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Full Length Research Paper

Calcium-induced stabilization of α-amylase against guanidine hydrochloride denaturation

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Guanidine hydrochloride (GdnHCl) denaturation of native and Ca-depleted *Bacillus licheniformis* α-amylase (BLA) was investigated both in the absence and presence of 2 mM calcium chloride ($CaCl_2$) using circular dichroism, fluorescence spectroscopy and biological activity. In both states (Ca-depleted and native form), the protein was denatured to a considerable extent in the absence of 2 mM $CaCl_2$ with concomitant loss of biological activity upon increasing GdnHCl concentration. On the other hand, this effect was significantly reduced when 2 mM $CaCl_2$ was included in the incubation mixture as revealed by a higher relative mean residue ellipticity, higher relative fluorescence intensity, smaller change in emission maximum and lesser reduction in biological activity. Interestingly, using these probes, 2 mM $CaCl_2$ seemed to offer the same degree of stability to Ca-depleted BLA as that observed with native BLA in the absence of 2 mM $CaCl_2$. All these results suggest calcium-induced stabilization of BLA against GdnHCl denaturation.

Key words: α-Amylase, *Bacillus licheniformis*, calcium, denaturation, guanidine hydrochloride, circular dichroism, intrinsic fluorescence.

INTRODUCTION

Enzymes catalytic efficiency is much higher than ordinary inorganic catalysts used in industries, which makes them a suitable substitute in industrial processes. However, there is a major drawback of enzyme stability that limits their application after repeated usage in industries and upon storage. Thus, it is of utmost importance to explore avenues which help in maintaining the stability of these enzymes. α-Amylases are extensively used in industries and have carved their niche in present day biotechnology. Their commercial applications include production of maltodextrins, baking, brewing, alcohol, detergent and textile industries (Pandey et al., 2000; Ammar et al., 2002; Varavinita et al., 2002; Bessler et al., 2003; Nagarajan et al., 2006). Whilst α-amylases have been isolated from various origins such as fungal, bacterial, plant and animal sources (Darnis et al., 1999; Teotia and Gupta, 2001; Jensen et al., 2003; Wanderley et al., 2004; Hmidet et al., 2008; Kiran and Chandra, 2008; Tripathi et al., 2008), the well-studied α-amylases are those from the *Bacillus* genus (Zhi et al., 2004; Liu and Xu, 2008; Liu et al., 2008). In particular, α-amylase from *Bacillus licheniformis* (BLA) has fomented researchers due to its unusual thermostability (T_m~103°C) (Fitter and Haber-Pohlmeier, 2004) while being present in a mesophilic organism (Duy and Fitter, 2005) and curiosity to unravel the mechanism of its inactivation. This hyperthermostable property of the enzyme has been highly exploited in the industry involving frequent use of temperature above 100°C such as starch processing industry (Varavinita et al., 2002; Bessler et al., 2003). BLA is a single polypeptide

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Abbreviations: BLA, *Bacillus licheniformis* α-amylase; **GdnHCl**, guanidine hydrochloride; **$CaCl_2$**, calcium chloride; **MRE**, mean residue ellipticity; **EGTA,** ethylene glycol-bis (2-aminoethyl-ether)-N, N, N', N'-tetraacetic acid; **CD,** circular dichroism.

chain of 483 amino acid residues and comprised of three domains with three calcium binding sites, two of which are situated on domain B, while the third is found at the interface of domains A and C as revealed by its crystal structure (Machius et al., 1998).

In view of the high resistance offered by thermostable proteins against chemical denaturants (Griffin et al., 2003), it would be of interest to study the denaturation behaviour of BLA against chemical denaturants. A comparison between heat-denatured and 8.0 M GdnHCl-denatured states of α-amylases from BLA and *Bacillus amyloliquefaciens* has shown that thermally unfolded structures are more compact than fully denatured states induced by GdnHCl. Furthermore, removal of calcium from its specific binding sites on the enzyme has also been shown to reduce its melting temperature tremendously (Fitter and Haber-Pohlmeier, 2004) and the enzyme remains unfolded even at 3.0 M urea in renaturation experiments (Nazmi et al., 2006). Although calcium has been shown to provide stability against thermal and urea denaturation, no attempt has been made to study the effect of calcium on GdnHCl denaturation of BLA. Here, we report the effect of calcium on GdnHCl denaturation of native and Ca-depleted BLA using circular dichroism, fluorescence spectroscopy and biological activity.

