Role of Disulfide Bridges in Structure-Activity Relationship of Plant Lipases from Wheat germ and Rice bran

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Disulfide bonds of wheat germ lipase (WGL) and rice bran lipase (RBL) were reduced with sodium borohydride (NaBH₄) and dithiothreitol (DTT) under non-denaturing conditions to assess the activity, conformation and stability of reduced form with those of native enzyme by using kinetics, far UV-CD, fluorescence, thermal denaturation studies and microcalorimetric measurements. Activity of reduced lipases was found to decrease in a sequential manner involving at least two steps in both the lipases. The CD spectrum in the far UV-region indicates that overall conformation was drastically affected upon reduction. A decrease in tryptophanyl fluorescence was observed without any shift in the emission maximum. The apparent thermal denaturation temperature [Tm(app)] of the reduced WGL and RBL was lowered by 6°C and 12°C, respectively, from the native enzyme. Reduction and carboxymethylation of all four cysteines caused extensive unfolding of the enzymes resulting in the loss of activity, conformation and thermal stability significantly indicating that the disulfide bonds have a major role in stabilizing the native conformation and stability of these two lipases.

Keywords: lipase; wheat germ; rice bran; disulfide bond; activity; conformational stability; thermal stability.

Introduction

Lipases (triacylglycerol hydrolases, EC 3.1.1.3), present in diverse organisms including animals, fungi and bacteria, have been studied extensively. However, the knowledge of plant lipases in terms of their industrial potential is still rather limited as compared to mammalian and microbial lipases. One of the common features among many folded proteins is the presence of disulfide bonds between cysteine residues (Thornton, 1981), particularly those evolved to function extracellularly, where an oxidizing environment favours disulfide bond formation. Although these bonds generally contribute to the stability of the native proteins, it is often found that removing disulfide bridges frequently results in the destabilization of these proteins (Price-Carter & Hull, 1998). The vital part of disulfide chemistry is to understand the mechanism by which crosslinks stabilize or destabilize native conformation of proteins. Therefore, knowledge of their number, reactivity and availability to reducing agents is important from the biotechnological point of view.

The role of disulfide bridges has not been established in relation to the conformation and stability of plant lipases to understand the physiological role and explore biotechnological applications. In this connection, two important plant lipases have been chosen in the present study to investigate the effect of reduction of disulfide bonds on the activity, conformation and stability by reducing agents, Sodium borohydride (NaBH₄) and Dithiothreitol (DTT). Wheat germ lipase (WGL) is an esterase and was found to be highly active on triacetin and tributyrin and inactive on long-chain triacylglycerols. Initial studies on wheat germ lipase were focused mainly on the isolation and physico-chemical characterization of different hydrolases, effect of metal ions, certain inhibitors and temperature on activity, their structural and thermal stability in presence of different cosolvents and denaturants (Rajendran et al, 1990; Sudhindra Rao et al, 1991; Rajeshwara & Prakash, 1994; Rajeshwara & Prakash, 1996). Similarly studies on rice bran lipase aimed at isolation, purification, inactivation and activity and stability in presence of added third component (Funatsu et al, 1971; Shastry & Rao, 1971; Aizono et al, 1971; Aizono et al, 1973; Shastry & Rao, 1976; Aizono et al, 1976; Fujiki et al, 1978; Rajeshwara et al, 1996) were carried out. To give an insight into the structural and functional role of disulfide bonds, the overall conformation of individual lipases was evaluated by circular dichroic spectroscopy and
reactivity of cysteine sulphydryls towards alkylation reagents coupled with activity measurements, fluorescence spectroscopy and thermal denaturation temperature analysis of both disulfide-intact and disulfide-reduced enzymes.

Materials and Methods

Lipase from wheat germ (Type I), Lysozyme, Triacetin, Triton X-100, Urea, DTT, NaBH₄, DTNB, Iodoacetic acid, and 2-Mercaptoethanol were obtained from Sigma Chemical Co., St. Louis, Missouri, USA. Buffer salts were obtained from E-Merck India Ltd, Calcium chloride was from CDH (P) Ltd. New Delhi. All other chemicals used were of analytical grade. Quartz-double distilled water was used throughout.

