (Fig. 1c, d). On increasing the GdnHCl concentration, a significant change occurred in intensity as well as emission maxima for BGLI, while BGLII showed a change in intensity with only a marginal shift in emission maxima (Figs. 4 and 5).

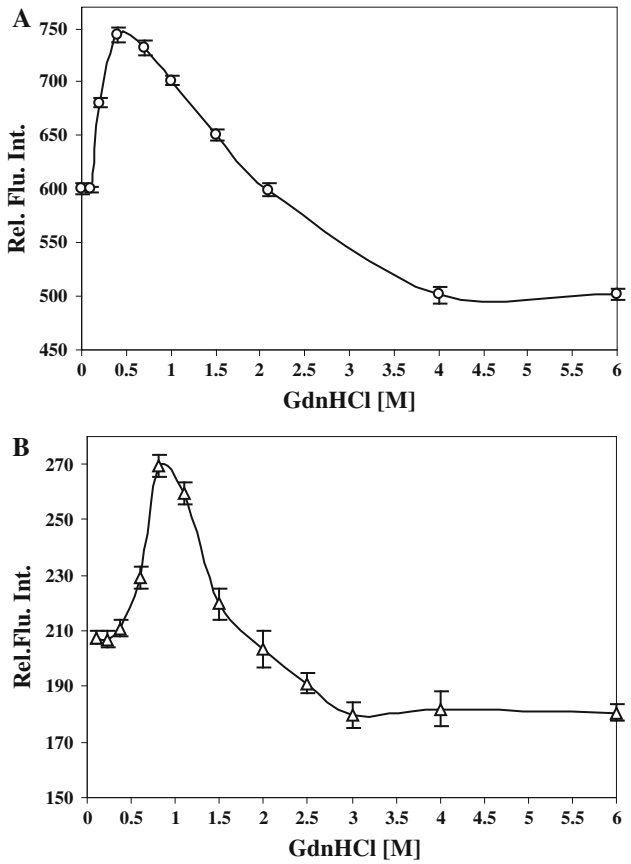

Fig. 4 Intrinsic fluorescence emission spectra of GdnHCl-induced unfolding of BGLI (**a**) and BGLII (**b**). The protein concentrations were 0.5 μM in 10 mM sodium phosphate buffer, pH 7, for BGLI and 0.25 μM in 25 mM sodium phosphate buffer, pH 7, for BGLII. The emission spectra were recorded between 300 and 400 nm with the excitation wavelength of 295 nm at 25°C. As observed, relative fluorescence intensity of both proteins increased up to denaturant concentrations of 0.5 M and 0.75 M for BGLI and BGLII, respectively

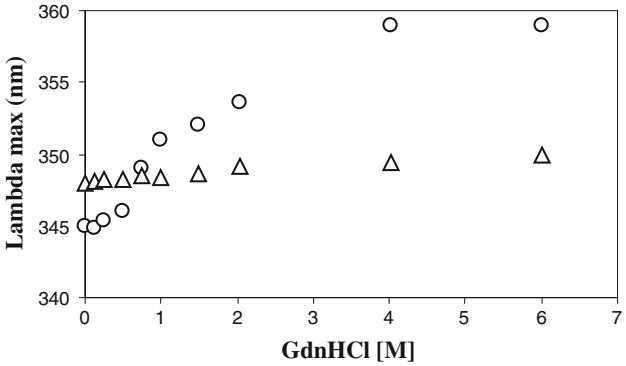

Fig. 5 Fluorescence emission maxima wavelengths (λ_{max}) as a function of GdnHCl concentration for BGLI (*circles*) and BGLII (*triangles*) during unfolding. BGLI and BGLII were titrated with different concentrations of denaturant (0.125, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 4.0, and 6.0 M) and incubated at 25°C. A red shift of 15 nm is observed for BGLI, while the shift in BGLII is marginal

The intrinsic tryptophan fluorescence of BGLI showed a 25% initial increase in intensity up to 0.5 M of denaturant after which there was a gradual decrease, indicating the exposure of tryptophan residues to aqueous environment. The fluorescence intensity also increased up to 0.75 M. At 4 M GdnHCl there was a maximum red shift to 359 nm in BGLI with a drastic fall in the intensity indicative of more exposure of tryptophan residues to a polar environment (Fig. 5). For BGLII, the initial increase in the fluorescence intensity up to 0.75 M was followed by a rapid decrease, which tailed off at 3 M concentration of the denaturant, indicating complete exposure of the tryptophan residues to the polar environment.