MATERIALS AND METHODS

Materials

α-Amylase from BLA (Lot 018K7008), 3,5-dinitrosalicylic acid, guanidine hydrochloride (GdnHCl) (≥99% pure) and ethylene glycol-bis (2-aminoethyl-ether)-N, N, N', N'-tetraacetic acid (EGTA) were purchased from Sigma Chemical Co., USA. Maltose and starch from potato were procured from R & M Chemicals, UK and Fluka BioChemika, Germany, respectively. Tris base was obtained from AMRESCO®, USA, while calcium chloride ($CaCl_2$) was the product of SYSTERM®, Malaysia. All other chemicals used were of analytical grade purity.

Analytical procedures

Protein concentration was determined spectrophotometrically on a Shimadzu double-beam spectrophotometer, model UV-2450, using a molar extinction coefficient of 139,690 $M^{-1}cm^{-1}$ at 280 nm (Nazmi et al., 2006). The concentration of stock GdnHCl solution was determined from the data of Nozaki (1972) as described by Pace et al. (1989).

Preparation of Ca-depleted BLA

To obtain Ca-depleted BLA, native BLA (commercial preparation) was dissolved and dialyzed against 40 mM Tris / 20 mM EGTA buffer, pH 7.2 overnight with a minimum of three changes with the same buffer (Nazmi et al., 2006). This step was followed by the removal of EGTA from the sample by dialysis against 20 mM Tris-HCl buffer, pH 7.5. All steps were carried out at 4°C and plastic vessels were used instead of glass to avoid Ca^{2+} contamination.

Circular dichroism

Circular dichroism (CD) measurements in the far-UV region (200 - 250 nm) were recorded on a Jasco J-810 spectropolarimeter equipped with a thermostatically controlled cell holder under constant nitrogen flow. The instrument was calibrated with (+)-10-camphorsulfonic acid and the response time was 1 sec with a scan speed of 100 nm/min. The spectra were recorded in a 0.2 cm path length cell with a protein concentration of 0.1 mg/ml, averaged over three scans and corrected with suitable blanks. Results are expressed as mean residue ellipticity (MRE) in $deg.cm^2.dmol^{-1}$ using the relation:

$$MRE = \theta_{obs} \times (MRW / 10 \times l \times c)$$

where θ_{obs} is the measured ellipticity at 222 nm in millidegrees; l is the optical path length of the cell in cm; c is the protein concentration in mg/ml and MRW is the mean residue weight (molecular weight of the protein divided by the total number of amino acid residues).

Fluorescence spectroscopy

Intrinsic fluorescence was measured on a Hitachi fluorescence spectrophotometer, model F-2500 using a quartz cuvette of 1 cm path length. The protein concentration used was 0.1 µM. The slits were fixed at 10 nm each for excitation and emission spectra and excitation wavelength of 280 nm was used to excite both tyrosine and tryptophan residues. Fluorescence spectra were recorded in the wavelength range of 300 – 400 nm.

Denaturation studies

Denaturation experiments were carried out in the same way as described earlier (Muzammil et al., 2000). All solutions for denaturation experiments were prepared in 20 mM Tris-HCl buffer, pH 7.5. Different volumes of buffer were added first to BLA solution (final concentration: 0.1 mg/ml for CD and 0.1 µM for fluorescence measurements) taken in different tubes followed by the addition of different volumes of stock (6.7 M) GdnHCl solution to obtain the desired concentration of denaturant. The tubes containing final solution mixture (5.0 ml) were incubated overnight at room temperature (25°C) before CD / fluorescence measurements. For experiments involving calcium, 2 mM $CaCl_2$ was included in all solutions. Results are expressed as relative MRE by taking the MRE of BLA in the absence of GdnHCl as 100%. Similarly, values of fluorescence intensity were transformed into relative fluorescence intensity by taking the fluorescence intensity of BLA in the absence of GdnHCl as 100% at its emission maximum. The data were plotted as relative MRE / relative fluorescence intensity versus GdnHCl concentration.