Purification of Wheat Germ Lipase (WGL)

Commercial wheat germ lipase was further purified to homogeneity by the method of Rajeshwara & Prakash (1994). Crude enzyme was subjected to ammonium sulphate fractionation followed by Sephadex G-25 column chromatography of the supernatant. The enzyme fraction eluting as the void volume was pooled, concentrated, dialyzed free of salts and lyophilized.

Purification of Rice Bran Lipase (RBL)

Rice bran lipase was isolated and purified by modified procedure of Rajeshwara & Prakash (1995) as follows: The total protein extract from the defatted rice bran flour was subjected to selective ammonium sulphate precipitation. The pellet obtained was suspended in 50 mM tris-HCl buffer, pH 7.5 was dialyzed exhaustively at 4°C. The clear supernatant obtained after centrifugation was loaded on to pre-equilibrated DEAE-sepharose CL-6B anion exchange column. The enzyme fraction eluted at 0.2 M KCl gradient was pooled, concentrated, dialyzed free of salt. The clear solution was then loaded on to Sephadex G-75 column pre-equilibrated with 50 mM sodium phosphate buffer, pH 7.4. The enzyme fraction was pooled, concentrated, dialyzed free of salts and lyophilized. The enzyme thus obtained was stored at -20°C in a desiccator and used for further studies.

Protein Concentration

Protein concentration of lipases was determined using an absorption coefficient (E₁%₁cm) of 15.5±0.5 at 278 nm for wheat germ lipase and 15.25±0.5 at 276 nm for rice bran lipase.

Lipase Assay

Lipase activity of both native and disulfide-reduced enzymes was monitored by following the modified procedure of Tietz & Fiereck (1996) as described by Sudhindra Rao et al., (1991) using triacetin as substrate in Mettler Toledo DL12 titrator.

Estimation of Disulfide Bonds

The number of disulfide bonds present in wheat germ lipase and rice bran lipase was estimated according to the standard procedures of Habeeb (1966). Lysozyme was throughout used as the standard.

Estimation of Percentage Reduction of Disulfide Bonds

Time dependent reduction and estimation of percentage disulfide reduced was carried out according to the procedures of Singh (1996).

Circular Dichroic Spectroscopy

CD spectra of lipases were recorded in a Jasco J20-C automatic recording spectropolarimeter at 30±0.5°C with the slits programmed to give 1 nm bandwidth. The far UV-CD spectra of the native and reduced lipases were measured at 0.2 mg/ml with a 1 mm cell in the wavelength range of 200-260 nm. NaBH₄ in the reduced enzymes was removed by exhaustive dialysis against respective buffers prior to CD measurements. The far UV-CD spectral data were expressed in mean residue ellipticity (degree cm² dmol⁻¹) (Chen & Yang, 1971).

Fluorescence Spectroscopy

Intrinsic tryptophan fluorescence spectra of lipases were recorded with a protein concentration of 0.1 mg/ml using Shimadzu RF-5000 recording spectrofluorimeter at 25-30°C. The emission spectrum of native and disulfide reduced protein was recorded in the range of 300-400 nm, 10 sec after excitation at 284 nm and 285 nm for wheat germ lipase and rice bran lipase, respectively.

Size-exclusion Chromatography using Fast Protein Liquid Chromatography (FPLC)

Size-exclusion chromatography of both native and disulfide reduced WGL and RBL was carried out on Superdex-75 column using FPLC. Both the enzymes were incubated in presence of 5 and 10 mM DTT concentrations under nitrogen at 37°C for 2 hrs in Innova 4000 incubator shaker with gentle stirring.
Both native and disulfide-reduced samples were dialyzed with 3-4 changes against respective buffers and centrifuged at 10,000 rpm for 10 min. Clear supernatant (500 μl) was loaded on to Superdex-75 column. The protein was eluted at a flow rate of 0.5 ml/min in a 60 min run. Blanks containing 10 and 20 mM DTT were also run to give necessary corrections.

