Does Recovery in the Spectral Characteristics of GdnHCl-Denatured *Bacillus licheniformis* α-Amylase Due to Added Calcium Point towards Protein Stabilization?

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Treatment of *Bacillus licheniformis* α-amylase (BLA) with guanidine hydrochloride (GdnHCl) produced both denatured and aggregated forms of the enzyme as studied by circular dichroism, fluorescence, UV difference spectroscopy, size exclusion chromatography (SEC), and enzymatic activity. The presence of CaCl$_2$ in the incubation mixture produced significant recovery in spectral signals, being complete in presence of 10 mM CaCl$_2$, as well as in enzymatic activity, which is indicative of protein stabilization. However, the SEC results obtained with GdnHCl-denatured BLA both in the absence and the presence of 10 mM CaCl$_2$ suggested significant aggregation of the protein in the absence of CaCl$_2$ and disaggregation in its presence. Although partial structural stabilization with significant retention of enzymatic activity was observed in the presence of calcium, it was far from the native state, as reflected by spectral probes. Hence, spectral results as to BLA stabilization should be treated with caution in the presence of aggregation.

Key words: *Bacillus licheniformis* α-amylase; calcium-induced stabilization; enzymatic activity; guanidine hydrochloride denaturation; protein aggregation

α-Amylases (α-1,4-glucan-4-glucanohydrolases, EC 3.2.1.1), being members of the endo-amylase family, catalyze the hydrolysis of 1,4-α-D-glycosidic linkages in starch and related carbohydrates.1-3) These enzymes have great significance in biotechnology due to their use in the production of maltodextrins, baking, brewing, alcohol, detergent, and textile industries.4-6) *Bacillus licheniformis* α-amylase (BLA) is a highly thermostable enzyme produced by a mesophilic bacterium, and is even more thermostable than the related enzyme produced by a thermophilic organism, *Bacillus steato-thermophilus*, though these enzymes possess highly homologous primary and tertiary structures.7) Because of its remarkably high thermal stability, BLA has been used as a model to determine the mechanism and structural requirements for high thermal stability.8)

The primary structure of BLA is characterized by the presence of three domains encompassing all 483 amino acid residues.9) The crystal structure of BLA has shown the presence of two calcium binding sites in domain B and one at the interface of domains A and C.10) Calcium is known to play important roles in maintaining the structural integrity and the enzymatic activity of BLA, as is evident in the increase in the melting temperature (T$_m$) as well as the mid transition concentration of urea denaturation in the presence of calcium.11-16) Furthermore, removal of calcium from its specific binding sites on the enzyme has been found to decrease its stability and enzymatic activity.11,17) Even at a concentration of 3.0 M urea, the enzyme remains largely unfolded, though it is otherwise folded in the presence of calcium.14) In addition, the enzyme has been found to form aggregates upon thermal denaturation and in the presence of lower guanidine hydrochloride (GdnHCl) concentrations.15,18)

Most structural stability studies have been performed with calcium-depleted BLA or with BLA in presence of CaCl$_2$.11-18) A commercial BLA preparation (native BLA), which is partially saturated with calcium, has rarely been used in structural stability studies. In an earlier study, we found that the addition of 2 mM CaCl$_2$ to calcium-depleted BLA conferred the same degree of stability as that exhibited by the intrinsic calcium present in native BLA.19) Even native BLA did not show complete reversibility of structural transition in presence of 2 mM CaCl$_2$ in GdnHCl denaturation experiments.19) Although Nazmi et al.14) identified an effect of increasing calcium concentrations on urea-denatured BLA, studies of GdnHCl-denatured BLA in presence of various CaCl$_2$ concentrations are lacking. Furthermore, the effect of calcium on the aggregation state of native BLA has not been studied to date. Hence, we studied the effects of various CaCl$_2$ concentrations on the GdnHCl-denaturation of native BLA in order to check the completion of reversibility of transition as well as protein aggregation during GdnHCl denaturation.

Materials and Methods

Materials. *Bacillus licheniformis* α-amylase (BLA) (lot 018K7018V), acrylamide, N-acetyl-L-tryptophanamide (NATA), GdnHCl (lot 120MS431V), 3,5-dinitrosalicylic acid, starch from potatoes, and Sephacryl S-200 HR were purchased from Sigma

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Abbreviations: BLA, *Bacillus licheniformis* α-amylase; CaCl$_2$, calcium chloride; CD, circular dichroism; GdnHCl, guanidine hydrochloride; MRE, mean residue ellipticity; NATA, N-acetyl-L-tryptophanamide; SEC, size exclusion chromatography
Aldrich (St. Louis, MO). Maltose and calcium chloride (CaCl$_2$) were from R & M Chemicals (Essex, UK) and System* (Shah Alam, Malaysia) respectively. Tris base and Tris–HCl were from Amresco (Irvine, CA) and Promega (Madison, WI) respectively. All other chemicals used were of analytical grade. A commercial BLA preparation (partially saturated with calcium) was used throughout these studies, and is termed as BLA/native BLA.