α-Amylase assay

Enzymatic assay was performed according to the method of Bernfeld (1951) in 20 mM sodium phosphate buffer, pH 6.9 containing 6 mM sodium chloride at 25°C using starch as the substrate. The reaction mixture containing 0.1 ml of enzyme solution (44 µg/ml), 0.4 ml of activity buffer and 0.5 ml of starch solution (10

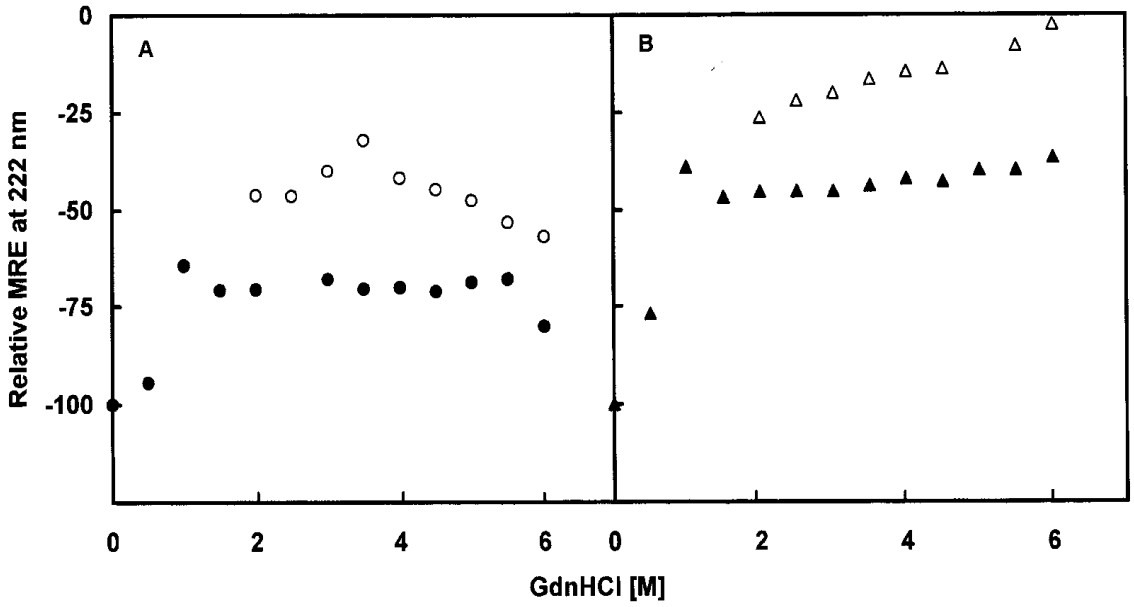

Figure 1. GdnHCl denaturation of BLA in 0.02 M Tris-HCl buffer, pH 7.5 in the absence (○/Δ) and presence (●/▲) of 2 mM $CaCl_2$ as followed by MRE measurements at 222 nm. (A) CD data of native BLA were transformed into relative MRE as described in Materials and Methods and plotted against GdnHCl concentration. (B) Relative MRE of Ca-depleted BLA plotted against GdnHCl concentration.

mg/ml) was incubated for 3 min at 25°C followed by the addition of 1.0 ml of 1% (w/v) 3, 5-dinitrosalicylic acid. The tubes were subsequently transferred to a boiling water bath for 5 min incubation, cooled to room temperature and diluted with 10.0 ml of water. Absorbance was read at 540 nm against appropriate blank to measure the concentration of reducing sugars produced using maltose standard curve. Specific activity of BLA was calculated using the formula:

$$\text{Specific activity of BLA (Unit mg}^{-1}) = \frac{\text{Maltose released (µmoles)}}{\text{Enzyme in reaction mixture (mg)} \times 3 \text{ min}}$$

Enzymatic activity was also measured in the presence of different GdnHCl concentrations. For experiments involving GdnHCl with or without calcium, enzyme solution was incubated with desired concentrations of GdnHCl with or without 2 mM $CaCl_2$ overnight. Specific activity of BLA at different GdnHCl concentrations was expressed as percentage (%) specific activity by taking the activity of the BLA in the absence of GdnHCl as 100%.

RESULTS AND DISCUSSION

GdnHCl denaturation of native and Ca-depleted BLA was studied both in the absence and presence of 2 mM $CaCl_2$ using MRE, intrinsic fluorescence and biological activity as probes.

