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Succinylation-induced Conformational Destabilization of Lysozyme as Studied by Guanidine Hydrochloride Denaturation

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Using 100-fold molar excess of succinic anhydride, about 99% of lysine residues of hen egg white lysozyme (HEWL) were modified. Succinylated (S_{99}) HEWL showed both charge and size homogeneity as judged by PAGE and gel filtration, respectively. Hydrodynamic parameters such as Stokes radius and frictional ratio (f/f_o) showed more expanded conformation of S_{99} HEWL compared to native HEWL as evident from the increase in Stokes radius (from 1.36 to 1.86 nm) and f/f_o (from 0.86 to 1.15) values. Guanidine hydrochloride (GdnHCl) denaturation studies using fluorescence spectroscopy connoted a marked decrease in conformational stability of HEWL upon succinylation. Complete denaturation of S_{99} HEWL was achieved at lower GdnHCl concentration (~3.8 M) compared to native HEWL (~5 M). Furthermore, free energy of stabilization ($\Delta G_D^{H_2O}$) value also showed a notable decrease from 8,559 and 7,956 cal/mol (for native HEWL) to 4,404 and 4,669 cal/mol (for succinylated HEWL) using excitation at 280 and 295 nm, respectively. Both expanded conformation and decreased $\Delta G_D^{H_2O}$ can be attributed to the increase in the net negative charge on the protein upon succinylation. All these results manifested the importance of positively charged lysine residues in maintaining the conformational stability of HEWL through electrostatic interactions.

Key words: conformational stability, denaturation, fluorescence spectroscopy, hen egg white lysozyme, succinylation.

Abbreviations: BSA, bovine serum albumin; DMSO, dimethylsulphoxide; GdnHCl, guanidine hydrochloride; HEWL, hen egg white lysozyme; TNBS, trinitrobenzenesulphonic acid.

Acquisition of native globular conformation (3D structure) of a protein is governed by its linear amino acid sequence (*1*) and biological environment surrounding it (*2*, *3*). However, the stability of protein's 3D structure is endorsed by various intramolecular forces, such as hydrogen bonds, *van der Waals*, electrostatic and hydrophobic interactions involving various amino acid side chains as well as their milieu in the native structure (*4*, *5*). Majority of the proteins have been found to be marginally stable than their unfolded forms (*6*). Enhancement of enzyme stability to withstand severe reaction conditions (extreme temperature, pH and pressure) and presence of destabilizing agents (salt, alkali, denaturant and surfactant) has become a crucial industrial demand (*7*). The stability of a protein can be increased by either decreasing the free energy of native (N) or folded conformation or increasing the free energy of denatured (D) or unfolded conformation (*8*) by changing the amino acid sequence of the protein (*9*). Currently, intensive research has been made to produce stable mutant forms of enzymes using site-directed mutagenesis. However, the process is hampered due to lack of information about the importance of a particular amino acid type towards the enzyme stability and its suitability for replacement with another amino acid without changing its molecular properties. Several mutations need to be carried out in a protein on a hit and trial basis and yet, it may result in either an increased (*10*, *11*) or decreased (*12–14*) stability. Single amino acid substitutions have been found difficult to predict in advance although some do result in enhanced stability (*15*).

Of the various forces, hydrogen bonding and hydrophobic effect have been shown to make significant contribution to the protein stability using mutant proteins (*4*). Being secondary contributor to protein's stability through salt bridges, charged residues have also been found important in manipulating protein stability as revealed by charge-reversal mutation studies. Both enhanced (*16*) and decreased (*17*) stability have been demonstrated by charge-reversal mutations.

Hen egg white lysozyme (HEWL) (EC 3.2.1.17), a well-characterized globular protein (*18–21*) is made up of a single polypeptide chain of 129 amino acid residues arranged in the form of two domains and stabilized by four disulphide bonds. Due to its muramidase action on the peptidoglycan layers of bacterial cells (*22*), it is used extensively as a natural preservative in food and dairy industries (*23–25*). All six lysine residues of HEWL are located at the molecular surface (*26*, *27*). This offers advantage of being a suitable candidate for modification or substitution. Succinylation of lysine residues of HEWL had been performed earlier to study the effect

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of modification on the activity, immunochemical properties (*28*, *29*), adsorption (*30*), conformational changes (*31*) and thermostability (*32*) of the enzyme. However, effect of succinylation on conformational stability of HEWL against chemical denaturants has not been studied so far to the best of our knowledge. The present work describes GdnHCl denaturation of native and succinylated HEWLs, in order to show the importance of lysine residues in conformational stability of HEWL and their suitability towards substitution and charge reversal mutations.

EXPERIMENTAL PROCEDURES

Materials—HEWL (Lot 096K1237), different marker proteins such as bovine serum albumin (BSA) (Lot 015K0591), carbonic anhydrase (Lot 99H0669), α-chymotrypsinogen A, type II (Lot 16H7075), myoglobin (Lot 064K7006) and cytochrome c (Lot 27H7065), *Micrococcus lysodeikticus*, Sephadex G-75, blue dextran and guanidine hydrochloride (GdnHCl) (purity ≥ 99%) were purchased from Sigma Chemical Co., USA. Succinic anhydride was the product of Fluka, Germany. Trinitrobenzenesulphonic acid (TNBS) was supplied by Pierce Chemical Co., USA. All other reagents used were of analytical grade. All the experiments were performed at 28°C unless otherwise stated.

Analytical Methods—Protein concentration was routinely determined by the method of Lowry *et al*. (*33*) using BSA as the standard. The concentration of GdnHCl stock solution was determined from the data of Nozaki (1972), as suggested by Pace *et al*. (*34*, *35*). Enzymatic activity of both native and succinylated HEWL was determined by measuring rate of lysis of *M. lysodeikticus* suspension as described in the Sigma Technical Bulletin (*36*).