**Analytical procedures.** The protein concentration was determined spectrophotometrically on a Shimadzu double-beam spectrophotometer, model UV-2450 (Shimadzu, Kyoto, Japan), using a molar extinction coefficient of 139,690 M$^{-1}$cm$^{-1}$ at 280 nm, and a molar extinction coefficient of 5630 M$^{-1}$cm$^{-1}$ at 280 nm was used to determine the NATA concentration.$^{20}$ The concentration of stock GdnHCl solution was determined by the method of Pace et al.$^{21}$

**Circular dichroism.** Circular dichroism (CD) measurements in the far-UV region (200–250 nm) were made at 25°C on a Jasco spectropolarimeter, model J-815 (Jasco, Tokyo, Japan) equipped with a thermostatically controlled cell holder under constant nitrogen flow after calibration of the instrument with (+)-10-camphorsulfonic acid. Spectra were recorded at a protein concentration of 1.7 M in a 1-mm path length cuvette at a scan speed of 100 nm/min and a response time of 1 s. Each spectrum was the average of three scans, and the spectra were corrected with suitable blanks. Results are expressed as mean residue ellipticity (MRE) in deg-cm$^2$-mol$^{-1}$-dm$^{-1}$, as obtained using the following equation:

$$\text{MRE} = \theta \times \text{MRW} / (10 \times c \times l) \quad (1)$$

where $\theta$ is the measured ellipticity in millidegrees; MRW is the mean residue weight, obtained by dividing the molecular weight of the protein (55,200 Da) with the number of amino acid residues of the protein (483),$^{26}$ is the concentration of the protein in mg/mL, and $l$ is the optical path length in centimeters.

**Fluorescence spectroscopy.** Fluorescence measurements were performed on a Jasco fluorescence spectrophotometer, model FP 6500 using quartz cuvette of 1-cm path length at a protein concentration of 0.1 M. The excitation and emission slits were fixed at 10 nm each, and the emission spectra were recorded in a wavelength range of 300–400 nm after excitation of the protein solution at 280 nm. The fluorescence spectra were corrected using appropriate blanks.

Synchronous fluorescence spectra were recorded in wavelength ranges of 250–350 nm and 250–400 nm following wavelength intervals ($\Delta\lambda$) of 15 nm and 60 nm respectively. Three-dimensional (3-D) fluorescence spectra were recorded in a wavelength range of 220–500 nm by exciting the protein solution in a wavelength range of 220–400 nm at 10 nm increments. The number of scanning curves was 19.

**UV difference spectroscopy.** UV absorption spectra of BLA under different experimental conditions were recorded at 25°C on a Shimadzu double-beam spectrophotometer, model UV-2450, in a wavelength range of 250–350 nm at a protein concentration of 5.2 M. UV difference spectra were obtained by subtracting the absorbance values of native BLA at each wavelength from the absorbance values of GdnHCl-denatured BLA, obtained both in the absence and the presence of 10 mM CaCl$_2$.

**Acrylamide quenching.** Acrylamide quenching experiments were carried out both in the absence and the presence of 2 mM/10 mM CaCl$_2$ by adding increasing volumes (45–450 μL) of stock acrylamide solution (2 M) to different tubes containing a constant volume (0.5 mL) of native BLA/GdnHCl-denatured BLA/NATA solution. The total volume in each tube was made up to 3.0 mL with the respective buffers. The final concentrations of BLA and NATA were 0.1 μM and 1.7 μM respectively. For experiments involving GdnHCl-denatured samples, all solutions (protein, acrylamide, and buffer) contained the same amount of GdnHCl, and the protein samples with GdnHCl were pre-incubated for 12 h at 25°C. Fluorescence intensity at the emission maximum was recorded after exciting the samples at 295 nm, at slit widths of 10 nm. Fluorescence intensity values were corrected with suitable blanks, and the data were analyzed according to the Stern-Volmer equation.$^{22}$

$$F_0/F = 1 + K_{SV}[Q] \quad (2)$$

where $F_0$ and $F$ are the fluorescence intensities at the emission maximum in the absence and the presence of a quencher (acrylamide) respectively; $K_{SV}$ is the Stern-Volmer constant, and [Q] is the molar concentration of the quencher (acrylamide). The values of $K_{SV}$ were calculated by least-squares analysis of Stern-Volmer plots, which were obtained by plotting $F_0/F$ versus [Q].

**Preparation of GdnHCl-denatured BLA.** GdnHCl-denatured BLA samples were prepared by dissolving a constant amount of the protein in 10 mL of 20 mM Tris–HCl buffer, pH 7.5, containing a fixed GdnHCl concentration. The solutions were incubated at 25°C for 12 h to achieve equilibrium, and were used in subsequent experiments.

**Denaturation experiments.** All solutions used in the denaturation experiments were prepared in 20 mM Tris–HCl buffer, pH 7.5. For experiments involving calcium, a fixed concentration (2.5, or 10 mM) of CaCl$_2$ was included in all solutions. Various volumes of the buffer were added first to 0.5 mL BLA solution, taken in different tubes. Then increasing volumes of stock (7 mM) GdnHCl solution were added to these tubes in order to achieve the desired GdnHCl concentrations. The final mixture (5.0 mL) was shaken well and incubated overnight at room temperature (about 25°C) to achieve equilibrium before CD, fluorescence, and absorption measurements. The values of MRE, fluorescence intensity, and absorbance were transferred into relative MRE, relative fluorescence intensity, and molar difference extinction coefficient as described earlier,$^{19,23}$ and were plotted against GdnHCl concentration.