Circular dichroism

Denaturation of native and Ca-depleted BLA was followed by ellipticity measurements at 222 nm with increasing GdnHCl concentrations both in the absence and presence of 2 mM $CaCl_2$. Ellipticity values were transformed into relative MRE at 222 nm as described in 'Materials and Methods' and plotted against GdnHCl concentration (Figure 1A and B). As can be seen from Figure 1A, native BLA showed a significant decrease in MRE (54%) at 2.0 M GdnHCl compared to native protein. It should be noted that MRE data could not be collected at GdnHCl concentrations < 2.0 M due to significant precipitation. A further decrease of 14% in MRE was noted in the GdnHCl concentration range, 2.0 – 3.5 M. Decrease in MRE was indicative of the reduction in the α-helical content of native BLA suggesting denaturation of the protein. Similar decrease in MRE with increasing GdnHCl concentrations has been reported for a number of proteins (Lai et al., 1997; Kumar et al., 2005; Usha and Ramasami, 2008). However, beyond 3.5 M GdnHCl, a gradual increase of 25% in MRE was noted up to 6.0 M GdnHCl. Although increase in MRE value within this range suggested some reversal in helical content, nevertheless, MRE value observed at 6.0 M GdnHCl was still much lesser (43%) when compared to the MRE value of native BLA, suggesting protein denaturation. Anomalous behavior of GdnHCl on protein structure has been described for a number of proteins (Hagihara et al., 1993; Mayr and Schmid, 1993) where guanidinium ions were suggested to stabilize native protein conformation at lower concentrations. However, we observed slight refolding at higher GdnHCl concentrations. It seems probable that guanidinium ions might have replaced intrinsic calcium in the calcium binding site of BLA and provided

greater stability in that region, which may account for the increase in helical content. Furthermore, chloride binding site is also located in the vicinity of calcium binding site and active site of BLA (Machius et al., 1995). Interestingly, addition of 2 mM $CaCl_2$ in the incubation mixture resulted in an increase in MRE values at all the GdnHCl concentrations used. Furthermore, MRE values remained constant within the GdnHCl concentration range: 1.5 – 5.5 M which was 34% lesser than the MRE observed with native protein. In other words, presence of 2 mM $CaCl_2$ offered a greater stability to native BLA against GdnHCl denaturation throughout the concentration range used.

Native BLA does not represent a well-defined state of the protein as it might be partially saturated with calcium. In view of this, denaturation profile observed with native BLA in the absence of calcium as shown in Figure 1A may not represent the actual denaturation profile of the pure protein. Therefore, native commercial preparation was treated with 20 mM EGTA to remove bound calcium from the protein in order to obtain Ca-depleted BLA. Figure 1B shows GdnHCl denaturation profile of Ca-depleted BLA both in the absence and presence of 2 mM $CaCl_2$. Ca-depleted BLA also showed precipitation in the lower GdnHCl concentration range that is, < 2.0 M. Therefore, denaturation was studied in the GdnHCl concentration range of 2.0 – 6.0 M in the absence of 2 mM $CaCl_2$. A marked reduction in MRE (74%) was observed at 2.0 M GdnHCl compared to the value obtained in its absence. This decrease was significantly higher than the decrease (54%) observed with native BLA (Figure 1A). There was a gradual decrease in MRE from 26% at 2.0 M GdnHCl to 2% at 6.0 M GdnHCl (Figure 1B). This again indicates gradual loss of α-helical content in Ca-depleted BLA in the above mentioned range of GdnHCl. However, this loss was more pronounced as compared to native BLA (Figure 1A) in which the intrinsic calcium offered certain degree of stability to the protein against GdnHCl denaturation. Complete unfolding of BLA at 6.0 M GdnHCl has also been observed earlier (Strucksberg et al., 2007). Presence of 2 mM $CaCl_2$ in the incubation mixture produced significant reversal in MRE values which was more or less the same as reflected from the similar values of MRE within GdnHCl concentration range, 1.5 - 6.0 M. A comparison of the results obtained with native and Ca-depleted BLAs shown in Figure 1A and B suggested that native BLA has partially bound calcium which provided some stability since Ca-depleted BLA showed approximately total loss in MRE (98%) at 6.0 M GdnHCl. Furthermore, values of MRE observed with Ca-depleted BLA in presence of 2 mM $CaCl_2$ were found comparable to those observed with native BLA in the absence of 2 mM $CaCl_2$. This signifies that addition of 2 mM $CaCl_2$ to Ca-depleted BLA was sufficient to confer same degree of stability to that exhibited by intrinsic calcium present in native BLA. Irrespective of the values of MRE obtained in presence of 2 mM $CaCl_2$ with both native and Ca-depleted BLAs, both preparations showed significant reversal in MRE at all the GdnHCl concentrations used. This unequivocally showed the stability conferred by 2 mM $CaCl_2$ to BLA against GdnHCl denaturation.

Intrinsic fluorescence

In proteins, there are only three intrinsic fluorophores, namely tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) with larger to smaller quantum yield respectively. In general, studies involving Trp fluorescence are practiced due to low quantum yield of Phe and quenching of Tyr fluorescence by neighboring Trp residues. Being a class B protein (Weber, 1960) with multiple Trp residues, fluorescence emission in BLA is solely attributed to Trp residues. Hence, the effect of calcium on GdnHCl-induced denaturation of BLA was studied by detecting fluorescence signals upon excitation at 280 nm.