**Thermal Denaturation Studies**

The thermal denaturation behaviour of the native as well as reduced lipases was followed using Gilford Response II Spectrophotometer. The change in the absorbance of about 0.3 ml protein having an absorbance of 0.3-0.4 in each case was monitored at 287 nm as a function of temperature in the range 25-95°C with 1°C increment with appropriate blanks. Apparent thermal denaturation temperature \( T_{\text{app}} \) was calculated either by first derivative plot of absorbance or van’t Hoff plot (Pace & Scholtz, 1997).

**Differential Scanning Calorimetry**

Differential scanning calorimetric (DSC) experiments were performed on a Microcal MC-2 Ultrasensitive differential scanning calorimeter. Protein solutions were incubated with the indicated concentrations of DTT for 30 min at 37°C. Buffer containing an indicated concentration of DTT was used in the reference cell. Protein concentration of 2.5×10\(^{-4}\) and 2.0×10\(^{-4}\) M was used for WGL and RBL, respectively. Samples and buffer solutions were degassed with gentle stirring under vacuum in a Microcal cell cleaning and degassing station. Experiments were performed in the range 30-90°C at a scan rate of 1.5°C per min. Normalized heat capacity \( (C_p) \) data were corrected for buffer baseline. Raw data were analyzed using Origin™ (version 2.9) scientific plotting software and curve fit by using non-two state fit with fixed \( T_m \) for both the lipases.

**Results**

**Estimation of Disulfide Bonds**

The number of disulfide bonds of both wheat germ lipase and rice bran lipase was estimated according to the methods described earlier. Under non-denaturing conditions, both WGL and RBL did not show any free cysteine residues. In denatured but in the absence of reducing agent, no cysteine was detected where as in the presence of denaturant and a trace amount of the thiol reagent, β-mercaptoethanol, the unfolded and reduced enzymes, WGL and RBL, showed the presence of four cysteine residues each. These four cysteines take part in the formation of two disulfide bonds each in wheat germ lipase and rice bran lipase.

**Effect of Disulfide Bond Reduction on the Activity of Lipases**

The stability offered by the disulfide bonds on the function of WGL and RBL was studied by the activity measurements using reducing agent, NaBH\(_4\). A sharp decrease in the activity of WGL was observed up to 25 mM NaBH\(_4\) concentration where the enzyme activity drops from 87%-47% (Fig. 1). From 25 mM-150 mM, WGL gradually becomes inactive and at 150 mM NaBH\(_4\) concentration, enzyme was found to retain only 13% of initial activity. On the other hand, the rate of inactivation in RBL was much faster when compared to WGL, in the range 0-100 mM NaBH\(_4\) concentration. At 10, 25, 50 and 75 mM NaBH\(_4\) concentrations, RBL was found to retain 80, 60, 40 and 18% of original activity, respectively and becomes totally inactive at 100 mM NaBH\(_4\) concentration. The data suggest that loss of activity is directly proportional to the concentration of the reducing agent and it takes place at little higher concentrations in the absence of denaturant to reach 100% inactivation.

**Effect of Disulfide Bond Reduction on the Conformation of Lipases**

Reductive unfolding of lipases, whether or not, results in any conformational changes was...
investigated by far UV-CD spectroscopy to correlate the disulfide reduction with protein conformation. Table I presents the secondary structural elements of native and disulfide-reduced WGL and RBL, at different incubation time intervals in the presence of 0.2 M and 0.1 M concentrations of NaBH₄, respectively. In WGL, a 4% increase in the α-helix and 6% increase in β-structure from the control values of 16% and 44%, respectively, were observed at 10 min incubation time. No significant change in the α-helix and β-structure was observed at 40 and 60 min incubation time. However, aperiodic structure decreases by 10% and 3% at 10 min and 60 min incubation, respectively, without any gross change at 40 min. On the other hand, the α-helix of reduced RBL increased to 20, 17 and 17% at 10, 40 and 60 min of incubation time, respectively, from a control value of 15%. The β-structure was not altered at 10 min whereas it was found to increase by 3% at 40 and 60 min of incubation time as compared to 45% in the case of native enzyme. The aperiodic structure was found to decrease in all three cases by 5% from a control value of 40%. This alteration in the secondary structure of reduced enzymes was due to the change in the local conformation around the half-cysteines that are involved in the disulfide bond formation in the native protein.