Because of some difficulties, it was not possible to calculate the center of mass for each of the fluorescence emission curves during the unfolding process. Therefore characterization of the unfolding process by means of the change in the center of mass could not be reported. However, the unfolding process has been characterized in terms of the red shift of λ_{max} of fluorescence emission for both isozymes.

Alkali-induced unfolding

Change in secondary structures In order to gain further insights into conformational changes during unfolding of BGLI and BGLII, denaturation was carried out with alkali. During alkali-induced unfolding, dramatic structural reorganization or pH hysteresis was observed in the case of BGLI (Fig. 6a). For BGLI, both the shape and intensity of CD spectra changed as pH increased. At pH 7, the proteins showed typical double minima at 220 and 208 nm. As the pH increased to a value of 8, the CD signal at 220 nm

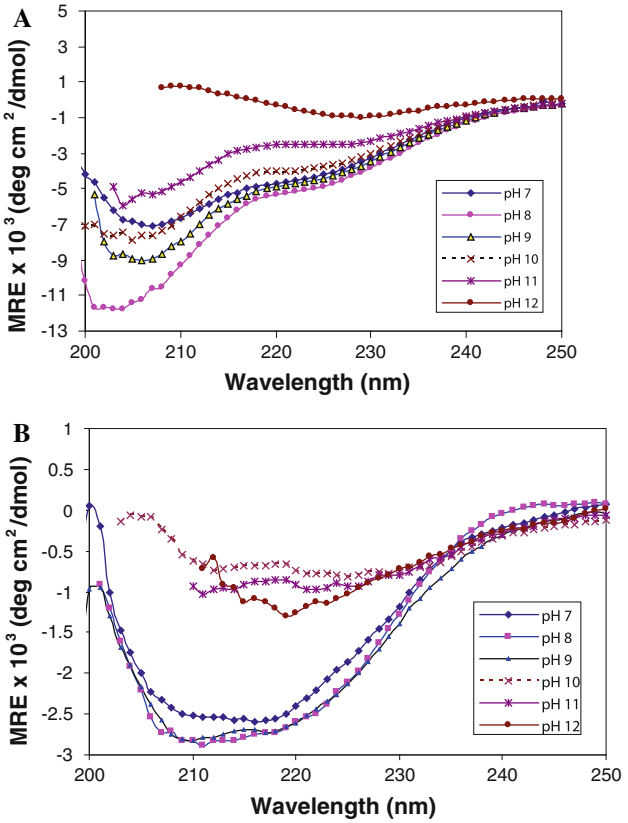

Fig. 6 Unfolding transitions induced by alkaline pH (7–12) for BGLI (**a**) and BGLII (**b**) monitored by far-UV CD spectra. The samples were titrated in their respective buffers of different pH values and incubated at 25°C. Both proteins show reorganization of structure up to pH 9 followed by a steady decrease in secondary structure for BGLI and a rapid transition for BGLII along the unfolding pathway

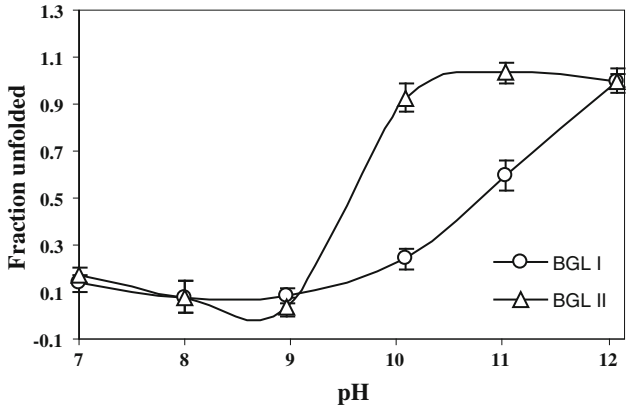

Fig. 7 Alkaline pH-induced unfolding transition curve for BGLI (*circles*) and BGLII (*triangles*). MRE values are plotted against different pH values. $D_{50\%}$ for BGLI occurred at pH 11, while for BGLII it was observed at pH 10