Figure 2A shows the effect of increasing GdnHCl concentrations on the relative fluorescence intensity of native BLA at 336 nm in the absence and presence of 2 mM $CaCl_2$ when excited at 280 nm. As can be seen from the Figure 2A, a significant decrease (~ 38%) in fluorescence intensity of native BLA was observed up to 1.0 M GdnHCl in the absence of 2 mM $CaCl_2$. This decrease in fluorescence intensity remained constant up to 4.0 M GdnHCl. Increasing GdnHCl concentrations beyond 4.0 M led to a gradual increase in fluorescence intensity reaching 88% at 6.0 M GdnHCl. On the other hand, fluorescence intensity signal showed smaller variation (~7%) in the presence of 2 mM $CaCl_2$ throughout the concentration range of GdnHCl used (Figure 2A). In other words, decrease in fluorescence intensity observed in the absence of 2 mM $CaCl_2$ was almost abolished upon addition of 2 mM $CaCl_2$.

GdnHCl denaturation of Ca-depleted BLA both in the absence and presence of 2 mM $CaCl_2$ as monitored by fluorescence intensity at 338 nm is shown in Figure 2B. A drastic decrease in relative fluorescence intensity (57%) was observed at 1.0 M GdnHCl followed by a slow increase in fluorescence intensity reaching to a value of 63% at 6.0 M GdnHCl. Addition of 2 mM $CaCl_2$ in the incubation mixture stabilized BLA against GdnHCl denaturation as revealed by the higher fluorescence intensity compared to those observed in the absence of 2 mM $CaCl_2$ throughout the GdnHCl concentration range. Specifically, the reversal in fluorescence intensity was more pronounced (34%) at 0.5 M GdnHCl followed by 15 – 20% at higher GdnHCl concentrations. Surprisingly, this pattern was similar to the denaturation pattern obtained with native BLA but in the absence of 2 mM $CaCl_2$ suggesting similar degree of stability conferred by intrinsic calcium in native BLA.

In addition to the change in relative fluorescence

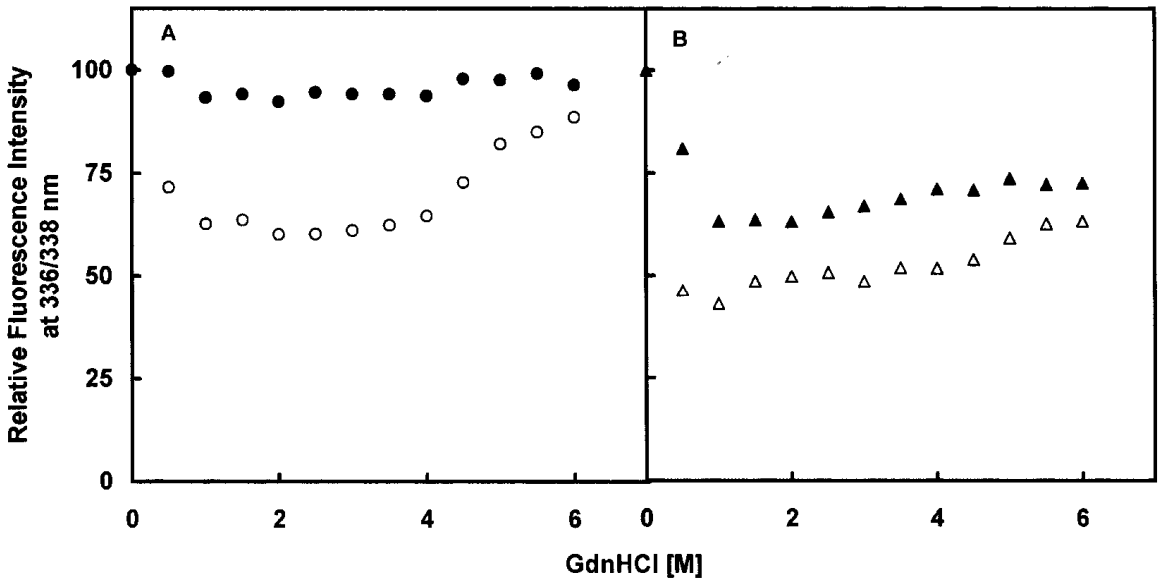

Figure 2. GdnHCl denaturation of BLA in 0.02 M Tris-HCl buffer, pH 7.5 in the absence (○/Δ) and presence (●/▲) of 2 mM $CaCl_2$ as followed by fluorescence measurements at 336/338 nm upon excitation at 280 nm. (A) Fluorescence intensity data of native BLA were transformed into relative fluorescence intensity as described in Materials and Methods and plotted against GdnHCl concentration. (B) Relative fluorescence intensity of Ca-depleted BLA plotted against GdnHCl concentration.

