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Effect of Succinylation of Antibodies on Their Conformation and Interaction with the Antigen

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Abstract—Using succinic anhydride, a succinylated derivative of anti-urease IgG having 49 ± 6% modification was prepared and its physicochemical and immunological properties were studied. IgG undergoes substantial changes in its native conformation on succinylation, which was mainly attributed to electrostatic destabilization of the native protein conformation. The modified IgG exhibited a decrease in its cross-reactivity with urease. This decrease is attributed to the conformational change in IgG upon succinylation and/or is due to the disruption of the lysine residues in the antigen-binding site of IgG upon succinylation, which may be involved in binding the antigen. IgG was able to bind to the specific antigen although its conformation was partially modified. Therefore, partial modification of the conformation of the antigen-binding site of IgG is permissible in order to bind to the antigen.

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Key words: antibodies, succinylation, conformation, immunological properties

The interaction of an antibody with its antigen is highly specific and involves noncovalent interactions such as hydrophobic interactions, electrostatic interactions, and hydrogen bonding [1]. An antibody molecule combines with its antigen through the complementarity determining regions (CDRs). Since the repertoire of the antigen to which it combines is large, it is understandable that the amino acid residues comprising the CDRs have greater functional versatility [2]. These residues have both polar and non-polar groups in them and generally a non-polar end is buried and a polar end protrudes out. These polar atoms have the potential of forming hydrogen bonds, and additional stability of the complexes is provided by hydrophobic interactions [3]. Furthermore, the residues of the CDRs should posses a flexible side chain to improve complementarity of the interacting surfaces.

Acylation of lysine residues of proteins has been extensively used by protein chemists for investigating different aspects of protein structure and function [4]. Among others, this modification has been used for studying the involvement of lysine residues in ligand binding to protein [5], for increasing the specificity of proteolytic digestion of protein [6], and in preparing protein derivatives with desirable biological properties [7]. Considerable conformational changes have been reported to occur after acylation of the lysine residues of various proteins [8, 9].

A number of studies suggest that lysine residues in the antigen-binding site of an immunoglobin molecule might be involved in binding the antigen [10-12]. In the present study, we have chemically modified anti-urease antibodies by succinic anhydride. The reaction of proteins with succinic anhydride results in the incorporation of succinyl groups mainly at the amino groups of lysine residues (ε-amino) and α-amino groups of the N-terminal residues [13]. In our study, we used antibodies whose lysine residues were modified up to 49 ± 6%. Succinylation of IgG was performed with the aim of understanding the role of the lysine residues in the protein structure and in binding with the antigen. Urease was used as the antigen as it is a large protein with a molecular mass of 480 kD and therefore highly immunogenic. Urease is an important enzyme as it is used in the analysis of urea and hence useful in diagnosing possible kidney disease, determination of water quality, sea water analysis, and in agricultural chemistry [14].

Abbreviations: CD) circular dichroism; CDRs) complementarity determining regions; ELISA) enzyme linked immunosorbent assay; PBS) phosphate buffered saline; TMB) tetramethyl benzidine; TNBS) 2,4,6-trinitrobenzenesulfonic acid.

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MATERIALS AND METHODS

Jack bean urease (type IX) and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma (USA). Succinic anhydride was a product of Qualigens Fine Chemicals (India). Microtiter plates were obtained from Granier (USA). Goat anti-rabbit IgG–peroxidase conjugate and tetramethyl benzidine (TMB)/H_2O_2 were supplied by Genei Laboratories (India).

The commercial urease was homogenous on the basis of size as determined by SDS-PAGE [15]; therefore, it was used in the experiments without further purification. The activity of urease was measured using urea as the substrate [16].

Healthy rabbits were injected subcutaneously with 500 μg of urease using Freund's adjuvant. The animals were boosted on day 21 and subsequently bled after a week for monitoring the production of urease-specific antibodies.

The generation of urease-specific antibodies was measured in the sera of urease-immunized rabbits by ELISA. Ninety-six-well microtiter plates were coated overnight with 100 μl of urease (10 μg/ml) in 0.05 M sodium bicarbonate buffer, pH 9.6, at 4°C. After extensive washing with phosphate-buffered saline (PBS)-Tween 20, 100 μl of blocking buffer (5% skimmed milk in PBS-Tween 20) was applied to the wells and the plates incubated at 37°C for 2 h. After removal of the blocking buffer, serially diluted test and control sera were added and binding allowed to proceed at 37°C for 2 h. The microtiter plates were then washed and incubated with 100 μl of horseradish peroxidase-conjugated goat anti-rabbit IgG at 37°C for 2 h. After the usual washing steps, the peroxidase reaction was initiated by the addition of the substrate TMB/H_2O_2, arrested by the addition of 4 N H_2SO_4, and absorbance measured at 492 nm in an ELISA reader. Urease was immunogenic and readily elicited the formation of antibodies in rabbits. The antiserum of one rabbit that exhibited the highest titer was used in all the experiments.