Effect of Disulfide Bond Reduction on the Intrinsic Tryptophan Fluorescence of Lipases

Fluorescence spectroscopy was carried out to probe the perturbation of tryptophanyl residues as a result of reductive unfolding of lipases in the presence of DTT. Fluorescence emission intensity of the reduced WGL decreased as the concentration of the reducing agent increased (Fig. 2A). The change in the fluorescence emission intensity, using model compound data, between native and reduced WGL is shown in Fig. 2B. The decrease in the fluorescence emission intensity is gradual due to sequential unfolding of the enzyme over a range 5-20 mM DTT concentration. In the case of RBL, the decrease in the fluorescence emission intensity is sharp at 5 mM DTT concentration when compared to WGL and the decrease becomes gradual at 10, 15 and 20 mM DTT concentrations [Fig. 3(A & B)]. This decrease in the intrinsic tryptophan fluorescence may be attributed to the quenching of the unfolded enzyme by the reducing agent. However, the reduction of disulfide bonds did not alter the fluorescence emission maximum in all the concentration of DTT used, indicating partial exposure of the tryptophanyl residues to the bulk solvent due to reductive unfolding.

<table>
<thead>
<tr>
<th>Incubation Time (min)</th>
<th>α-Helix (%)</th>
<th>β-Structure (%)</th>
<th>Aperiodic (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat germ lipase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control^</td>
<td>16±1</td>
<td>44±2</td>
<td>40±2</td>
</tr>
<tr>
<td>10</td>
<td>20±1</td>
<td>50±2</td>
<td>30±2</td>
</tr>
<tr>
<td>40</td>
<td>18±1</td>
<td>41±2</td>
<td>41±2</td>
</tr>
<tr>
<td>60</td>
<td>18±1</td>
<td>45±2</td>
<td>37±2</td>
</tr>
<tr>
<td>Rice bran lipase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control^</td>
<td>15±1</td>
<td>45±2</td>
<td>40±2</td>
</tr>
<tr>
<td>10</td>
<td>20±1</td>
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<td>35±2</td>
</tr>
<tr>
<td>40</td>
<td>17±1</td>
<td>48±2</td>
<td>35±2</td>
</tr>
<tr>
<td>60</td>
<td>17±1</td>
<td>48±2</td>
<td>35±2</td>
</tr>
</tbody>
</table>

^ Native protein in 0.02 M sodium phosphate buffer, pH 7.0 and 0.05 M sodium phosphate buffer, pH 7.4 for wheat germ lipase and rice bran lipase, respectively.

Fig. 2 — (A) Fluorescence emission spectra of (a) native and reduced wheat germ lipase in presence of (b) 5 mM, (c) 10 mM and (d) 20 mM and DTT concentrations in 0.02 M sodium phosphate buffer, pH 7.0 at 284 nm in the range 300-400 nm. (B) Change in fluorescence intensity of wheat germ lipase as a function of DTT concentration.
**Time-course of Inactivation of Lipases**

Time course of inactivation and per cent reduction to quantify the number of disulfide bonds reduced as a function of time in WGL and RBL by NaBH₄ (Fig. 4). The time-course of inactivation of both the enzymes followed a stepwise unfolding. In case of WGL, in the first step, between 0-40 min, enzyme loses its activity rapidly and retains only 17% of its original activity due to the reduction of one disulfide bond. Second step, between 40-80 min, is comparatively a slower one where the residual specific activity decreases to 5% from 17% at which time the second disulfide bond was broken resulting in the further loss of activity. The last step, between 80-120 min, involves the total inactivation of the enzyme indicating the complete reduction of the disulfide bonds.