**Size exclusion chromatography.** Size exclusion chromatography (SEC) of the various BLA samples was performed on AKTAprime plus (Uppsala, Sweden) equipped with a Sephacryl S-200 HR column (1.0 × 40 cm). The column was equilibrated under various experimental conditions (both in the absence and the presence of GdnHCl and CaCl$_2$) with the following buffers:

Reagent mixture A: 20 mM Tris–HCl buffer, pH 7.5 (Buffer A)
Reagent mixture A$: 20$ mM Tris–HCl buffer, pH 7.5 containing 10 mM CaCl$_2$ (Buffer B)
Reagent mixture B: Buffer A + 3 mM GdnHCl
Reagent mixture B$: Buffer B + 3$ mM GdnHCl
Reagent mixture C: Buffer A + 4 mM GdnHCl
Reagent mixture C$: Buffer B + 4$ mM GdnHCl
Reagent mixture D: Buffer A + 5 mM GdnHCl
Reagent mixture D$: Buffer B + 5$ mM GdnHCl
Reagent mixture E: Buffer A + 6 mM GdnHCl
Reagent mixture E$: Buffer B + 6$ mM GdnHCl

BLA samples (1.25 mg/500 μL) prepared under various conditions (as described above) in the absence and the presence of GdnHCl and 10 mM CaCl$_2$ were injected into the column and eluted at a flow rate of 0.2 mL/min. Each experiment was repeated 2–4 times to check reproducibility. The peak area under each peak in the chromatogram was analyzed by MagicPlot 2.3 software (http://www.magicplot.com).

**Enzymatic activity.** The enzymatic activity of BLA was checked under native and denatured conditions in the absence and the presence of 10 mM CaCl$_2$ by the method of Bernfeld.$^{24}$ The experiments were carried out in activity buffer (20 mM Tris–HCl buffer, pH 7.5) at 25°C using potato starch as substrate. To 0.1 mL of the stock enzyme solution (0.72 μM) taken in three different tubes, 0.4 mL of activity buffer and 0.5 mL of the substrate solution (1% starch, w/v) were mixed and incubated for 3 min at 25°C. The reaction was stopped by the addition of 1.0 mL of 1% (w/v) 3,5-dinitrosalicylic acid, and the tubes were kept in a boiling water bath for 5 min. The absorbance of the colored solution was monitored at 540 nm against an appropriate blank after cooling of the mixture to room temperature and dilution with 10.0 mL of water. The concentration of reducing sugars was determined using the maltose standard curve. Enzyme specific activity was calculated in the way described earlier.$^{18}$
Various denatured/unfolded forms of BLA, used for activity measurements, were prepared by incubating the enzyme with the desired concentrations of GdnHCl in the absence and the presence of 10 mM CaCl$_2$ for 12 h at 25 °C. All the solutions needed for enzyme assay contained the desired concentrations of GdnHCl and CaCl$_2$ (if required). For refolding studies, 6 M GdnHCl-denatured enzyme solution was diluted with the activity buffer (with and without 10 mM CaCl$_2$) to achieve the desired concentrations of GdnHCl, and was incubated overnight at 25 °C before activity measurements. In a separate experiment, 6 M GdnHCl-denatured enzyme solution was dialyzed against the activity buffer (with and without 10 mM CaCl$_2$) for 15 h at 25 °C, followed by activity measurements. The BLA specific activity obtained with different enzyme preparations was transformed into percentage (%), specific activity, taking the specific activity of native BLA to be 100%.

Results and Discussion

Far-UV CD, intrinsic, synchronous, and 3-D fluorescence, the UV difference spectral signal, and enzymatic activity were used as probes to study the effects of various CaCl$_2$ concentrations on the GdnHCl denaturation of BLA. SEC was also employed to monitor protein aggregation and calcium-induced stabilization of BLA.

Far-UV CD

Figure 1 shows the GdnHCl-induced denaturation of BLA as monitored by MRE$_{222\text{nm}}$ measurements in the absence and the presence of various (2, 5, and 10 mM) CaCl$_2$ concentrations. Significant precipitation was noticed in BLA at lower GdnHCl concentrations (<2 M). There was a significant decrease in the MRE$_{222\text{nm}}$ value in a GdnHCl concentration range of 2–3 M, reaching a minimum (about 67% decrease in MRE$_{222\text{nm}}$) at 3 M GdnHCl. The decrease in MRE$_{222\text{nm}}$ is indicative of the disruption of the α-helical structure of BLA, suggesting protein denaturation. However, at higher GdnHCl concentrations (>3 M), significant increases in MRE$_{222\text{nm}}$ were observed upon 6 M GdnHCl, being 34% from 3–6 M GdnHCl. Such increases in MRE$_{222\text{nm}}$ might be due to either refolding in the protein molecule or protein aggregation.14,15 These results are similar to our previously published report19 suggesting that the protein is stabilized at higher GdnHCl concentrations. On the other hand, Strucksberg et al.15 have reported protein aggregation at lower (<1.0 M) GdnHCl concentrations, and complete unfolding of BLA at higher GdnHCl concentrations, based on their MRE results. The main difference between our study and that of Strucksberg et al.15 was the use of MOPS buffer and 2 mM EDTA in the incubation buffer, which must have removed all the intrinsic calcium bound to BLA, leading to Ca-depleted BLA in their study.15

The presence of CaCl$_2$ in the incubation mixture was not only successful in abolishing protein precipitation at lower GdnHCl concentrations, but also minimized the changes in MRE$_{222\text{nm}}$ values induced by GdnHCl. Normalization of MRE$_{222\text{nm}}$ values was more significant at higher CaCl$_2$ concentrations, as only a 4% change in the MRE$_{222\text{nm}}$ value was observed in the presence of 10 mM CaCl$_2$ throughout the range of GdnHCl concentrations.