The rabbit serum samples that exhibited a good titer of anti-urease antibodies were saturated with ammonium sulfate to 40% concentration. The precipitate thus obtained was separated by centrifugation and dissolved in minimal volume of sodium phosphate buffer (10 mM, pH 7.0) and then was dialyzed against the same buffer. This IgG-containing sample was further purified by ion-exchange chromatography on DEAE-cellulose matrix equilibrated with the same buffer. The impurities bound to the matrix, while pure IgG was left in the supernatant. The purity of IgG was ascertained by SDS-PAGE [15]. Two bands were visible in the SDS-PAGE of IgG, and their molecular masses corresponded to those of heavy (50 kD) and light chain (25 kD) of IgG. The cross-reactivity of the purified IgG with urease was also proved by ELISA.

Anti-urease IgG was succinylated as described in the literature [17]. The IgG was dialyzed against 0.2 M Tris-HCl buffer, pH 8.6, and treated with the appropriate amount of succinic anhydride so that the molar ratio of the anhydride to the protein was 500 : 1. The mixture was then stirred for 12 h at 4°C. The succinylated protein was then dialyzed against 10 mM sodium phosphate buffer, pH 7.0, to remove excess succinic anhydride. The extent of modification of IgG was determined by the TNBS method [18]. The extent of modification was calculated using Eq. (1), from the straight line plots between the concentration of IgG and absorbance at 335 nm:

$$\text{modification degree } (\%) = 100\cdot(1 - m/m_o), \quad (1)$$

where m and m_o represent the slopes of the straight lines obtained for succinylated and native IgG, respectively.

Electrophoresis of the native and succinylated IgG was performed in 6% (w/v) polyacrylamide gel using 0.02 M Tris-glycine buffer, pH 8.2, under non-denaturing conditions [15]. About 60 μg of each protein was loaded and a current of 3-4 mA per well was applied for nearly 2 h. The gel was stained with staining solution containing 0.25% (w/v) Coomassie brilliant blue R-250 in 40% (v/v) methanol and 10% (v/v) acetic acid, and destained with 10% (v/v) acetic acid solution containing 10% (v/v) methanol at 37°C.

Fluorescence was measured using a Shimadzu model RF-540 spectrofluorimeter (Japan) equipped with a DR-3 recorder at 25.0 ± 0.1°C. The fluorescence was recorded in the wavelength range 300-400 nm with excitation of the protein solution at 280 nm for total protein fluorescence. The slits were set at 10 nm for both excitation and emission. The path length of the sample cuvette was 1 cm.

Circular dichroism (CD) was measured using a Jasco model J-720 spectropolarimeter equipped with a microcomputer. The instrument was calibrated with d-10-camphorsulfonic acid. All measurements were carried out at 25°C. Far-UV (200-250 nm) and near-UV (250-300 nm) CD spectra were taken at 0.5 mg/ml protein concentration with a 1 cm path length cell. The results are expressed as CD (mdeg) in accordance with the formula (2):

$$\text{CD (mdeg)} = \text{MRE}\cdot(10\cdot n\cdot l\cdot C_p), \quad (2)$$

where MRE is the mean residual ellipticity in deg·cm^2·dmol^{-1}, n is the number of amino acid residues, l is the path length of the cell in cm, and C_p is the molar concentration.

Immunological cross-reactivity of the native and succinylated IgG with urease were compared by ELISA and quantitative precipitin titration. ELISA was performed as described earlier except that instead of adding the sera, increasing amount of the native and succinylated IgG in the range of 50-500 ng was added to the microtiter plate. The extent of antigen–antibody reaction was determined by the precipitation titration method [19]. To a fixed amount of native and succinylated IgG (0.5 mg) in differ-

ent centrifuge tubes, increasing concentration of urease were added and the final volume was adjusted to 1 ml by the addition of 10 mM sodium phosphate buffer, pH 7.0. The tubes were incubated at 37°C for 3 h, and then at 4°C for 12 h. After centrifugation at 20,000*g* for 10 min, the resulting precipitate in the tubes was washed twice with 0.15 M NaCl and dissolved in 1 ml of 0.1 M NaOH. The protein concentration in each tube was determined [20].