Succinylation of HEWL—Succinylation of HEWL was performed following the method of Klotz (*37*) with slight modification as described earlier (*29*). About 340 mg of solid succinic anhydride (100-fold molar excess over protein) was dissolved in 1 ml of dimethylsulphoxide (DMSO) (*38*) and added in aliquots to a continuously stirred protein solution (containing 500 mg HEWL in 25 ml of 0.1 M sodium phosphate buffer, pH 8.0) over a period of 20 min. The pH of the reaction mixture was maintained at pH 8.0 by simultaneous addition of 1 N sodium hydroxide solution. The reaction was allowed to proceed at 4°C for 30 min. Upon completion of the reaction, the protein solution was thoroughly dialysed against water followed by 0.1 M sodium phosphate buffer, pH 7.0 for 48 h. The extent of modification in the modified preparation was determined by the TNBS reaction method (*39*) as described in the Pierce Technical Bulletin (*40*).

Polyacrylamide Gel Electrophoresis—The charge homogeneity of the modified preparation was checked with native polyacrylamide gel electrophoresis (PAGE) under acidic condition, using β-alanine-acetic acid buffer, pH 4.5, on 15% (w/v) polyacrylamide gel following the method of Reisfeld *et al*. (*41*). After electrophoresis, the gel was stained with coomassie brilliant blue R-250, as suggested elsewhere (*42*).

Analytical Gel Filtration—Analytical gel filtration was performed following standard procedure as described earlier by Tayyab *et al*. (*43*) using a Sephadex G-75 column (2.34 × 64 cm) equilibrated with 0.06 M sodium phosphate buffer, pH 7.0 containing 0.02% sodium azide. The void volume, V_o and inner volume, V_i of the column were determined by passing blue dextran and potassium ferricyanide, respectively. The column was calibrated with marker proteins of known Stokes radii (*44*), BSA (3.63 nm), carbonic anhydrase (2.30 nm), α-chymotrypsinogen A, (2.20 nm), myoglobin (1.90 nm) and cytochrome c (1.70 nm). The elution volume of each marker protein was determined in triplicate and then normalized in terms of distribution coefficient, K_d and available distribution coefficient, K_{av} according to standard procedures as described earlier (*43*). The data were analysed in the same way as described by Laurent and Killander (*45*) and Ackers (*46*). Stokes radii of both native and modified HEWLs were computed from their elution volumes on the calibrated column.

GdnHCl Denaturation Studies—Denaturation experiments were carried out in the same way as described earlier (*47*). Solutions for denaturation experiments were prepared in 0.06 M sodium phosphate buffer, pH 7.0. To 0.5 ml stock solution of native (or modified) HEWL preparation (final concentration = 1 μM) taken in different tubes, different volumes of buffer were added first. This was followed by the addition of different volumes of stock GdnHCl solution (7.0 M) to get the desired concentration of denaturant in the final solution mixture (5.0 ml). The contents were mixed well and incubated for 12 h at 25°C. Fluorescence spectra were recorded on a Hitachi fluorescence spectrophotometer, model F-2500 with a 1 cm path-length cell in the wavelength range of 300–400 nm by exciting the protein solution at 280 and 295 nm. Excitation and emission slits were fixed at 10 nm each.

Data Analysis—Denaturation data were analysed by assuming two-state mechanism (*47*). Apparent fraction of denatured form, F_D, was calculated by normalizing denaturation curves using the following Equation 1.

$$F_D = \frac{Y - Y_N}{Y_D - Y_N} \quad (1)$$

where Y indicates observed variable parameter at a given denaturant concentration. Y_N and Y_D are the variable characteristics of the native and denatured states, respectively and these values were obtained by linear extrapolation of pre- and post-transition regions. The apparent equilibrium constant, K_D was calculated using values of F_D ranging from 0.25 to 0.75. The calculation for K_D values between the native and denatured states of protein at a given denaturant concentration, is given by Equation 2

$$K_D = \frac{F_D}{(1 - F_D)} \quad (2)$$

Free-energy change (ΔG_D) was calculated from K_D values using Equation 3

$$\Delta G_D = -RT \ln K_D \quad (3)$$

where R is the gas constant (1.987 cal/deg/mol) and T is the absolute temperature. The free energy of stabilization, $\Delta G_D^{H_2O}$ was determined using least squares

analysis of ΔG_D values versus denaturant concentration, [D] to fit the data to the following Equation 4

$$\Delta G_D = \Delta G_D^{H_2O} - m\,[D] \quad (4)$$

where '*m*' is the slope of the linear plot and is a measure of dependence of ΔG_D on denaturant concentration. $\Delta G_D^{H_2O}$ values were obtained from the intercept on *y*-axis.