On the other hand, in RBL, the breaking of first disulfide bond in the first step, between 5-10 min, resulted in the loss of 35% of specific activity. The second step of inactivation, between 20-30 min, involves the breaking of second disulfide bond and loss of 70% activity whereas in the last step, between 30-40 min, RBL becomes totally inactive indicating total reduction of disulfide bonds. The plateau of per cent disulfide reduction curve of WGL after 60 min and RBL after 40 min indicates that there is no disulfide bond available for further reduction.

**Stability of both Lipases in DTT**

The effect of disulfide bond reduction on the size of lipases was checked by size-exclusion chromatography on Superdex-75 column using FPLC; (Fig. 5). Both native and disulfide-reduced WGL in presence of 5 and 10 mM DTT eluted with an elution volume of 8.04 ml (Fig. 5A). Similar observation was also made in the case of RBL where the elution volume of disulfide-reduced enzyme in presence of 5 and 10 mM DTT remained unchanged (11.50 ml) as that of control in buffer alone (Fig. 5B). However, in both the cases, the intensity of the peak marginally decreases as compared to control. These data clearly indicate that either WGL or RBL is reduced upto 10 mM DTT studied. In the case of WGL, no change appears in the elution volume indicating that there are no aggregates, whereas in the case of RBL there is a small leading shoulder possibly arising out of sulphydryl interaction and aggregation eventhough the percentage is very very low. These data reinforce the concept that these lipases are structurally intact in the presence of DTT whereas there is a perturbation in the microenvironment of tryptophanyl residues in presence of 5 and 10 mM DTT, both in the case of WGL and RBL although comparatively the extent is much less in WGL as compared to RBL (Fig. 3).

![Fig. 3](image-url)  
**Fig. 3** — (A) Fluorescence emission spectra of (a) native and reduced rice bran lipase in presence of (b) 5 mM, (c) 10 mM, (d) 15 mM and (e) 20 mM DTT concentration in 0.05 M sodium phosphate buffer, pH 7.4 at 285 nm in the range 300-400 nm. (B) Change in fluorescence intensity of rice bran lipase as a function of DTT concentration.

![Fig. 4](image-url)  
**Fig. 4** — Time-dependent activity (○, ■) and per cent disulfide reduction (●, □) of wheat germ lipase (○, ●) and rice bran lipase (■, □) as a function of incubation time in presence of 0.2 M and 0.1 M NaBH₄, respectively. A protein concentration of 5 × 10⁻⁵ M for wheat germ lipase and 6 × 10⁻⁵ M for rice bran lipase, was used.
Effect of Disulfide Bond Reduction on the Thermal Stability of Lipases

The above data indicate that the conformational change induced by the disulfide bond reduction may be locally limited to the region close to the disulfide bond. Therefore, investigations were carried out to find, whether or not, such a local destabilization affects the overall stability of these two enzymes against thermal denaturation by thermal transition temperature measurements. The $T_{m(\text{app})}$ values of both WGL and RBL decrease with an increase in the NaBH$_4$ concentration (Table 2). In the case of WGL, a gradual decrease in $T_{m(\text{app})}$ was observed, maximum being 6°C at 75 mM NaBH$_4$ concentration from a control value of 55±1°C. Similarly in the case of RBL, an 8°C decrease at 50 mM and 12°C decrease at 75 mM was observed, from a control value of 67±1°C. This is a clear indication that the thermal stability of the native enzyme is strictly due to the presence of disulfide bonds and their breakage leads to the inactivation of the enzymes.