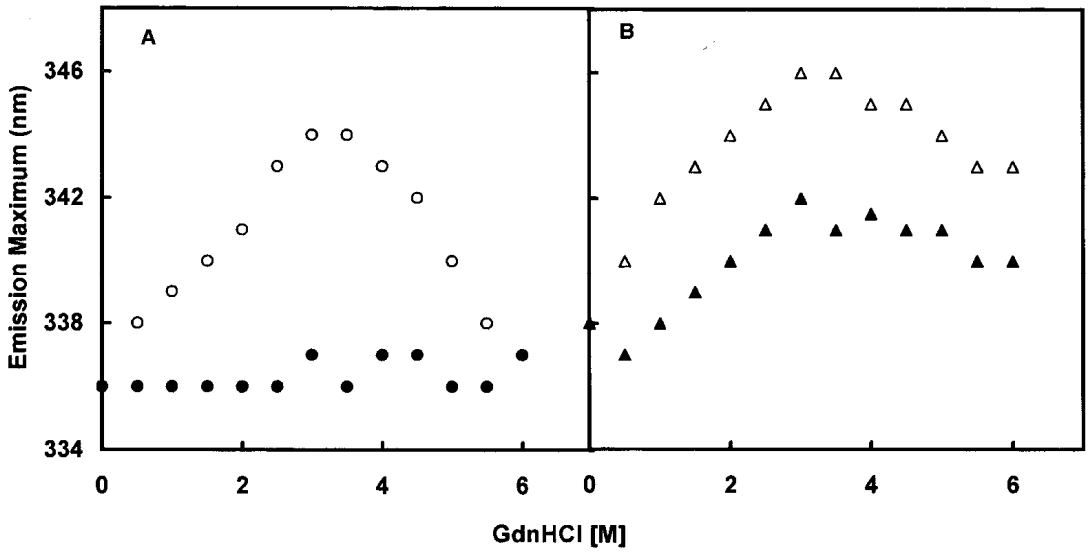

Figure 3. GdnHCl denaturation of BLA in 0.02 M Tris-HCl buffer, pH 7.5 in the absence (○/Δ) and presence (●/▲) of 2 mM $CaCl_2$ as followed by change in emission maximum upon excitation at 280 nm. (A) Variation in emission maximum of native BLA plotted against GdnHCl concentration. (B) Plot showing variation in emission maximum of Ca-depleted BLA against GdnHCl concentration.

intensity observed with native BLA in the absence of 2 mM $CaCl_2$, emission maximum (336 nm) was also shifted (8 nm) towards higher wavelength (red shift) up to 3.5 M GdnHCl and reverted back to lower wavelength above 3.5 M GdnHCl (Figure 3A). At 6.0 M GdnHCl, emission maximum of native BLA was found to be at 337 nm (Figure 3A). Both increase in fluorescence intensity and normalization of the red shift in emission maximum at higher GdnHCl concentrations may be explained with the similar phenomenon of guanidinium ion-induced stabiliza-

tion of calcium binding site. Furthermore, the calcium binding segment encompassing residues 182 - 192 also contains two Trp residues (Declerck et al., 2002) whose environment might have been affected by the binding of guanidinium ions and led to the reversal in the shift of emission maximum. Presence of 2 mM $CaCl_2$ in the reaction mixture abolished this effect as emission maximum remained indifferent throughout the GdnHCl concentration range (Figure 3A). This behavior of shift in emission maximum in the absence of 2 mM $CaCl_2$ and its abolishment upon addition of 2 mM $CaCl_2$ was similar to the pattern of relative fluorescence intensity signal shown in Figure 2A. The shift in emission maximum was also observed with Ca-depleted BLA upon GdnHCl denaturation (Figure 3B). Similar red shift of 8 nm was observed at 3.5 M GdnHCl which was reduced to 5 nm at 6.0 M GdnHCl. Addition of 2 mM $CaCl_2$ though significantly reduced the extent of red shift at all GdnHCl concentrations but failed to abolish the shift in emission maximum completely as observed with native BLA in presence of 2 mM $CaCl_2$ (Figure 3A).