RESULTS AND DISCUSSION

Succinylation of HEWL—Reaction of TNBS with amino groups of a protein results in the formation of yellow coloured trinitrophenyl derivatives which absorb maximally at 335 nm (*39*). Therefore, any decrease in the colour intensity of TNBS reaction is indicative of amino groups' modification. Results of TNBS reaction with native HEWL and HEWL preparation treated with 100-fold molar excess of succinic anhydride are shown in Fig. 1. As can be seen from the figure, absorbance at 335 nm increased linearly with the increase in protein concentration for native HEWL and followed the given straight line equation:

$$(\text{Absorbance})_{335\,nm} = 2.73\,(\text{Amount of protein, mg}) + 0.02 \quad (5)$$

On the other hand, succinic anhydride-treated protein produced either no colour or very little colour in the whole range of protein concentration used which was higher than the range used with native HEWL. A least squares analysis of the data gave the following straight line equation:

$$(\text{Absorbance})_{335\,nm} = 0.04\,(\text{Amount of protein, mg}) + 0.02 \quad (6)$$

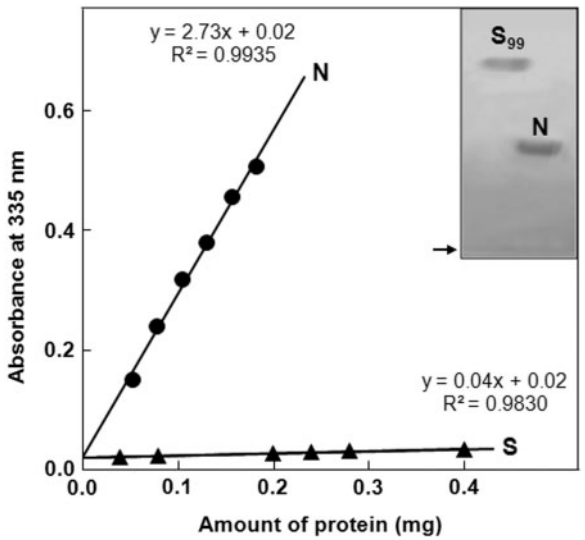

Fig. 1. **Quantification of modification in succinylated HEWL preparation by TNBS reaction method.** Native, 'N' HEWL (filled circle) and HEWL treated with 100 molar excess of succinic anhydride, 'S' HEWL (filled triangle). Straight lines were drawn using least squares analysis. Inset shows PAGE pattern of native, 'N' and 99% succinylated, 'S_{99}' HEWL preparations performed according to the method of Reisfeld *et al*. (*41*) on 15% polyacrylamide gel. The arrow shows the position of the tracking dye, methylene blue. About 10 µl of the sample containing 10 µg of protein was loaded in each well and electrophoresis was carried out in β alanine–acetic acid buffer, pH 4.5 for 2 h. The gel was then stained with 0.2% (w/v) coomassie brilliant blue R-250 and destained in 5% methanol, 7% acetic acid solution following the method of Laemmli (*42*).

Such a drastic decrease in TNBS reaction in the treated preparation indicated that the reaction had mainly occurred at amino groups (*48*). Using the slope values of TNBS reaction obtained with native and treated HEWLs (Fig. 1), the extent of modification was determined as 99% (*49*). Taking the total number of amino groups in HEWL as 7 (6 ε-amino and 1 α-amino) (*50*), the number of amino groups modified was calculated as 7 (Table 1). This seems understandable as lysine residues of HEWL are found located at the surface of the protein (*26*, *27*). Although the reaction of succinic anhydride with HEWL can modify amino, tyrosyl, seryl, threonyl and imidazole groups of HEWL (*37*), the specificity of the reaction was believed mainly towards amino groups under slightly alkaline condition used (*51*). Furthermore, use of extensive dialysis of modified preparation against water was also responsible to reverse the modification of seryl, threonyl and imidazole groups (*52*).

Purity of the modified HEWL was checked by PAGE under acidic condition (*41*) [due to the basic nature of the protein (*27*)] as described in 'EXPERIMENTAL PROCEDURES section'. Inset of Fig. 1 shows electrophoretic pattern of native (N) and modified (S_{99}) HEWLs, where both proteins migrated in the form of a single major band, suggesting charge homogeneity of both preparations. However, S_{99} preparation showed retardation in its cathodic mobility compared to native protein, as the relative mobility value of 0.22 was obtained with S_{99} preparation compared to 0.56 obtained for native HEWL (Table 1). This decrease in relative mobility of S_{99} preparation seems justifiable in view of the replacement of a positive charge on lysine residues with a negative charge upon succinylation (*37*). Therefore, net positive charge on S_{99} preparation would be less compared to native HEWL, which was responsible for the lower cathodic mobility observed with S_{99} preparation. Native HEWL has an isoelectric point of 11.1, which is shifted to 4.5–4.7 upon succinylation of its lysine residues (*26*, *30*, *53*). It means that S_{99} preparation possessed a net charge close to zero at pH 4.5 (experimental pH of PAGE) compared to positively charged native HEWL. Using titration curves of native and succinylated HEWLs (*53*), a net charge of +11 and +1 was calculated for native and S_{99} HEWL preparations respectively at pH 4.5, which supported our PAGE results.

Table 1. **Modification of amino groups of HEWL.**

Modifying reagent	Molar excess of reagent	Extent of modification (%)	No. of amino groups modified[a]	Relative mobility (R_m)
–	0	0	0	0.56
Succinic anhydride	100	99	7	0.22

[a]Taking the total number of amino groups as 7 (6 ε-amino + 1 α-amino).

Both native and 99% succinylated HEWL preparations were eluted in the form of a single symmetrical peak from a Sephadex G-75 column (2.34 × 64 cm) (Fig. 2), suggesting size homogeneity of the preparations. Thus, the modified preparation (S_{99} HEWL) was homogenous both with respect to charge and size.

Conformational Changes in Succinylated HEWL—Analytical gel filtration was performed using Sephadex G-75 column (2.34 × 64 cm) to evaluate the effect of succinylation on the conformation of HEWL by determining hydrodynamic parameters such as Stokes radius and frictional ratio. V_o and V_i of the column were determined to be 79 and 146 ml, respectively. Different marker proteins, *i.e*. BSA, carbonic anhydrase, α-chymotrypsinogen A, myoglobin and cytochrome c had elution volumes of 92, 132, 137, 152 and 168 ml, respectively as indicated by numbers 1–5 with arrows in Fig. 2. Elution profiles of both native (N) and modified (S_{99}) HEWLs are also shown in Fig. 2. As evident from the figure, modified (S_{99}) HEWL eluted much earlier with an elution volume of 156 ml than native HEWL which had an elution volume of 186 ml (Table 2). A marked decrease in elution volume (30 ml) of S_{99} HEWL was suggestive of the increase in hydrodynamic volume of the modified derivative.