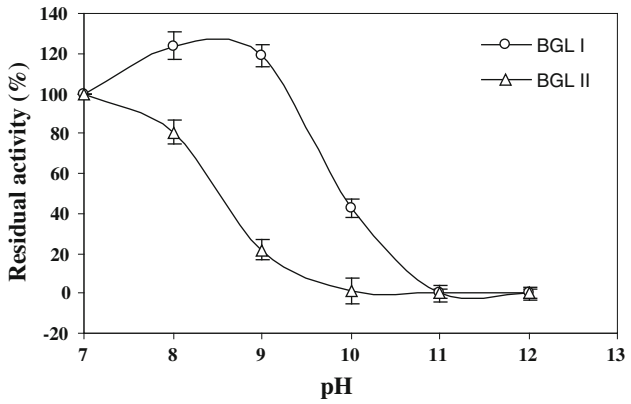

Fig. 8 Percent residual enzyme activity as a function of alkaline pH for BGLI (*circles*) and BGLII (*triangles*). *β*-Glucosidase activity was measured at 50°C on *p*NPG substrate. BGLI lost activity at pH 11, while BGLII had no activity at pH 10

increased, while the signal at 208 nm showed a shift to 202 nm with a large enhancement in signal for BGLI. A further increase in pH to 9 drove the CD signal towards the native conformation of the protein, and the CD signal showed a red shift to 205 from 202 nm. At pH 10, loss in helicity occurred. Increase to pH 11 resulted in loss of the secondary structures, and at pH 12 the enzyme had little structural organization with no CD signal. For BGLII, the signal showed a marginal enhancement at pH values of 8 and 9 (Fig. 6b). There was a rapid unfolding when the pH was raised to 10, and the enzyme showed little structural organization above this value. A plot of MRE values at 222 nm against different pH values indicated the midpoint of transition at pH 11.0 for BGLI and 10.0 for BGLII (Fig. 7). The enzymatic activity data showed that BGLI retained the active site geometry up to pH 9, while rapidly losing 60% of its activity at pH 11. The enzyme was completely inactivated at pH 12, indicating melting of the structure. For BGLII, the activity data indicated 80% loss in enzyme activity up to pH 9 and complete alkali denaturation at pH 10 (Fig. 8).

Change in tryptophan environment The intrinsic tryptophan fluorescence of alkali-induced unfolding for BGLI and BGLII is shown in Fig. 9. The fluorescence intensity increased as pH was raised from 7 to 9, reaching a maximum value at pH 9. The increase indicates that the change in the tryptophan environment occurs on alkalization of the protein. Increase in intensity suggested that as pH was raised, the tryptophans were being moved to a more non-polar environment or away from a self-quencher, which might be deprotonated carboxyl, deprotonated imidazole, or tyrosinate anion (Halfman and Nishida 1971).

Thermal unfolding

Change in secondary structures As both BGLI and BGLII are thermo-tolerant enzymes, conformational changes induced by temperature to obtain information about structural organization and the stability of the

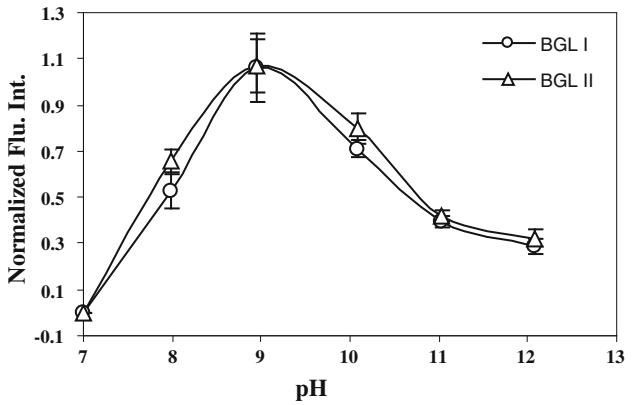

Fig. 9 Intrinsic fluorescence emission spectra of BGLI (*circles*) and BGLII (*triangles*) at different pH values. The fluorescence spectra were taken at 25°C with an excitation wavelength of 295 nm. Fluorescence intensity of both proteins increased up to pH 9 after which there was a sharp decrease indicative of formation of some nonnative intermediate during unfolding. The data set was normalized by using the expression $||X_i|| = \left\{\frac{(x_i - x_{i,\min})}{(x_{i,\max} - x_i)}\right\}$, where X_i is any data point of x-series while X_i, min and X_i, max are the minimum and maximum values