Differential Scanning Calorimetric Studies

The DSC profile of both native and disulfide reduced WGL and RBL is shown in Fig. 5. Native WGL shows two transitions, one at 57 and other at 65°C. (Fig. 6A). In the presence of 5 mM and 10 mM DTT, $T_{m1}$ does change significantly whereas $T_{m2}$ was found to increase by 4°C. Above 65°C, slowly the protein starts precipitating as evidenced by the scattered points. In case of RBL, similar two transitions were observed in the presence and absence of DTT, as shown in (Fig. 6B). Native enzyme showed a transition at 59°C ($T_{m1}$) and the other at 71°C ($T_{m2}$) whereas these transitions of reduced enzyme were found to decrease by 8 and 9°C, respectively, in presence of 10 mM DTT. It is also evident from the figure that excess heat capacity of the reduced enzymes was found to increase in both the cases. The thermodynamic parameters of native and disulfide-reduced lipases are listed in Table 3.

Discussion

Our data clearly indicate that the reduction of disulfide bonds of lipases from wheat germ and rice bran by NaBH$_4$ results in the loss of activity and is a concentration dependent phenomenon. Under non-

Table 2 — $T_{m(\text{app})}$ values of native and disulfide reduced wheat germ lipase and rice bran lipase as a function of NaBH$_4$ concentration.

<table>
<thead>
<tr>
<th>NaBH$_4$ concentration (mM)</th>
<th>$T_{m(\text{app})}$(°C)</th>
<th>Wheat germ lipase</th>
<th>Rice bran lipase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>55</td>
<td>67</td>
</tr>
<tr>
<td>10</td>
<td>53</td>
<td>61</td>
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<td>59</td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>49</td>
<td>55</td>
<td></td>
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</table>

*Native protein in 0.02 M sodium phosphate buffer, pH 7.0 and 0.05 M sodium phosphate buffer, pH 7.4 for wheat germ lipase and rice bran lipase, respectively.
Denaturing conditions, the enzymes were found to unfold at slightly higher concentrations of reducing agents as evident from the earlier studies of Chang (1997). Complete unfolding is achieved at effective concentrations of reducing agent, which results in the total loss of biological activity. The loss of activity is directly related to the extent of disulfide bond reduction (Thornton, 1981).

It is evident from the perturbations in the far UV-CD spectrum that the disulfide bonds are not critically important for catalytic function of these lipases but essential for the maintenance of the native conformation of the enzymes. Gradual decrease in the fluorescence emission intensity without affecting the emission maximum clearly supports the partial exposure of the tryptophanyl residues to the bulk solvent supporting the partial unfolding of the enzyme. The studies of Zhu et al. (1995) show that in the case of phospholipase A2, removal of the disulfide bridge C11-C77 results in a large decrease in stability and a large conformational perturbation. This conformational instability may be attributed to the local destabilization around half cystines in lipases from wheat germ and rice bran.

The reductive unfolding of WGL and RBL takes place in a sequential manner as evidenced by kinetics of inactivation. The first step in the sequential reduction process is slightly faster in both the cases taking around 6-8 min whereas the second step is quite slower resulting in the complete reduction of disulfide bonds as evidenced by the plateau after 100 min in WGL and 40 min in RBL indicating the complete inactivation of the enzyme. Similar observations were also made in the case of tick anticoagulant peptide, hirudin and bovine ribonuclease A (Chang, 1997) and a-lactalbumin (Segawa et al., 1992). This clearly shows the reductive unfolding pathway in lipases is not a two state but a multistep process resulting in the formation of scrambled intermediates. No change in the size of the molecule and no aggregation of the enzyme due to disulfide bond reduction clearly support the fact that the loss of enzyme activity is mainly due to the disruption of secondary structure as a result of

![Fig. 6](https://example.com/fig6.png)

**Fig. 6** — (A) Differential scanning calorimetric profile of wheat germ lipase in the (a) absence and presence of (b) 5 mM and (c) 10 mM DTT in 0.02 M sodium phosphate buffer, pH 7.0. A protein concentration of 2.5×10⁻⁴ M was used for the experiment. (B) Differential scanning calorimetric profile of rice bran lipase in the (a) absence and (b) 10 mM DTT concentration in 0.05 M sodium phosphate buffer, pH 7.4. A protein concentration of 2×10⁻⁴ M was used for the experiment.