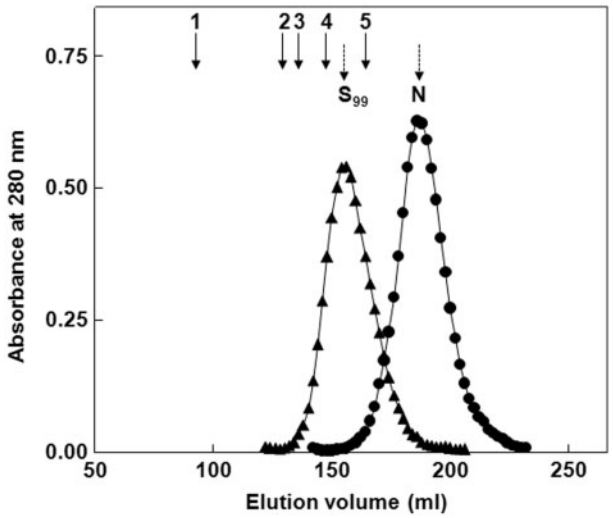

Fig. 2. **Elution profiles of native, 'N' (filled circle) and 99% succinylated, 'S_{99}' (filled triangle) HEWLs on Sephadex G-75 column (2.34 × 64 cm) equilibrated with 0.06 M sodium phosphate buffer, pH 7.0 containing 0.02% sodium azide.** Arrows show the position of elution volumes of marker proteins: 1. BSA; 2. carbonic anhydrase; 3. α-chymotrypsinogen A; 4. myoglobin and 5. cytochrome c. Sample volume of 2.0 ml containing about 8 mg protein was applied on to the column and eluted at 20 ml/h. Fractions of 2.0 ml were collected and monitored for absorbance at 280 nm.

Elution volumes of marker proteins, native and modified HEWL preparations were transformed into K_d and K_{av} values following standard procedures (*43*) and treated according to Laurent and Killander (*45*) and Ackers (*46*). Values of K_d, K_{av}, $(-\log K_{av})^{1/2}$ and erfc^{-1} K_d for native and modified HEWL preparations are shown in Table 2. Following straight line equations, Equations 7 and 8 were obtained for Laurent and Killander's plot and Acker's plot, respectively as shown in Fig. 3A and B:

$$(-\log K_{av})^{1/2} = 0.266 \text{ Stokes radius, nm} + 0.093 \quad (7)$$

$$\text{Stokes radius, nm} = 2.329 \text{ erfc}^{-1} K_d + 0.815 \quad (8)$$

Table 2. **Analytical gel filtration data for native and 99% succinylated HEWLs on Sephadex G-75 column (2.34 × 64 cm) at pH 7.0, ionic strength 0.15.**

HEWL preparation	V_e (ml)	K_d	K_{av}	$(-\log K_{av})^{1/2}$	erfc^{-1} K_d
Native	186	0.733	0.626	0.451	0.241
99% Succinylated	156	0.527	0.450	0.589	0.446

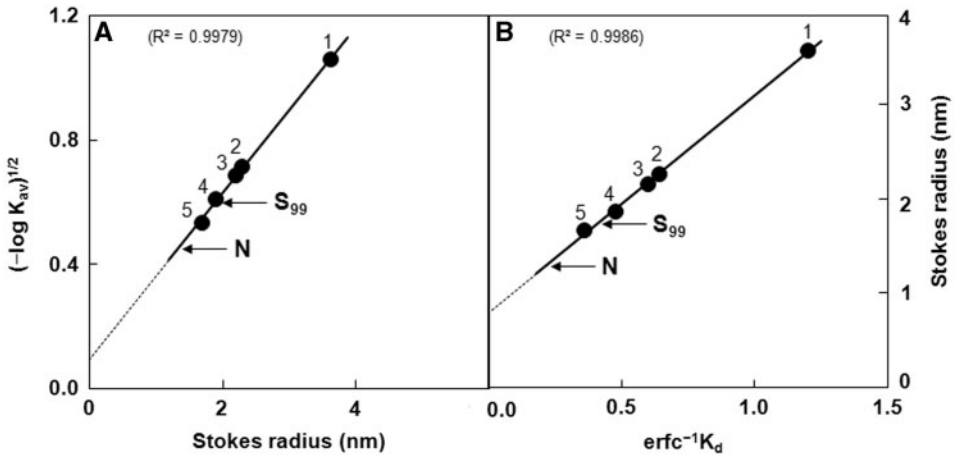

Fig. 3. **Determination of Stokes radii of native and 99% succinylated HEWLs according to (A) Laurent and Killander (*45*) and (B) Ackers (*46*).** Numbers 1–5 refer to marker proteins as indicated in the legend to Fig. 2. Positions of native, 'N' and modified, 'S_{99}' HEWL preparations are shown by arrows. Straight lines were drawn using least squares analysis.