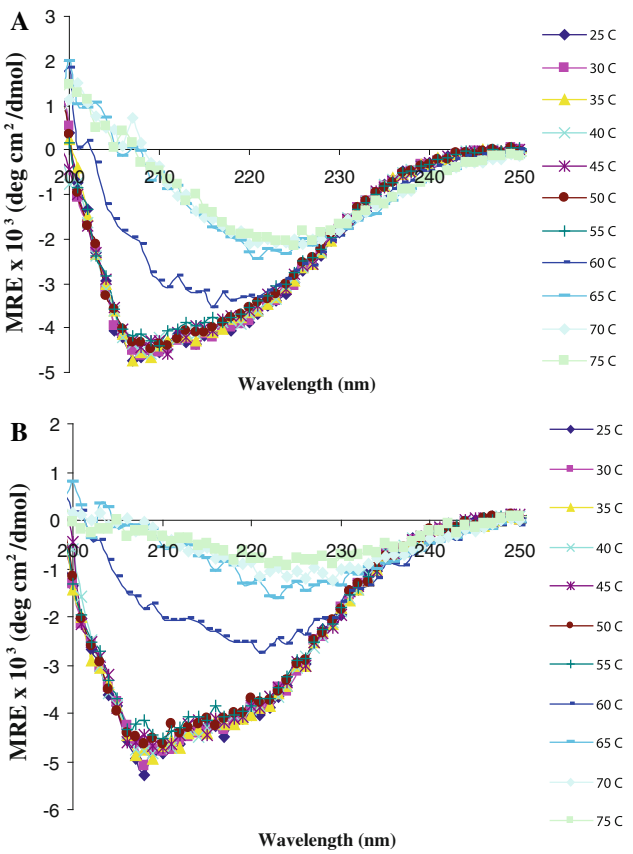

Fig. 10 Far-UV CD spectra of thermally denatured BGLI (**a**) and BGLII (**b**). CD spectra were taken on a Chirascan CD polarimeter between 25 and 75°C at 5°C increments. The spectra were recorded between 200 and 260 nm

enzymes over a temperature range of 25–75°C were studied. Thermal denaturation was carried out at constant heating rates, and the unfolding process was monitored by recording far-UV CD spectra between 200 and 260 nm. The CD signal presented the same features for both the proteins and showed almost no change, either in shape or intensity, up to 55°C as shown in Fig. 10a and b. This suggested that the proteins retained their native-like secondary structures. The tertiary structures were also retained as both the proteins retained β-glucosidase activity up to 55°C. However, when the temperature was raised to 60°C, loss of the secondary structure started to occur as was observed by a decrease in the CD signal. The loss in signal at 208 nm was greater than the disappearance of the CD signal at 222 nm, indicating that β-sheets melted earlier than α-helices. It is interesting to note that the decrease in the shape and intensity of the CD signal was greater in the case of BGLII than BGLI. This suggests that BGLII started to lose its secondary structure and unfolded earlier than BGLI, suggesting more conformational rigidity of the latter. As the temperature reached 65°C, there was loss in CD signal in both cases indicating melting of the residual secondary structures and complete unfolding.

Analysis of the temperature-induced denaturation curves indicated different denaturation profiles, although both maintained a sigmoidal shape (Fig. 11). The curve showed marginal changes for BGLI up to 60°C followed by opening of the structure between 61 and 65°C. For BGLII, no change occured up to 55°C. However, at 58°C almost half of the protein molecules were in the unfolded state. BGLII showed a sharper and earlier unfolding transition

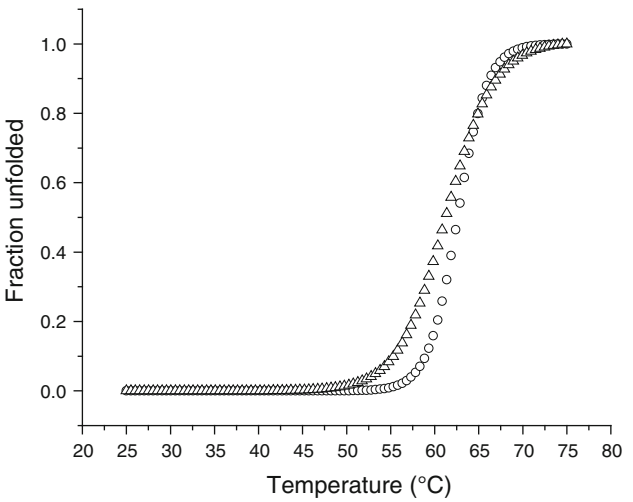

Fig. 11 Thermal-unfolding transitions as measured for BGLI (*circles*) and BGLII (*triangles*) by far-UV CD spectroscopy. BGLII unfolds at an early stage, $D_{50\%} = 58$°C, while BGLI shows rigidity towards unfolding, $D_{50\%} = 63$°C. The averaged experimental data points as measured during unfolding transitions were fitted with a sigmoidal Boltzman function using Origin ProV 8 software

than BGLI. When the temperature was raised to 70°C, the proteins were completely unfolded as seen by the disappearance of the CD signal for both BGLI and BGLII.