<table>
<thead>
<tr>
<th>OTI (mM)</th>
<th>Temp (°C)</th>
<th>ΔH_{cal} (kJ mol⁻¹)</th>
<th>ΔH_{vap} (kJ mol⁻¹)</th>
<th>Temp (°C)</th>
<th>ΔH_{cal} (kJ mol⁻¹)</th>
<th>ΔH_{vap} (kJ mol⁻¹)</th>
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<tbody>
<tr>
<td>Wheat germ lipase</td>
<td>0</td>
<td>57±0.5</td>
<td>123±26</td>
<td>282±14</td>
<td>65±0.5</td>
<td>157±8</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>58±0.5</td>
<td>292±15</td>
<td>197±10</td>
<td>69±0.5</td>
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<tr>
<td></td>
<td>10</td>
<td>58±0.5</td>
<td>391±20</td>
<td>183±9</td>
<td>69±0.5</td>
<td>104±5</td>
</tr>
<tr>
<td>Rice bran lipase</td>
<td>0</td>
<td>59±0.5</td>
<td>46±2</td>
<td>209±10</td>
<td>71±0.5</td>
<td>121±6.0</td>
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<td></td>
<td>10</td>
<td>51±0.5</td>
<td>71±4</td>
<td>414±21</td>
<td>62±0.5</td>
<td>56±3.0</td>
</tr>
</tbody>
</table>

**Table 3** — Thermodynamic parameters of WGL and RBL as a function of DTT concentration. [Here the T_m1 refers to the lower temperature peak and T_m2 refers the higher temperature peak in the bimodal differential pattern].
modification of cysteiny1 residues, as evidenced by size-exclusion chromatography data.

Disulfide bonds increase the conformational stability mainly by constraining the unfolded conformation of the protein and thereby decreasing conformational entropy (Pace, 1990). The thermal denaturation temperature analysis of native and disulfide reduced WGL and RBL indicated the destabilization of the native conformation of these enzymes as evidenced by the decrease in the $T_{\text{m(app)}}$ values and DSC data. Reduced RBL was found to be more unstable in terms of thermal stability as compared to WGL. Similar observations were also made in ribonuclease T$_1$ (Pace et al., 1988), B-1, 4-endoglucanase E (McGinnis & Wilson, 1993) and ovalbumin (Takahashi et al., 1991). This can also be supported by the studies in bovine pancreatic trypsin inhibitor (States et al., 1987) where the breaking of disulfide bond between 5 and 15 or 14 and 38 cysteine residues lowers the melting temperature by over 50°C from 95°C. The differential or sequential reduction and fluctuating local conformation of the reduced form around the cysteine residues may be related to the decreased stability against thermal denaturation (Takahashi et al., 1991) The stabilizing effect of a disulfide bond in a protein can be attributed predominantly to an increase in the entropy difference between the folded and unfolded states of the disulfide bonded protein versus the reduced protein (Pace, 1990; Wetzel, 1987). The presence of disulfide bonds in proteins or the breaking of such bonds affects the fluorescence of tryptophan and tyrosyl residues. Reduction of disulfide bonds may result either in the increase of fluorescence as in the case of lysozyme (Steiner, 1964; Churchich, 1966) and ribonuclease A (Cowgill, 1966) or decrease in the fluorescence as observed in avidin (Green, 1964). The decrease in the fluorescence emission intensity in both WGL and RBL may be ascribed to modifications of the microenvironment of the fluorescent residues and a minor conformational change that might have accounted for the results.

In conclusion, the disulfide bonds in wheat germ lipase and rice bran lipase are essential for maintaining the overall conformation and activity of the enzyme, and reduction of disulfide bonds affect the microenvironment around cysteine residues that are involved in the formation of disulfide bonds in the native proteins to a large extent. An understanding of the mechanism of stabilization/destabilization of the enzyme paves way for a possible role of chemical modification of cysteine residues in stabilizing/destabilizing lipases in general.

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References