Table 3. **Hydrodynamic properties of native and 99% succinylated HEWLs at pH 7.0, ionic strength 0.15.**

HEWL preparation	Stokes radius (nm)			Frictional ratio (f/f_o)
	From Eq. 8	From Eq. 7	Mean	
Native	1.38	1.34	1.36	0.86
99% Succinylated	1.85	1.86	1.86	1.15

Table 3 shows values of Stokes radius for native and S_{99} HEWLs as determined from Equations 7 and 8 along with their mean values. The Stokes radius of native HEWL (1.36 nm) obtained from gel filtration data was much lower than the value (1.9 nm) derived from diffusion coefficient (*54*). This was due to the abnormal elution behaviour of native HEWL on Sephadex G-75 column as it eluted much later than expected which was in agreement with a previous report (*55*), suggesting possible interaction of the enzyme with Sephadex gel (*56*). Decrease in elution volume of succinylated HEWL compared to native enzyme (Table 2) can be ascribed to the increase in molecular size of the enzyme, which was evident from the Stokes radius of S_{99} HEWL (1.86 nm), a value higher than 1.36 nm, obtained for native HEWL (Table 3).

Values of Stokes radius were used to calculate frictional ratio (f/f_o) for both native and modified HEWL preparations using the following formula (*57*):

$$f/f_o = r/(3\bar{v}_2\,M/4\pi N)^{1/3} \quad (9)$$

where r is the Stokes radius of the protein (in cm), $\bar{v}_2$ is the partial specific volume of the protein, taken as 0.703 ml/g for both native and modified HEWLs (*58*), N is the Avogadro's number (6.02×10^{23}/mole) and M is the molecular weight of the protein, taken as 14,307 Dalton (*20*) for native HEWL and 15,000 Dalton for S_{99} HEWL after considering the molecular weight contribution of succinyl groups of the protein. Values of f/f_o thus obtained for native and modified HEWLs are also listed in Table 3. A lower f/f_o value of native HEWL (0.86) compared to other globular proteins with f/f_o value as 1.0, can be attributed to the use of anomalous Stokes radius value (1.36 nm) of HEWL obtained from the calibrated Sephadex G-75 column.

There was an increase in both Stokes radius (from 1.36 to 1.86 nm) and frictional ratio (from 0.86 to 1.15) of HEWL upon succinylation (Table 3), which was indicative of gross conformational change (molecular expansion) in succinylated HEWL. This was in accordance with previous results on succinylation-induced conformational changes in other proteins (*31*, *48*, *59*, *60*). Succinylation of lysine residues converts a positive charge into a negative charge, thus increases the net negative charge on a protein by 2 units per lysine residue modified (*37*). This increase in net negative charge destabilizes the native protein conformation through electrostatic repulsion between similar charges, thereby causes expansion in its conformation and asymmetry (*31*, *59*, *60*). Our results of increase in Stokes radius and frictional ratio of S_{99} HEWL supported this contention.

Effect of Succinylation on Conformational Stability of HEWL—GdnHCl denaturation of both native and 99% succinylated HEWLs were carried out in 0.06 M sodium phosphate buffer, pH 7.0 and fluorescence spectra were recorded after excitation of protein solution at 280 nm (to excite both tyrosine (Tyr) and tryptophan (Trp) residues) and 295 nm (to excite Trp residues only). Figure 4 shows fluorescence spectra of both native (A and C) and modified (S_{99}) (B and D) HEWLs upon excitation at 280 nm (A and B) and 295 nm (C and D), respectively. Native and S_{99} HEWLs showed a similar emission maximum of 340 and 340.5 nm, respectively upon excitation at 280 nm (Fig. 4A and B); 334 and 334.5 nm, respectively upon excitation at 295 nm (Fig. 4C and D). There was an increase in fluorescence intensity of both native and S_{99} HEWL preparations, accompanied by a red shift in emission maximum with increasing GdnHCl concentrations. This seems understandable since more tryptophan residues in HEWL will be exposed to solvent as a result of denaturation (*61*, *62*). Moreover, tryptophan residues in HEWL are believed to be the major contributor to the emission spectra, as contribution of tyrosine residues has been reported as negligible (*63*). The increase in fluorescence and magnitude of red shift was smaller at both lower and higher GdnHCl concentrations for both HEWL preparations. The fluorescence spectra obtained with native HEWL (upon excitation at 280 nm) were in good agreement with previous reports (*61*, *64*).

Values of emission maximum obtained from selected fluorescence spectra of both native (N) and modified (S_{99}) HEWL preparations upon excitation at 280 and 295 nm were plotted against GdnHCl concentrations (Fig. 5A and B). As evident from the figure, denaturation of the HEWL preparations followed a two-state, single-step transition model as described earlier for native HEWL (*61*, *64*, *65*). The transition of native HEWL started at 3.0 and 3.3 M GdnHCl and completed around 5.2 and 4.9 M GdnHCl with a mid-point occurring around 4.3 and 4.1 M upon excitation at 280 and 295 nm, respectively (Fig. 5A and B). On the other hand, S_{99} HEWL showed differences in the transition characteristics compared to native HEWL, as it started much earlier around 1.6 and 2.0 M GdnHCl and completed at 3.7 M GdnHCl with a mid-point falling around 2.7 and 2.9 M GdnHCl for excitation at 280 and 295 nm, respectively. The early transition obtained with S_{99} HEWL compared to native HEWL was indicative of decrease in conformational stability of the modified HEWL.