RESULTS AND DISCUSSION

The reactions of proteins with succinic anhydride can modify the amino, tyrosyl, seryl, and threonyl groups. However, under the alkaline conditions as used in this study, the acylation reaction is believed to be quite specific for the amino groups of proteins [13, 21]. The fact that the reaction had occurred at amino groups of the IgG was evident from the decrease in TNBS reaction of the succinylated preparation (Fig. 1). The percent modification was calculated as described in the methods section, and it was 49 ± 6%. Both native and succinylated IgG preparations gave only one protein band in polyacrylamide gel electrophoresis, indicating homogeneity in chemical modification (Fig. 1, inset). The relative mobility of the IgG increased slightly upon succinylation, since introduction of each succinyl group replaces a positive charge on lysine (near pH 8.0) by a negative charge. The thickness and darkness of the band corresponding to succinylated IgG decreased due to poor binding of Coomassie dye to the succinylated IgG.

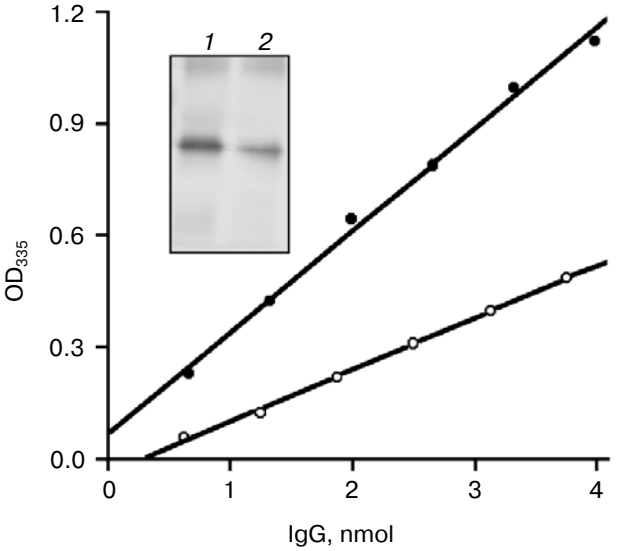

Fig. 1. TNBS reaction and polyacrylamide gel electrophoresis of native and succinylated IgG. Plots of TNBS color intensity versus protein concentration for native IgG (•), and IgG treated with 500-fold molar excess of succinic anhydride (o). The inset shows the electrophoretic pattern of native IgG (lane *1*) and succinylated IgG (lane *2*) in 6% (w/v) polyacrylamide gel.

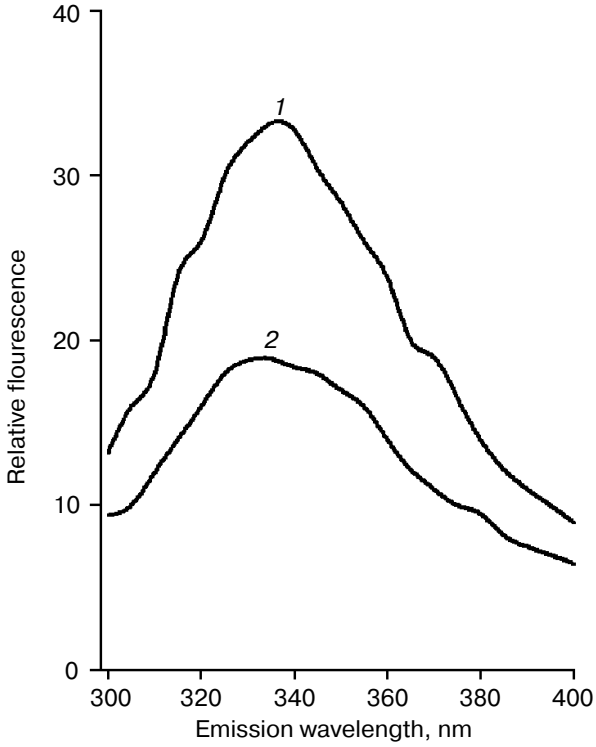

Fig. 2. Fluorescence spectra of native (*1*) and succinylated (*2*) IgG. The emission spectra of 0.5 mg/ml IgG in 10 mM sodium phosphate buffer, pH 7.0, were measured in the wavelength range 300-400 nm with excitation of the proteins at 280 nm. Slits were 10 nm for both excitation and emission.

Conformational changes in IgG upon succinylation. The fluorescence emission spectra of native and succinylated IgG are shown in Fig. 2. The emission maximum of native and succinylated IgG were 338 and 333 nm, respectively. Therefore, there is a hypsochromic shift of 5 nm in the emission maximum of IgG upon succinylation. Succinylation of IgG also produces a marked quenching in the emission spectrum, which is probably due to changes in the microenvironment around the tryptophan residues as a result of unfolding in the protein and/or introduction of carboxyl groups (contributed by succinyl groups) in the vicinity of tryptophans. The later factor has actually been found to be responsible for similar quenching in case of succinylated egg albumin [22]. The hypsochromic shift in the emission maximum of IgG upon succinylation arises because of the emission contribution from the remaining buried tryptophans in the IgG after the emission intensity of the solvent exposed tryptophans is quenched by either of the above two reasons.