Intrinsic fluorescence

The GdnHCl denaturation of BLA in the absence and the presence of various CaCl$_2$ concentrations, as monitored by intrinsic fluorescence measurements upon excitation at 280 nm, is shown in Fig. 2A. A significant decrease (52%) in fluorescence intensity was noted at 1 M GdnHCl. This was maintained at 4 M GdnHCl, and showed anomalous behavior thereafter. In a previous study,19 we found a 38% decrease in the fluorescence intensity of BLA at 1.0 M GdnHCl. Such a difference in the percentage decrease in fluorescence intensity can be attributed to the presence of various amounts of bound calcium in the various BLA samples. Within a GdnHCl concentration range of 4–6 M, the decrease in fluorescence intensity became smaller, reaching 29% at 6 M GdnHCl (Fig. 2A), suggesting burial of Tyr and Trp residues in the nonpolar environment. The addition of CaCl$_2$ to the incubation mixture produced significant reversal in fluorescence intensity throughout the GdnHCl concentration range, being more significant at higher CaCl$_2$ concentrations. In the presence of 10 mM CaCl$_2$, the fluorescence signal showed a little variation (about 4%) throughout the GdnHCl concentration range. These results are in accordance with the CD results described above, showing almost complete recovery in the CD spectral signal in the presence of 10 mM CaCl$_2$.

The fluorescence spectrum of BLA also showed a significant shift in the emission maximum upon GdnHCl denaturation, as shown in Fig. 2B. There was a significant red shift (12 nm) in the emission maximum, from 337 to 349 nm, up to a concentration of 3.5 M GdnHCl, which is suggestive of a change in the microenvironments of the Tyr and Trp residues from nonpolar to polar, indicating protein denaturation.19 In the earlier study, we noticed a red shift of 8 nm at 3.5 M GdnHCl.19 Such a difference of 4 nm red shift, can be ascribed to the use of various BLA samples, which might had different amounts of intrinsic calcium bound to the protein. On the other hand, the emission maximum started to normalize with increasing GdnHCl concentrations beyond 3.5 M, reaching 339 nm at 6 M GdnHCl. Such normalization of the emission maximum is suggestive of burial of the Tyr and Trp residues in a nonpolar environment. Both protein refolding and aggregation might account for this change. Contrary to our results, Strucksberg et al.15 found complete unfolding of BLA (calcium-depleted) at a concentration of

![Fig. 1. GdnHCl-Induced Structural Changes in BLA as Studied by MRE Measurements at 222 nm in 20 mM Tris–HCl Buffer, pH 7.5 in the Absence (○) and the Presence of 2 mM (●), 5 mM (▲), and 10 mM (■) CaCl$_2$.](image-url)
1.5 M GdnHCl based on the emission maximum results (a red shift of about 14 nm), and found complete recovery of the emission maximum in the refolding experiments. The presence of intrinsic calcium in our BLA sample appears to be responsible for the requirement of higher GdnHCl concentration (3.5 M) to achieve a similar red shift in the emission maximum. In other words, these results indicate significant stability of BLA against GdnHCl denaturation by bound calcium. Furthermore, the addition of increasing concentrations (2, 5, and 10 mM) of CaCl\textsubscript{2} also normalized the GdnHCl-induced changes in the emission maximum of BLA to the original value, since the presence of 5 mM CaCl\textsubscript{2} was sufficient enough to abolish the spectral perturbations caused by GdnHCl on the emission maximum of BLA (Fig. 2B).

**UV difference spectroscopy**

Figure 3A shows the GdnHCl denaturation results for BLA as monitored by the UV difference spectral signal at 286 nm ($\Delta\varepsilon_{286 \text{ nm}}$). Similarly to the far-UV CD results shown in Fig. 1, the $\Delta\varepsilon_{286 \text{ nm}}$ value of BLA could not be obtained at lower GdnHCl concentrations (<2.0 M) due to significant protein precipitation. The use of high protein concentrations, needed in both far-UV CD as well as UV difference spectral measurements, might account for the significant precipitation at lower GdnHCl concentrations. This is understandable, since no precipitation was observed when denaturation was monitored by fluorescence measurements, which require much lower protein concentrations. UV difference spectral results on the GdnHCl denaturation of BLA showed similar behavior as found for far-UV CD and fluorescence measurements, as the $\Delta\varepsilon_{286 \text{ nm}}$ value showed reversibility or normalization within concentrations of 3–6 M GdnHCl, indicating burial of aromatic chromophores. The addition of 10 mM CaCl\textsubscript{2} to the incubation mixture not only prevented protein precipitation at lower GdnHCl concentrations, but also produced weaker UV difference spectral signals over the whole GdnHCl concentration range, suggesting that the protein is stabilized at 10 mM CaCl\textsubscript{2}. This can be seen clearly in the UV difference spectra of BLA in the presence of 2 M GdnHCl obtained in the absence and the presence of 10 mM CaCl\textsubscript{2} (Fig. 3B). This difference spectrum is characterized by the presence of a shoulder at 280 nm, a negative peak at 286 nm, and another shoulder at 291 nm. The presence of spectral features at 280 nm and 286 nm is suggestive of changes in the environment around the Tyr residues, while the appearance of a negative shoulder at 291 nm indicates environmental perturbation around the Trp residues.\textsuperscript{25} In other