Change in tryptophan environment Further characterization of the change in the three-dimensional structure was done by inducing thermal-unfolding transitions monitored in terms of intrinsic tryptophan fluorescence. When a tryptophan or tyrosine is in a polar environment, there is a decrease in fluorescence with increase in temperature, T, whereas in a nonpolar environment there is little change. Therefore, deviation from a monotonic decrease in fluorescence with increasing T suggests that heating is inducing a conformational change because the polarity of the regions to which tryptophans or tyrosines are exposed must be changing. When the protein is in a polar solvent and there is an increase in fluorescence with temperature, it indicates that the tryptophans are being exposed to the solvent and unfolding is taking place (Freifelder 1982). The intrinsic fluorescence spectra of BGLI and BGLII are given in Fig. 12. The structural changes were studied in the temperature range of 25–75°C in 1° increments. As observed for BGLI, there was a monotonic decrease in intensity. No sharp transition was observed. However, the trend can be thought of as consisting of two zones, namely, zone I (25–55°C) and zone II (55–85°C). The midpoint of transition appeared to be somewhere after 55°C, however, it was not well demarcated. In the case of BGLII, the transition was sharp and distinct and could be easily demarcated into three zones: (1) a relatively long pretransition zone from 25 to 50°C, where fluorescence intensity showed almost no change, (2) a short and sharp transition zone from 50 to 60°C, and (3) a post-transition zone from 60 to 75°C. In the transition zone, the midpoint of transition was observed at 55.9°C. In the post-transition zone, the fluorescence intensity remained almost constant indicating that the tryptophan residues were fully exposed at this point, and the protein was in the fully unfolded conformation. Interestingly, a huge precipitate was observed in the case of BGLII only, while turbidity was seen for BGLI after the unfolding was completed, when turbidity of the samples was assessed by measuring absorbance at 360 nm. This indicates heavy aggregation of the former protein.

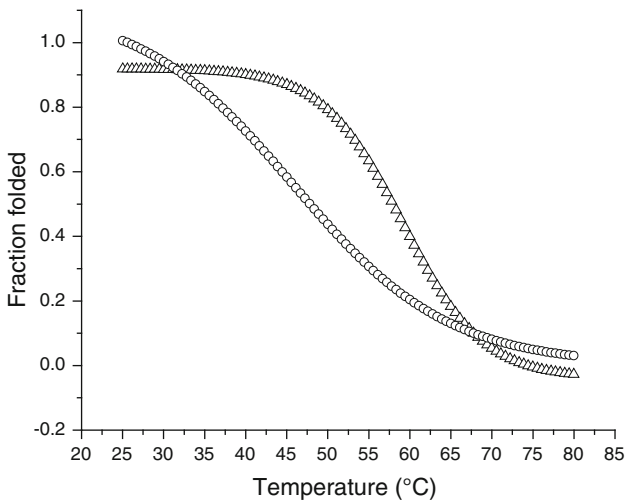

Fig. 12 Thermal unfolding of BGLI (*circles*) and BGLII (*triangles*). The process was monitored through measurement of intrinsic fluoresence emission at the λ_{max}. The unfolding was carried out in a Varian spectrofluorometer fitted with a peltier system. Temperature was increased at the rate of 1°C/min

Differential scanning calorimetry

In order to further characterize the stability of BGLI and BGLII, calorimetric studies of the samples were carried out in a differential scanning calorimeter. This is one of the most powerful methods for the characterization of protein stability (Plum and Breslauer 1995). For a thermodynamically reversible transition, the technique provides several pieces of information including the melting temperature, T_m, at which the maximum excess heat capacity is observed; enthalpy of denaturation, ΔH, the change in the heat capacity, ΔC_p, of the protein, and vant Hoff's enthalpy of the transition (thermal unfolding per cooperative unit). Since thermal unfolding of large multidomain proteins is thermodynamically irreversible due to aggregation, only a limited number of parameters (such as T_m and ΔH_{cal}) can be obtained by DSC.

Differential scanning calorimetry of BGLI showed a T_m of 56.07°C while BGLII showed a T_m of 54.02°C (Fig. 13a and b). The apparent enthalpy of unfolding for BGLI was found to be 85 kcal/mol, while for BGLII it was 25 kcal/mol.