Figure 6A and B show plots of fluorescence intensity at 360 nm obtained from the fluorescence spectra (Fig. 4) for both native (N) and succinylated (S_{99}) HEWLs upon excitation at 280 and 295 nm against GdnHCl concentration. These data (Fig. 6) were further evaluated by normalizing the fluorescence intensity into F_D values using Equation 1 as described in 'EXPERIMENTAL PROCEDURES section' and plotted them against GdnHCl concentration for both native (N) and modified (S_{99}) HEWL preparations (Fig. 7A and B). The denaturation curves showed a two-state, single-step transition, similar to those depicted in Fig. 5. The transition for native HEWL started at 3.4 and 3.5 M GdnHCl and ended around 5.2 and 4.7 M GdnHCl with a mid-point

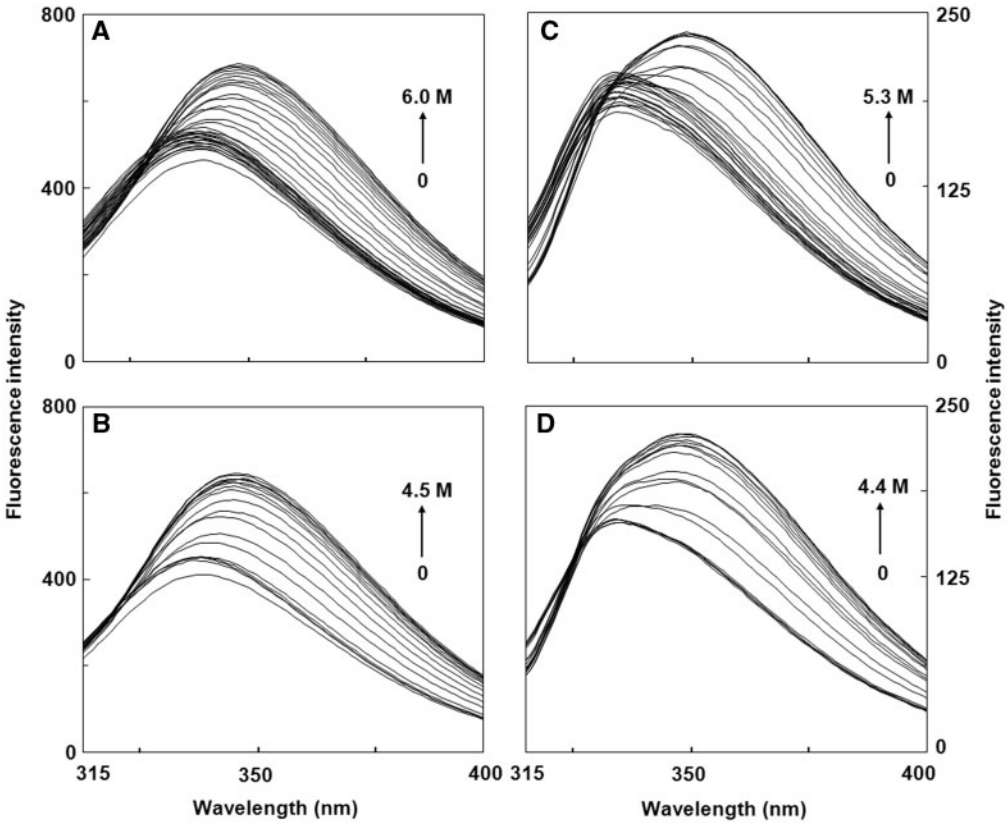

Fig. 4. **Fluorescence spectra of GdnHCl-induced denaturation of native (A and C) and 99% succinylated (B and D) HEWLs in 0.06 M sodium phosphate buffer, pH 7.0 at 25°C as followed by intrinsic fluorescence measurements upon excitation at 280 nm (A and B) and 295 nm (C and D).** Spectra from bottom to top shown by arrows correspond to increasing GdnHCl concentrations: (A) 0 to 6.0 M; (B) 0 to 4.5 M; (C) 0 to 5.3 M; (D) 0 to 4.4 M.

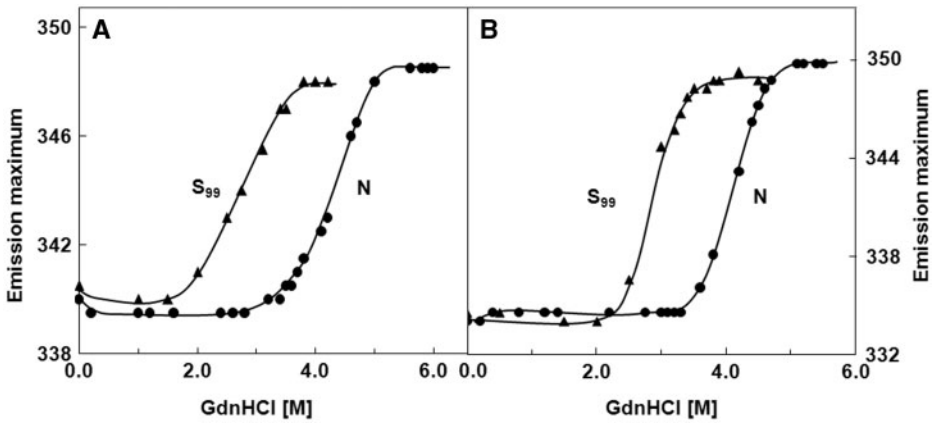

Fig. 5. **Plots showing emission maximum against GdnHCl concentration for the transition of native, 'N' (filled circle) and 99% succinylated, 'S_{99}' (filled triangle) HEWLs upon excitation at (A) 280 nm and (B) 295 nm.**

occurring around 4.1 M GdnHCl upon excitation at 280 nm (Fig. 7A) and 295 nm (Fig. 7B), respectively. Contrary to this, complete denaturation of the modified HEWL was achieved at lower GdnHCl concentration as evident from Fig. 7A and B. The transition of S_{99} HEWL started at 1.8 and 1.7 M GdnHCl and ended around 3.9 M GdnHCl with the mid-point at 2.9 M GdnHCl upon excitation at 280 and 295 nm, respectively (Fig. 7A and B). Values of start-, mid- and end-points of the transition determined for both native and S_{99} HEWLs upon excitation at 280 and 295 nm (Fig. 7) were found to be more or less similar to those obtained from Fig. 5 using emission maximum as a probe (Table 4). Moreover, the transition curves obtained in