![Fig. 2. GdnHCl-Induced Structural Changes in BLA as Studied by (A) Fluorescence Measurements at 337 nm and (B) Emission Maximum, upon Excitation at 280 nm. The experiments were performed in 20 mM Tris–HCl buffer, pH 7.5, in the absence (○) and the presence of 2 mM (●), 5 mM (▲), and 10 mM (■) CaCl\textsubscript{2}.](image1)

![Fig. 3. (A) GdnHCl-Induced Structural Changes in BLA as Studied by the Molar Difference Extinction Coefficient at 286 nm in 20 mM Tris–HCl Buffer, pH 7.5 in the Absence (○) and the Presence (●) of 10 mM CaCl\textsubscript{2}, and (B) UV Difference Spectra of 2 M GdnHCl-Denatured BLA in the Absence (○) and the Presence (●) of 10 mM CaCl\textsubscript{2}.](image2)
words, the microenvironments of both the Tyr and the Trp residue were significantly perturbed in the presence of 2 M GdnHCl. The amplitude of these signals was significantly reduced in the presence of 10 mM CaCl₂, suggesting that the protein is stabilized.

### Synchronous fluorescence

Synchronous fluorescence spectroscopy is a useful probe to monitor changes in the microenvironment of different fluorophors (Tyr and Trp) under a given set of conditions by simultaneous scanning of both excitation and emission monochromators of a fluorometer, while maintaining a fixed wavelength difference ($\Delta \lambda$) between them. Any shift in the emission maximum indicates a change in polarity around these fluorophors. The change in the microenvironment of the Tyr and Trp residues of a protein can be probed by synchronous fluorescence spectra if the wavelength interval ($\Delta \lambda$) is fixed at 15 nm and at 60 nm respectively.

The synchronous fluorescence spectral results (emission maximum) for native BLA at increasing GdnHCl concentrations in the absence and the presence of 10 mM CaCl₂ are shown in Fig. 4, with $\Delta \lambda$ fixed at 60 nm (A) and 15 nm (B). No significant shift in the emission maximum, attributed to Trp residues, was observed throughout the GdnHCl concentration range (Fig. 4A), whereas the emission maximum due to Tyr residues showed a significant blue shift (7 nm) from 308 nm to 301 nm in the presence of 3 M GdnHCl, followed by normalization at concentrations of 3–6 M GdnHCl. These results suggest significant changes in the microenvironment of the Tyr residues of the protein towards a more nonpolar environment at 3 M GdnHCl. BLA has 30 Tyr residues in the primary sequence, distributed as 21, 6, and 3 in domains A, B, and C respectively (PDB, entry code 1BLI). In view of this, it appears that domain A underwent major structural perturbation at 3 M GdnHCl. Normalization of the emission maximum at concentrations of 3–6 M GdnHCl may be ascribed to reorganization of these residues with respect to the original set-up. These results for higher GdnHCl concentrations (3.0–6.0 M) are in agreement with our CD, fluorescence, and UV difference spectral data, showing normalization in spectral behavior within this range. The presence of 10 mM CaCl₂ in the incubation mixture showed resistance towards any change in the emission maximum throughout the GdnHCl concentration range, suggesting that the protein is stabilized in the presence of 10 mM CaCl₂ against GdnHCl denaturation.

#### Three-dimensional fluorescence

Three-dimensional (3-D) fluorescence spectra provide additional information regarding the fluorescence characteristics of a protein by changing excitation and emission wavelengths simultaneously. Figure 5 shows 3-D fluorescence spectra and corresponding contour maps of native and 3 M GdnHCl-denatured BLAs in the absence and the presence of 10 mM CaCl₂. Two peaks, $a$ and $b$, which are commonly observed in 3-D fluorescence spectra, represent the Rayleigh scattering peak ($\lambda_{ex} = \lambda_{em}$) and second-order scattering peak ($\lambda_{em} = 2\lambda_{ex}$) respectively. Peak 1 mainly revealed the spectral behavior of the Trp and Tyr residues, and originated as a result of the $\pi \rightarrow \pi^*$ transition as the protein was excited at 280 nm. The occurrence of the second fluorescence peak, peak 2, upon excitation at 230 nm, can be ascribed to the $n \rightarrow \pi^*$ transition and represents the polypeptide backbone conformation. These peaks can be seen clearly in the contour maps of BLA under various experimental conditions. Table 1 shows peak positions in terms of $\lambda_{ex}/\lambda_{em}$ and the corresponding fluorescence intensity of each peak. A comparison between Fig. 5A; A' and 5B; B' suggests significant structural alteration in the peptide backbone conformation of BLA in the presence of 3 M GdnHCl, as peak 2 was completely abolished from the 3-D fluorescence spectra. Tang et al. reported the disappearance of the 220/336 nm peak of hemocyanin in the presence of 3.5 M GdnHCl. Furthermore, a red shift of 12 nm in emission maximum of peak 1 along with an approximately 36% decrease in fluorescence intensity was also noted. These results are suggestive of a change in the tertiary structure of the protein, leading to the transfer of the Tyr and the Trp residues from a nonpolar to a polar environment, indicating protein denaturation. The presence of 10 mM CaCl₂ in the incubation mixture restored peak 2 in 3 M GdnHCl-denatured BLA, though the intensity was much lower as compared to native BLA (Fig. 5A; A' and 5C; C'). Furthermore, both the intensity and the emission maximum of peak 1 were also normalized to a greater extent, approaching native BLA characteristics, as values of the intensity and emission maximum increased from 338.52 and 349 nm