BLA has been reported to contain 17 Trp residues, all of which are much more buried in the protein interior (Duy and Fitter, 2006). Fluorescence characteristics (fluorescence intensity and emission maximum) of a protein are sensitive to the environment around fluorophores. Decrease in fluorescence intensity and red shift in emission maximum can be ascribed to either the exposure of Trp residues to the solvent or change in microenvironment around Trp residues from nonpolar to polar (Svensson et al., 2003; Duy and Fitter, 2006). Therefore, it seems plausible to assume that decrease in relative fluorescence intensity up to 1.0 M GdnHCl concentration was due to the exposure of buried Trp residues to the solvent, whereas increase in fluorescence intensity above 1.0 M GdnHCl may be accounted for the formation of small hydrophobic loop-like structures surrounding Trp residues. This can be justified from the primary sequence of BLA where most of the neighboring residues around Trp are hydrophobic in nature (Declerck et al., 2002). Our emission maximum results (Figure 3) also support this contention, where the occurrence of red shift indicating exposure of Trp residues at initial GdnHCl concentrations and resurgence of emission maximum close to native BLA at higher GdnHCl concentrations were noticed.

Biological activity

Enzymatic activity of BLA in presence of different GdnHCl concentrations with and without 2 mM $CaCl_2$ was measured to investigate the effect of GdnHCl on its biological activity. Figure 4A and B shows the effect of increasing GdnHCl concentrations on the enzymatic activity of native and Ca-depleted BLAs both in the absence and presence of 2 mM $CaCl_2$. About 85% loss in enzymatic activity of native BLA was observed at 1.0 M GdnHCl in the absence of 2 mM $CaCl_2$ (Figure 4A), which remained constant up to 3.0 M and increased gradually thereafter, reaching 31% activity (69% loss) at 6.0 M GdnHCl. This pattern was similar to those presented in Figures 1A, 2A and 3A of native BLA in the absence of 2 mM $CaCl_2$. On the other hand, activity of native BLA in presence of 2 mM $CaCl_2$ showed relatively lesser loss at all GdnHCl concentrations used. For example, ~25% loss in enzymatic activity was observed at 1.0 M GdnHCl concentration in presence of 2 mM $CaCl_2$. There was a gradual decrease in enzymatic activity with increasing GdnHCl concentrations reaching to a value of 46% (54% decrease) at 6.0 M GdnHCl (Figure 4A). Effect of GdnHCl on the activity of Ca-depleted BLA both in the absence and presence of 2 mM $CaCl_2$ is shown in Figure 4B. A complete loss of enzymatic activity was observed at 1.0 M GdnHCl for Ca-depleted BLA in the absence of 2 mM $CaCl_2$, which persisted throughout the GdnHCl concentration range used (Strucksberg et al., 2007). In other words, removal of intrinsic calcium from BLA abolished the enzyme's ability to degrade starch even at lower concentrations of GdnHCl. Presence of 2 mM $CaCl_2$ in the incubation mixture offered some stability to the enzyme against GdnHCl denatu-ration as relatively higher percentage of enzymatic activity was observed in the whole GdnHCl concentration range, being 14% at 6.0 M GdnHCl. Even at 1.0 M GdnHCl, ~20% of enzymatic activity was observed.

Greater retention of enzymatic activity in both native and Ca-depleted BLAs at different GdnHCl concentrations but in presence of 2 mM $CaCl_2$ can be explained on the basis of stabilizing potential of calcium on enzyme structure. In view of the incomplete saturation of calcium binding sites of BLA in solution (Nazmi et al., 2006), addition of 2 mM $CaCl_2$ into the buffer system might have increased calcium binding to the native enzyme. This in turn might have enhanced substrate binding due to the induction of ordered conformation of BLA resulting in the extension of substrate binding site (Machius et al., 1998). In an earlier study, (Nazmi et al. 2006), native BLA was shown to retain substantial amount of secondary structure even at the highest urea concentration and this behavior was attributed to the presence of calcium in native BLA. A similar calcium-induced stabilizing effect can be speculated for GdnHCl denaturation of native BLA. Both aforementioned factors might be responsible for the higher activity of native BLA in presence of 2 mM $CaCl_2$. This explanation seems to be justified from our results on Ca-depleted BLA (devoid of intrinsic calcium) where absence of 2 mM $CaCl_2$ rendered the enzyme labile towards GdnHCl denaturation leading to complete loss of biological activity (Figure 4B). Addition of 2 mM $CaCl_2$ in the incubation mixture offered some degree of stability to the enzyme which was responsible for some enhancement of enzymatic activity. A comparison of