Dynamic light scattering

The dynamic light scattering data indicated that, for GdnHCl-induced denaturation, the hydrodynamic radius of BGLI increased from 5 to 7–8 nm for 2 M of the denaturant, while at 6 M, the radius increased to 18–24 nm. For BGLII, a slight increase in the radius to 9 nm was observed at 6 M concentration of the denaturant. When the denaturant was dialyzed out, BGLI showed formation of aggregates of smaller size (39–61 nm), while BGLII was found to be heavily aggregated (265–447 nm; Table 1). The hydrodynamic radius, obtained from DLS studies, of the alkaline pH–unfolded BGLI and BGLII showed that pH 11–unfolded BGLI had a larger size than the native enzyme (Table 1). In the case of BGLI, the size of the pH 11–unfolded protein was 171–265 nm, indicating heavy aggregation of the protein. For BGLII, the hydrodynamic

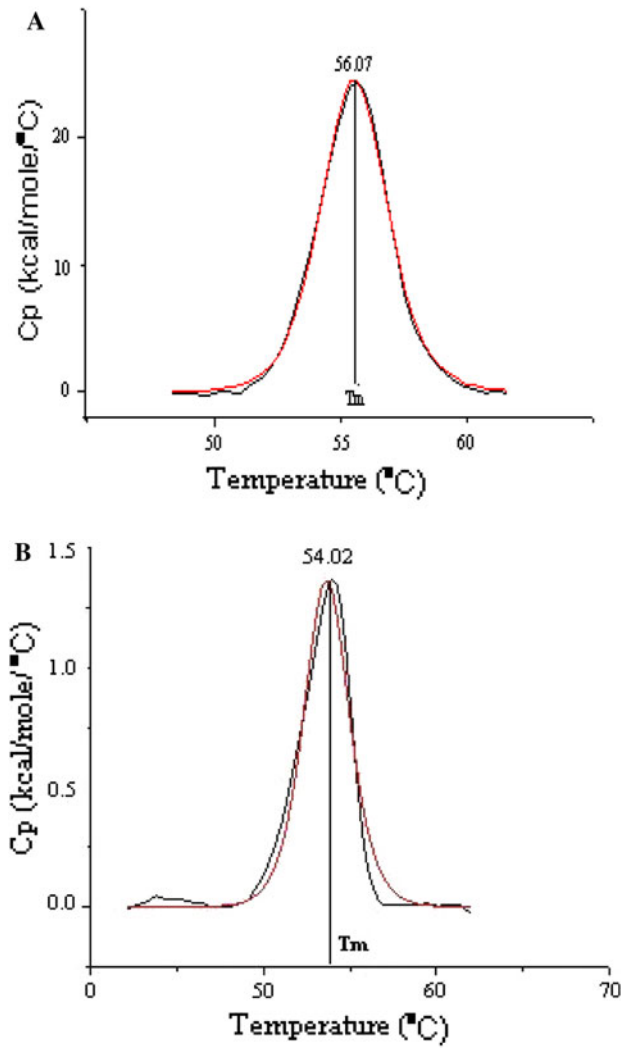

Fig. 13 Baseline-subtracted DSC scan of 5 μM BGLI (**a**) and 5 μM BGLII (**b**) in 10 mM sodium phosphate buffer, pH 7, scanned at a rate of 1°C/min. The *black line* indicates DSC scan, while the *red line* indicates the C_p fit

radius was 14–19 nm characteristic of formation of micro-aggregates. Thermally unfolded BGLI showed turbidity, while in the case of BGLII, a heavy precipitate was observed. The size of the thermally unfolded BGLI was 600 nm, indicating aggregation of the unfolded species that retained solubility. Most of the BGLII was precipitated under these conditions indicating heavy aggregation of the protein.

Discussion

Structural studies and unfolding transitions of proteins under different solvent conditions provide information about the conformation of protein molecules and the role of various stabilizing and destabilizing forces responsible for the proteins' unique three-dimensional structure (Timasheff 1993). The unique three-dimensional structure is a basic requirement for proper functioning of a protein. It has been demonstrated in many studies that the ability to keep this native and functional structure over a particular range of temperatures, pH levels, and salinities is an intrinsic property of the protein molecule itself, which is determined by the amino acid sequence (Anfinsen 1973). Outside of these conditions, the protein starts to unfold. Surprisingly this range of experimental conditions differs for different proteins from different sources. Even for homologous proteins, differences are observed in structural stability and unfolding behavior (Fitter and Haber-Pohlmeier 2004). However, isozymes that belong to the same organism and share similar folding are expected to show more similarity in stability and unfolding behavior. In the present communication, we have demonstrated the structural features and unfolding properties of two large *β*-glucosidases (BGLI and BGLII) from a thermo-tolerant yeast, *Pichia etchellsii*.