![Fig. 4. GdnHCl-Induced Structural Changes in BLA as Studied by Synchronous Fluorescence Spectra Showing Changes in the Emission Maximum in the Absence () and the Presence () of 10 mM CaCl₂. (A) $\Delta \lambda = 60$ nm and (B) $\Delta \lambda = 15$ nm.](image)
for 3 M GdnHCl-denatured BLA to 471.53 and 338 nm in the presence of 10 mM CaCl₂ (Table 1). This reversal in the peak position and intensity of peak 1 and the reappearance of peak 2 are suggestive of 10 mM CaCl₂-induced stabilization of BLA.

### Acrylamide quenching

Structural perturbations in GdnHCl-denatured BLAs were also studied in the absence and the presence of various CaCl₂ concentrations by acrylamide quenching experiments. Figure 6 shows Stern-Volmer plots for native and GdnHCl-denatured BLAs in the absence and the presence of 2 mM and 10 mM CaCl₂. Acrylamide quenching data for NATA are also included as a reference for complete exposure of Trp residues. The values of the Stern-Volmer constant, K_{SV}, were obtained from the slope of these plots (Table 2). A comparison of the K_{SV} value obtained with native BLA (2.2 M⁻¹) to

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**Table 1.** Three-Dimensional Fluorescence Spectral Characteristics of Native and 3 M GdnHCl-Denatured BLAs in the Absence and the Presence of 10 mM CaCl₂ as Studied in 20 mM Tris-HCl Buffer, pH 7.5

<table>
<thead>
<tr>
<th>System</th>
<th>Peak no.</th>
<th>Peak position [(\lambda_{em}/\lambda_{ex}) (nm/nm)]</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native BLA</td>
<td>a</td>
<td>250/250→350/350</td>
<td>76.59→163.55</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>250/500</td>
<td>146.23</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>280/337</td>
<td>531.91</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>230/336</td>
<td>739.88</td>
</tr>
<tr>
<td>3 M GdnHCl-denatured BLA (U)</td>
<td>a</td>
<td>250/250→350/350</td>
<td>205.49→246.58</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>250/500</td>
<td>333.67</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>280/349</td>
<td>338.52</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>250/500</td>
<td>328.70</td>
</tr>
<tr>
<td>(U) + 10 mM CaCl₂</td>
<td>a</td>
<td>250/250→350/350</td>
<td>203.09→239.86</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>250/500</td>
<td>471.53</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>280/338</td>
<td>45.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>230/337</td>
<td>471.53</td>
</tr>
</tbody>
</table>

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**Fig. 5.** Three-Dimensional Fluorescence Spectra and Corresponding Contour Maps of Native and 3 M GdnHCl-Denatured BLAs in the Absence and the Presence of 10 mM CaCl₂ as Studied in 20 mM Tris–HCl Buffer, pH 7.5, under Different Conditions.

A, A': native BLA; B, B': BLA + 3 M GdnHCl; C, C': BLA + 3 M GdnHCl + 10 mM CaCl₂.
that with NATA (17.0 m⁻¹) suggests significant burial of Trp residues in native BLA. This is also evident from the increase in the $K_{SV}$ value from 2.2 m⁻¹ for native BLA to 6.0 m⁻¹ for 3M GdnHCl-denatured BLA, suggesting that the Trp residues are exposed to polar environment in 3M GdnHCl-denatured BLA. Contrary to this, the $K_{SV}$ value, obtained with 6M GdnHCl-denatured BLA, was found to be lower than that of 3M GdnHCl-denatured BLA but higher than that of native BLA. Although the $K_{SV}$ values obtained with both 3M and 6M GdnHCl-denatured BLAs are significantly higher than that of native BLA and suggest protein denaturation, the lower $K_{SV}$ value obtained with 6M GdnHCl-denatured BLA may be ascribed to greater burial of Trp residues at higher GdnHCl concentrations. The presence of CaCl₂ in both the 3M and the 6M GdnHCl-denatured BLA led to significant decreases in $K_{SV}$ values, which were found to be higher with 10mM CaCl₂ than with 2mM CaCl₂ (Table 2). This decrease in $K_{SV}$ values indicate protein stabilization by CaCl₂ as the values approached that obtained with native BLA.

**Size exclusion chromatography**

In order to check aggregation and calcium-induced stabilization in different BLA preparations, size exclusion chromatography (SEC) of the samples was performed under various experimental conditions. Elution profiles of native and GdnHCl-denatured BLAs on Sephacryl S-200 HR column (1.0 x 40 cm) equilibrated with 20 mM Tris–HCl Buffer, pH 7.5, under different conditions.