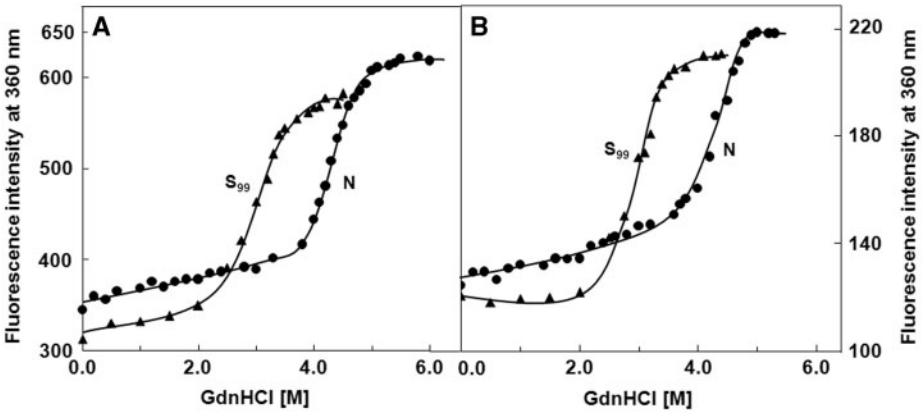

Fig. 6. **Plots of fluorescence intensity at 360 nm against GdnHCl concentration for the transition of native, 'N' (filled circle) and 99% succinylated, 'S_{99}' (filled triangle) HEWLs upon excitation at (A) 280 nm and (B) 295 nm.**

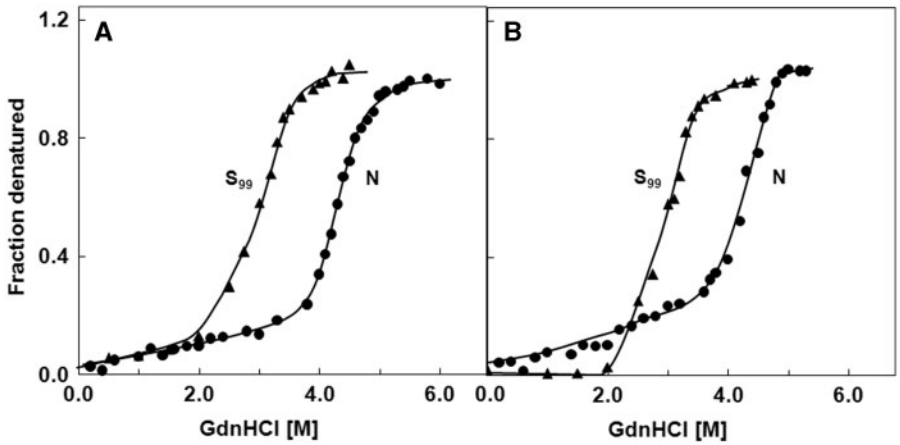

Fig. 7. **Normalized transition curves for GdnHCl denaturation (Fig. 6) in terms of F_D against GdnHCl concentration for the transition of native, 'N' (filled circle) and 99% succinylated, 'S_{99}' (filled triangle) HEWLs upon excitation at (A) 280 nm and (B) 295 nm.**

Table 4. **GdnHCl denaturation data of native and 99% succinylated HEWLs at pH 7.0.**

Transition	Native HEWL				99% Succinylated HEWL			
	Excitation at 280 nm		Excitation at 295 nm		Excitation at 280 nm		Excitation at 295 nm	
	Fig. 5A	Fig. 7A	Fig. 5B	Fig. 7B	Fig. 5A	Fig. 7A	Fig. 5B	Fig. 7B
Start-point [M]	3.0	3.4	3.3	3.5	1.6	1.8	2.0	1.7
Mid-point [M]	4.3	4.1	4.1	4.1	2.7	2.9	2.9	2.9
End-point [M]	5.2	5.2	4.9	4.7	3.7	3.9	3.7	3.9

this study with native HEWL (Figs 5 and 7) using fluorescence as a probe (in terms of start- and end-points of the transition) were akin to those reported for HEWL using other probe (*66*). That the modified HEWL had undergone significant conformational destabilization was corroborated from the marked decrease in denaturant concentration required to completely denature the modified protein compared to native HEWL (Figs 5 and 7).

In order to substantiate the above findings further, free energy of stabilization, $\Delta G_D^{H_2O}$ values were determined for both native and modified HEWL preparations using transition curves shown in Fig. 7A and B. K_D values were calculated from F_D values ranging from 0.25 to 0.75 using Equation 2 and transformed into ΔG_D values using Equation 3. Figure 8A and B show plots of ΔG_D against GdnHCl concentrations. The least squares analysis of the data (Equation 4)