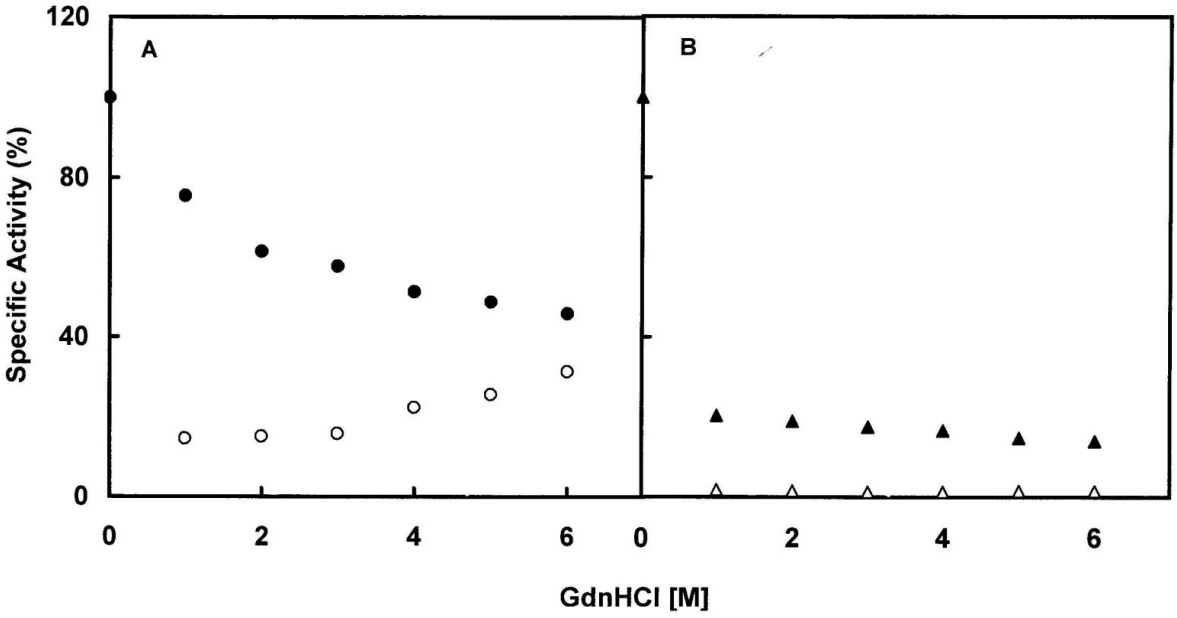

Figure 4. Effect of increasing GdnHCl concentrations on the specific activity of BLA in the absence (○/Δ) and presence (●/▲) of 2 mM $CaCl_2$. Specific activity of native BLA (A) and Ca-depleted BLA (B) were transformed into percentage specific activity as described in Materials and Methods and plotted against GdnHCl concentration.

three-dimensional structures of native and Ca-depleted forms of BLA showed differences in the structures of holo- and apo-enzymes (Machius et al., 1998). The segment between residues 182 and 192 containing metal-binding residue and essential *cis* peptide bond was found to be completely disordered in the apo form. This structural difference between native and Ca-depleted BLA could possibly account for the percentage difference in the biological activity of these states.

Presence of calcium offered stability to the enzyme against GdnHCl denaturation as can be judged from the indifferent behavior of CD, fluorescence, emission maximum signals and biological activity as shown in Figures 1, 2, 3 and 4, respectively. These results were in accordance to the previously published results on stabilization of BLA against thermal denaturation in presence of calcium (Fitter and Haber-Pohlmeier, 2004). Three calcium binding sites have been reported to fall within all the three domains: A, B and C of BLA. Unequivocally, two conserved calcium binding sites are located in domain B, whereas the third site is found at the interface between domains A and C (Machius et al., 1998). Taking this into consideration, it appears that calcium binding stabilizes all the three domains of BLA against GdnHCl denaturation. A structural comparison of apo- and holo- forms of BLA has also shown a major positive conformational change in domain B where two calcium binding sites are present, upon calcium binding (Machius et al., 1998).

Taken together, all these results suggest stabilizing potential of calcium towards GdnHCl denaturation of BLA. This is useful especially when such chemical denaturant is used as part of the purification steps to ensure the functionality of the enzyme without alteration in its structure.

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