**Table 2.** $K_{SV}$ Values Obtained from Acrylamide Quenching of Native and GdnHCl-Denatured BLAs, Studied in 20 mM Tris–HCl Buffer, pH 7.5, under Various Conditions

<table>
<thead>
<tr>
<th>Protein samples</th>
<th>$K_{SV}$ (m⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native BLA</td>
<td>2.2</td>
</tr>
<tr>
<td>3M GdnHCl-denatured BLA (U)</td>
<td>6.0</td>
</tr>
<tr>
<td>(U) + 2mM CaCl₂</td>
<td>3.9</td>
</tr>
<tr>
<td>(U) + 10mM CaCl₂</td>
<td>2.8</td>
</tr>
<tr>
<td>6M GdnHCl-denatured BLA (U')</td>
<td>4.5</td>
</tr>
<tr>
<td>(U') + 2mM CaCl₂</td>
<td>3.6</td>
</tr>
<tr>
<td>(U') + 10mM CaCl₂</td>
<td>2.6</td>
</tr>
<tr>
<td>NATA</td>
<td>17.0</td>
</tr>
</tbody>
</table>

Fig. 6. Stern-Volmer Plots of Acrylamide Quenching of Native and GdnHCl-Denatured BLAs as Studied in 20 mM Tris–HCl Buffer, pH 7.5, in the Absence and the Presence of CaCl₂. NATA (○), BLA (■), 3M GdnHCl-denatured BLA in the absence (●—●) and the presence of 2mM (●—●) and 10mM (●—●) CaCl₂, 6M GdnHCl-denatured BLA in the absence (▲—▲) and the presence of 2mM CaCl₂ (▲—▲) and 10mM CaCl₂ (▲—▲).

Fig. 7. Elution Profiles of Native and GdnHCl-Denatured BLAs in the Absence and the Presence of 10 mM CaCl₂ on a Sephacryl S-200 HR Column (1.0 x 40 cm) Equilibrated with 20 mM Tris–HCl Buffer, pH 7.5. A: native BLA, A': BLA + 10mM CaCl₂; B: BLA + 3M GdnHCl, B': BLA + 3M GdnHCl + 10mM CaCl₂; C: BLA + 4M GdnHCl, C': BLA + 4M GdnHCl + 10mM CaCl₂; D: BLA + 5M GdnHCl, D': BLA + 5M GdnHCl + 10mM CaCl₂; and E: BLA + 6M GdnHCl, E': BLA + 6M GdnHCl + 10mM CaCl₂. A sample size (1.25 mg/500 μL) was injected into the column, and elution was performed at a flow rate of 0.2 mL/min.
molecule. This is plausible, as GdnHCl is known to remove all non-covalent interactions in proteins. The appearance of a shoulder (marked ‘1’ in Fig. 7B) near the void volume (Ve) of the column (marked by an arrow) indicates the formation of protein aggregates in the presence of 3 M GdnHCl. In an earlier study, Strucksberg et al. confirmed the presence of aggregates of BLA (calcium-depleted), but at the lower (<1.0 M) GdnHCl concentrations, based on dynamic light scattering experiments. The distribution of the protein sample as the third component (25%) might represent retardation of a few GdnHCl-denatured BLA molecules due to interaction between BLA and the gel. Interaction of BLA with a gel was also suggested previously.

Figure 7B’ shows the elution profile of 3 M GdnHCl-denatured BLA in presence of 10 mM CaCl2. The presence of 10 mM CaCl2 in both the sample and the equilibrating buffer significantly affected the elution behavior of 3 M GdnHCl-denatured BLA, as is evident from the reduction of the shoulder and the increase in the area of peak 2 (Fig. 7B’). More specifically, the Ve of peak 2 (Fig. 7B’) slightly increased, from 12.67 mL (Ve/Vo = 1.31) to 13.38 mL (Ve/Vo = 1.36) in presence of 10 mM CaCl2 (Table 3). Furthermore, area of peak 2 increased from 57% to 67% (Table 3). The area of the peak 1 decreased greatly, from 20% to 9% (Table 3) in presence of 10 mM CaCl2. The peak ratio (peak 1/peak 2) calculation also showed a significant reduction, from 0.35 to 0.13, due to the reduced area of the shoulder (peak 1). All these characteristics, a significant reduction in the area of peak 1, an increase in the area of peak 2, and a slight increase in the Ve of peak 2 clearly suggest calcium-induced stabilization of BLA, which not only reduced aggregation but also made the protein stable against GdnHCl denaturation.

GdnHCl-induced aggregation in BLA was more evident when the protein was treated with 4–6 M GdnHCl (Fig. 7C–E). The elution profiles showed a prominent peak 1, with the elution volume falling to about 10.03–10.28 mL, which corresponds to a Ve/Vo value of 1.03–1.06 (Table 3). Based on the Ve/Vo value (about 1.0), peak 1 appears to represent BLA aggregates. This was confirmed by the significant reduction in peak 2, which represented GdnHCl-denatured BLA. Peak area calculations indicated the presence of 45–49% aggregates (peak 1) as compared to 35–42% GdnHCl-denatured BLA (peak 2) (Fig. 7C–E, Table 3). The presence of 10 mM CaCl2 in the incubation mixture markedly reduced the area under peak 1, from 45–49% to 25–33%, with a concomitant increase in the area of peak 2 from 35–42% to 53–61% (Fig. 7C’–E, Table 3). Furthermore, a significant reduction in the peak ratio in presence of 10 mM CaCl2 (Table 3) also confirmed lesser aggregation in GdnHCl-denatured BLA. These results clearly indicate that the presence of 10 mM CaCl2 significantly reduced the aggregation of BLA against GdnHCl denaturation.