Unfolding reactions by guanidine hydrochloride indicated that the structural integrity of BGLI and BGLII is maintained up to 0.5 M concentration of the denaturant, after which there is a rapid transition to unfolded states. The loss in the secondary structure is more rapid for BGLII than for BGLI, which is reflected in the different midpoints of transition, apparent C_m (BGLI = 1.20 ± 0.05 M and BGLII = 0.83 ± 0.03 M). The activity data showed the active site design of BGLI to be more prone to denaturation than BGLII. The active site geometry of BGLII was found to be more organized than BGLI up to 0.5 M of the denaturant. Tertiary structural changes, when monitored by intrinsic tryptophan fluorescence, indicated an initial increase in intensity up to 0.75 M in both cases, suggesting a similar kind of tryptophan arrangement, which is expected as both the proteins belong to the same category of enzymes. The emission maximum around 350 nm is the signature of the chromophore on the surface of the molecules (Burstein 1976; Kuwajima 1996). The DLS data indicated expansion of the polypeptide chain in the case of

Table 1 Hydrodynamic radii of native and denatured forms of BGLI and BGLII

Protein	Native (nm)	GdnHCl 2 M (nm)	GdnHCl 6 M (nm)	GdnHCl dialyzed (nm)	Alkaline pH (nm)	Therm. denatured
BGLI	5	7–8	18–24	39–61	171–265	600 nm
BGLII	6	6	9	265–447	14–19	Ppt.

Ppt Precipitate

BGLI (native $R_H = 5$ nm and 6 M denatured $R_H = 18$ nm), while a marginal increase was observed for BGLII (native $R_H = 6$ nm, 6 M denatured $R_H = 9$ nm). This may be due to heavy glycosylation of BGLI (32.20% g/g) as compared to BGLII (0.01% g/g), which might be responsible for the larger hydrodynamic radius. It was interesting to note that monomerization did not appear to occur as the size of the denatured proteins showed little difference from the native protein molecules for BGLII and an increase for BGLI. This might be due to the existence of protein molecules in the monomeric form under these conditions. This was confirmed by mass spectrometry, LC/MS, which showed a single peak at 97.6 kDa for BGLI and a peak at 94.4 kDa for BGLII (data not shown).

The existence of multimers/aggregates could not be established by mass spectrometry. However, when the oligomeric nature of the proteins was analyzed by zymogram, it confirmed our previous observation that BGLI existed as a homodimer and BGLII as a homotetramer. Hence, to compare equimolar amounts of the two isozymes, different protein concentrations were used. However, equal concentrations of the two proteins were used for DSC measurements to see whether the observed stability difference was due to different concentrations. The DSC data also indicated BGLI to be more stable than BGLII, melting at a higher temperature. Since our intent was not to investigate the effect of protein concentration on the stability of either of the proteins individually but only to address the stability issues between two homologous proteins, the concentration factor was ignored. Different biophysical methods were used to assess differential stability of the isozymes using concentrations appropriate to generate substantial signals. However, in order to study the denaturation of individual proteins by different methods, it becomes imperative to use the same protein concentration. The prolonged stay in the solution resulted in the aggregation of the proteins. It was also noted that the removal of the denaturant resulted in a larger increase in the hydrodynamic radius of BGLII than BGLI suggesting the former to be more hydrophobic than the latter. However, when the two proteins were separated by phenyl sepharose chromatography, BGLII did not bind to the column (data not shown). This might be explained by the fact that the hydrophobic patches on the surface of BGLII stick with each other to form a tetrameric assembly and hence result in reduced surface hydrophobicity of the protein.

The alkali-induced unfolding of BGLI and BGLII, when monitored by far-UV CD spectroscopy, revealed some interesting differences in the conformational behavior of the two proteins. BGLI showed more structural flexibility and dynamics than BGLII. The pH-induced hysteresis or reorganization of the main polypeptide chain of BGLI was greater than that of BGLII. An increase in pH to 8 resulted in the folding of the polypeptide chain towards a more random coil conformation. However, the activity data indicated the enzyme to be more organized, and instead there was a slight increase in the activity (20%) at pH 8. This might be due to a shift to more β turns rather than to a random coil conformation, a difference that is not easy to distinguish (Arakawaf and Timasheff 1984). Structural reorganization has been reported in lectins and glycoproteins (Burstein 1976; Regan 2003). Since both the proteins are hydrolases and have affinity for carbohydrates, this behavior is expected. Interestingly BGLII does show deviation from this behavior, which suggests its polypeptide chain to be more rigid to alkali hysteresis, and there was an abrupt change to an unfolded state. This structural reorganization is followed by disruption of the polypeptide chain into a random coil form. The effect of alkali indicated that electrostatic interactions have a more important role in the unfolding and reorganization of BGLI than of BGLII.