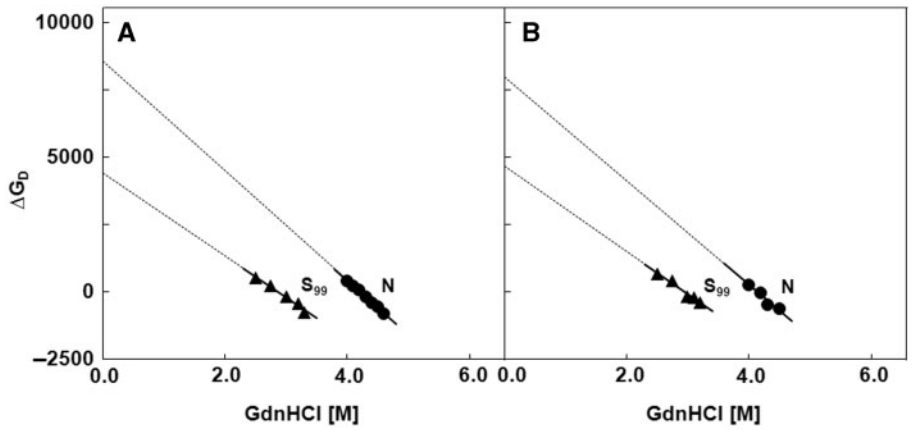

Fig. 8. **Plots of ΔG_D against GdnHCl concentration for the transition of native, 'N' (filled circle) and 99% succinylated, 'S_{99}' (filled triangle) HEWLs upon excitation at (A) 280 nm and (B) 295 nm.**

yielded $\Delta G_D^{H_2O}$ (intercept on *y*-axis) and '*m*' (slope of the linear plot) values for HEWL preparations. Values of 8,559 cal/mol and 2,033 cal/mol/M were obtained for $\Delta G_D^{H_2O}$ and '*m*' values of native HEWL using fluorescence data upon excitation at 280 nm (Fig. 8A). Similar values (7,956 cal/mol and 1,926 cal/mol/M) were obtained for $\Delta G_D^{H_2O}$ and '*m*' values of native HEWL when excitation at 295 nm was used (Fig. 8B). Values of $\Delta G_D^{H_2O}$ (8,559 and 7,956 cal/mol) obtained from the present work agreed well with the $\Delta G_D^{H_2O}$ values reported earlier for native HEWL (*65*, *66*) and lies within the range of 5–10 kcal/mol reported for many globular proteins (*6*). However, a pronounced decrease in $\Delta G_D^{H_2O}$ value (4,404 and 4,669 cal/mol) was noticed with S_{99} HEWL preparation compared to native HEWL. The '*m*' values were also decreased from 2,033 and 1,926 cal/mol/M (for native HEWL) to 1,539 and 1,592 cal/mol/M for S_{99} HEWL. Data of Fig. 5A and B were also normalized into F_D values and transformed into ΔG_D values to determine $\Delta G_D^{H_2O}$ of native and S_{99} HEWLs (Figures omitted for brevity). Similar values of $\Delta G_D^{H_2O}$ and '*m*' were obtained for both native (8,717 cal/mol and 2,011 cal/mol/M, respectively upon excitation at 280 nm; 9,119 cal/mol and 2,215 cal/mol/M, respectively upon excitation at 295 nm) and S_{99} (4,384 cal/mol and 1,600 cal/mol/M, respectively upon excitation at 280 nm; 4,366 cal/mol and 1,633 cal/mol/M, respectively upon excitation at 295 nm) HEWL preparations. The marked decrease in $\Delta G_D^{H_2O}$ and '*m*' values for S_{99} HEWL clearly suggested that modified HEWL was less stable than native HEWL. This seems to be justifiable since succinylation increased the electrostatic free energy in the modified protein (*59*). The conformational destabilization of the modified derivative correlated well with the expansion in its molecular size as reflected from the increase in Stokes radius and f/f_o values (Table 3).

Native state of proteins has been suggested to be stabilized by charge–charge interactions at neutral pH involving positively and negatively charged residues of proteins (*67* and other references therein). Lysozyme is a structurally stable protein (*53*). Shift in the isoelectric point of native HEWL (11.1) to a lower pH value (4.5–4.7) (*26*, *30*, *53*) upon succinylation was indicative of change in the net charge of the protein from positive to negative at neutral pH. This was expected as amino groups (intrinsic p*K* value around 10) in lysine residues of the protein were substituted with carboxyl groups (intrinsic p*K* value of 4.8) in the succinyl derivative. Furthermore, the net charge of both native and succinylated HEWL preparations was determined to be +8 and −9, respectively at pH 7.0 (experimental condition for denaturation) from the titration curves (*53*). Increase in net negative charge in succinylated HEWL might have disrupted the electrostatic network in the protein and destabilized the native protein conformation of HEWL. GdnHCl has been shown to produce different effects on conformational states of proteins. In case of cytochrome c, it stabilized the protein by shielding local negative charge repulsion caused by acetylation before it acted as a denaturant (*67*). However, the effect of GdnHCl on succinylated HEWL, as reported in this study was found to be different from that observed with cytochrome c in an earlier study (*67*) as unfolding transition of HEWL was significantly affected by succinylation (Figs 5–7). Furthermore, $\Delta G_D^{H_2O}$ values were also found to be different for both native and succinylated HEWLs. This clearly shows that HEWL had undergone significant conformational destabilization due to change in the overall net charge of the protein.

In addition, total loss of activity was also observed in 99% succinylated HEWL when determined with *M. lysodeikticus* suspension as the substrate. This result was in good agreement with a previous report in which 65% succinylation was found to abolish the enzyme activity completely (*29*). This loss in enzymatic activity can be ascribed to the modification of some positively charged lysine residues involved in the electrostatic interactions with negatively charged bacterial cell wall (*28*), or conformational changes or both.

Taken together, gross conformational change and destabilization of HEWL were greatly contributed by the conversion of positive charge of lysine residues into negative charge upon succinylation. These results revealed the importance of positively charged lysine

residues in maintaining the conformational stability of HEWL through electrostatic interactions. Hence, substitution of lysine residues of HEWL with negatively charged residues should not be taken as a suitable attempt to increase the stability of HEWL in mutant preparations.

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CONFLICT OF INTEREST

None declared.

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