### Enzymatic activity
Calcium-induced structural stabilization of BLA was also studied by measuring the enzymatic activity of native and GdnHCl-denatured BLA preparations in the absence and presence of 10 mM CaCl2. Figure 8A and B shows the enzymatic activity results obtained with native and denatured BLAs (3, 4, 5, and 6 M GdnHCl-denatured) in the absence and presence of 10 mM CaCl2 respectively. As is evident from the figure, about 91–96% activity was lost in all the GdnHCl-denatured BLA preparations in the absence of 10 mM CaCl2. On the other hand, the presence of 10 mM CaCl2 in the incubation mixture provided significant structural stabilization, as reflected by 41% remaining enzymatic activity in the 3 M GdnHCl-denatured BLA (Fig. 8B). Even 6 M GdnHCl-denatured BLA showed 26% remaining enzymatic activity in presence of 10 mM CaCl2. These results accord with an earlier report showing significant retention of enzymatic activity in GdnHCl-denatured BLAs in presence of 2 mM CaCl2. Quantitative differences in the magnitude of enzymatic activity found in the previous report and the present data can be ascribed to the use of different CaCl2 concentrations and to activity buffers. A recent report showing inhibition of enzymatic activity of α-amylase at higher CaCl2 concentrations (>2.5 mM) further confirms the decreased enzymatic activity in the present results. These results are in accordance with our SEC data (Fig. 7), as aggregation of the enzyme might account for the loss of activity.

### Table 3: Elution Characteristics of Native and GdnHCl-Denatured BLAs on a Sephacryl S-200 HR Column (1.0 × 40 cm) Equilibrated with 20 mM Tris–HCl Buffer, pH 7.5, under Various Conditions

<table>
<thead>
<tr>
<th>Protein samples</th>
<th>[CaCl2] = 0 mM</th>
<th>[CaCl2] = 10 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample ID</td>
<td>Peak no.</td>
</tr>
<tr>
<td>Native BLA</td>
<td>A</td>
<td>1</td>
</tr>
<tr>
<td>3 M GdnHCl-</td>
<td>B</td>
<td>1</td>
</tr>
<tr>
<td>denatured BLA</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>4 M GdnHCl-</td>
<td>C</td>
<td>1</td>
</tr>
<tr>
<td>denatured BLA</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>5 M GdnHCl-</td>
<td>D</td>
<td>1</td>
</tr>
<tr>
<td>denatured BLA</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>6 M GdnHCl-</td>
<td>E</td>
<td>1</td>
</tr>
<tr>
<td>denatured BLA</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
</tr>
</tbody>
</table>
enzymatic activity in the GdnHCl-denatured BLAs in the absence of calcium. Since 10 mM CaCl₂ was found to be effective in reducing aggregation in the GdnHCl-denatured BLAs (Fig. 7), significant retention of enzymatic activity in the presence of 10 mM CaCl₂, observed in these BLA preparations (Fig. 8B), is plausible in terms of stabilization of the tertiary structure of the enzyme.

Partial refolding experiments involving dilution of 6 M GdnHCl-denatured BLA showed no further improvement in enzyme stabilization. Rather a decrease in enzymatic activity was observed for the partially refolded enzyme preparations as compared to their counterparts, prepared by direct addition of GdnHCl. Whereas no significant enzymatic activity was observed with the partially refolded enzyme preparations in the absence of 10 mM CaCl₂, about 17–23% remaining enzymatic activity was found with the same enzyme preparations in the presence of 10 mM CaCl₂ (Fig. 8A and B). Prolonged incubation of these preparations under denaturing conditions at 25 °C might account for the reduced enzymatic activity of these preparations, since the activity of 6 M GdnHCl-denatured BLA also decreased by 9% (Fig. 8B). Refolding experiments involving the dialysis of 6 M GdnHCl-denatured BLA against 20 mM Tris–HCl buffer, pH 7.5 (with and without 10 mM CaCl₂) showed significant refolding of the enzyme as about 35% activity was recovered in the absence of 10 mM CaCl₂ as compared to 84% enzymatic activity with the dialyzed preparation in the presence of 10 mM CaCl₂. This is plausible, as aggregation of the enzyme in the absence of 10 mM CaCl₂ might be responsible for the reduced enzymatic activity.

Although the results described above showed significant stabilization of BLA in the presence of 10 mM CaCl₂, it was far from completion as indicated by the spectral results. The absence of any significant change in CD and fluorescence signals with increasing GdnHCl concentrations in presence of 10 mM CaCl₂ should not be considered proof of complete stabilization of the enzyme, which is rather to be attributed to protein aggregation. Therefore, these spectral data should be treated with caution in reference to protein stabilization.

Taken together, all these results suggest significant aggregation of BLA at higher GdnHCl concentrations, which affected spectral features in such a way as to suggest protein stabilization. Therefore, spectral results in the aggregation system should be treated with caution. Furthermore, the presence of CaCl₂ not only prevented aggregation in BLA, but also stabilized BLA against GdnHCl denaturation.

**Acknowledgments**

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**References**