In both cases, the relative fluorescence intensity change as a result of alkalization showed a similar trend. It was enhanced for both as the pH was raised from 7 to 9. This increase is indicative of the fact that tryptophans experience a less polar environment or move away from a nearby quencher. This pH dependency may be explained further by intramolecular quenching of the excited indole ring of Trp by the imidazole of His, which might have resulted in a marked decrease in the tryptophan fluorescence at low pH. At lower pH, some of the histidine residues may get protonated and intramolecularly interact with indole rings of the nearest tryptophan residues, thereby causing internal quenching of fluorescence. As the pH was increased from 7 to higher values, there might be deprotonation of selected histidine residues, which caused internal quenching of the tryptophan fluorescence. However, the fluorescence intensity gradually decreased on further increase of the pH, which might account for the alkaline denaturation of proteins. In the case of BGLI, the enzyme retained its activity up to a pH of about 9.0, whereas there was a decrease in the enzymatic activity of BGLII with the same increase in the pH of the solution. This information may signify that, in BGLI, the active site of the protein may not be influenced by histidine or tryptophan residues, which are involved in the internal quenching phenomenon. However in BGLII, the active site of the protein is sensitive to the change in the charged states of selective His or Trp residues, which are responsible for the change in fluorescence emission in the alkaline titration of the protein.

Monitoring of the thermal unfolding by far-UV CD spectroscopy corroborated the results obtained by GdnHCl- and alkali-effected unfolding. The far-UV CD spectra showed that the apparent midpoints of transition for BGLI and BGLII were about 63 and 58°C, respectively,

Table 2 Parameters for unfolding of BGLI and BGLII

Unfolding process	Unfolding parameters	
	BGLI	BGLII
GdnHCl unfolding	$C_m^{app} = 1.20 \pm 0.05$ M	$C_m^{app} = 0.83 \pm 0.03$ M
Alkali unfolding	$D_{50\%}$ = pH 11 ± 0.17	$D_{50\%}$ = pH 10 ± 0.40
Thermal unfolding	$D_{50\%}$ = 63 ± 0.12°C	$D_{50\%}$ = 58 ± 0.55°C
DSC	$T_m = 56.07 \pm 0.34$°C	$T_m = 54.02 \pm 36$°C
	ΔH = 85 kcal/mol	ΔH = 25 kcal/mol

indicating more rigidity in the former structure. The tertiary structures, when monitored by intrinsic fluorescence, showed a gradual decay in the fluorescence of BGLI as compared to BGLII. Both the proteins have maximum activity at 50°C (Wallecha and Mishra 2003). This indicates that the active site architecture of the protein is lost in the same manner in both cases during thermal unfolding. The melting temperatures could not be accurately determined through CD or fluorescence measurements. This was achieved by high sensitivity differential scanning calorimetry. The T_m for BGLI was found to be 56.07°C, while for BGLII it was 54.02°C. The enthalpy of unfolding was determined to be 85 kcal/mol for BGLI and 25 kcal/mol for BGLII. The summary of various biophysical parameters is provided in Table 2. These data again clearly support the fact that BGLI is structurally more rigid and stable than BGLII. When the sequence of BGLI was analyzed, it was found to contain four potential glycosylation sites. Analysis of glycans by a simple phenol–sulfuric acid method indicated BGLI to be more highly glycosylated (32.2% g/g) than BGLII (0.01% g/g). Thus different levels of glycosylation might be responsible for the different structural stability and unfolding behavior of the two isozymes. Although many studies have shown that glycosylation plays an important role in the stability of proteins, this was the first study on this family of enzymes, i.e., family 3 glycosylhydrolases.

The unfolding experiments were found to be irreversible for both isozymes. This may be due to the fact that both BGLI and II are aggregation prone, which prevents the unfolding transition for reversing. Enhanced surface hydrophobicity of partially denatured BGLI and BGLII reveals that the unfolding intermediates are capable of self-aggregation, leading to aggregate formation.

Conclusions

Isozymes share a substantial amount of their secondary and tertiary structural similarity. β-Glucosidase isozymes from the same source show markedly different behavior in terms of their stability and unfolding properties. The two enzymes investigated in this study show deviation in their unfolding properties induced by denaturants, alkali, or heat. This takes on significance as both the proteins are produced by a thermo-tolerant yeast under similar conditions of growth and show similarities in their biochemical and structural properties. However, BGLI is more heavily glycosylated than BGLII, which we believe to be responsible for their different unfolding behaviors.

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