The information obtained by fluorescence did not give a clear picture whether or not a conformational change is occurring in IgG upon succinylation. CD spectroscopy would be a complementary approach because it provides information about the protein structure at a submolecular level. The average secondary structure of native and modified IgG was studied by taking the far-UV CD

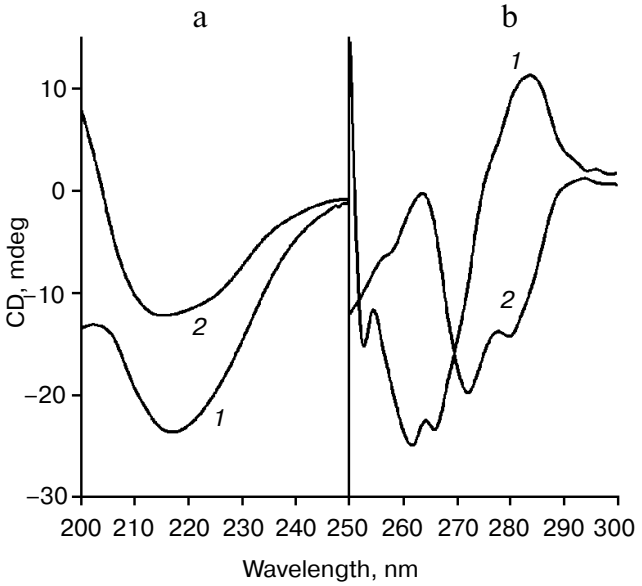

Fig. 3. CD spectra of native (*1*) and succinylated (*2*) IgG. The far-UV CD spectra (a) and the near-UV CD spectra (b) of IgG were measured in 10 mM sodium phosphate buffer, pH 7.0, using 0.5 mg/ml protein concentration.

spectra in 10 mM sodium phosphate buffer, pH 7.0 (Fig. 3a). The native and succinylated IgG preparations both showed a minimum at 216 nm. Both preparations also show a broad shoulder around 230 nm. However, succinylation of IgG resulted in a large decrease in the amplitude of the negative CD signal.

The far-UV CD spectrum of IgG is a superposition of the signals of the four structure elements: α-helix, β-sheet, β-turn, and randomly coiled conformations. Analysis of the CD spectra reveals that β-sheet is the most dominant secondary structure element present in native IgG. These results compare well with those reported in literature [23, 24]. The location of the minima and the broad shoulder does not change when IgG is succinylated, but their relative magnitude decreases. This implies that β-sheet content (secondary structure) of the protein decreases upon succinylation. The average tertiary structure of native and succinylated IgG was studied by taking the near-UV CD spectra (Fig. 3b). The near-UV CD spectrum of native IgG shows a positive band around 283 nm, which is exhibited by the aromatic chromophores, i.e. the tryptophans and the tyrosines in the protein. The spectrum also shows a broad negative band around 260 nm, consisting of three minima, i.e. at 252, 262, and 266 nm, which is due to the disulfide linkages in the protein. However, the CD spectra of succinylated IgG shows a broad negative band around 280 nm consisting of two minima at 272 and 280 nm, and a positive band around 263 nm; therefore, the microenvironment of the aromatic chromophores and the disulfide linkages has changed significantly. The near-UV CD spectra of the two preparations were very different, implying that IgG has undergone a significant change in its tertiary structure upon succinylation. Therefore, chemical modification of IgG by succinic anhydride results in the disruption of both secondary and tertiary structure of the molecule.

Succinylation of amino groups of proteins involves replacement of a proton with the succinyl group. Therefore, incorporation of each succinyl group would increase the net negative charge on the protein by two units around pH 7.0 [13]. It is therefore understandable that conformational changes occurring in a protein upon acylation have invariably been attributed to electrostatic destabilization of native protein conformation [25]. The succinylated IgG preparation would have substantially more negative charge or electrostatic free energy than the native protein. Since the various physicochemical measurements were performed at low ionic strength, i.e. 0.025, the contribution from electrostatic destabilization to protein conformation should be significant. Significant change in conformation of IgG upon succinylation as observed in our studies is likely to produce substantial changes in the molecular properties of the protein, including antigen-binding property.

Immunological properties of succinylated anti-urease IgG. Immunological cross-reactivity of the native and succinylated IgG with urease were compared by ELISA and quantitative precipitin titration. Both preparations bound to urease; however, the binding of succinylated IgG to urease was less compared with native IgG when the amount of IgG taken for ELISA was varied from 50-500 ng (Fig. 4). Therefore, the succinylated IgG prepara-

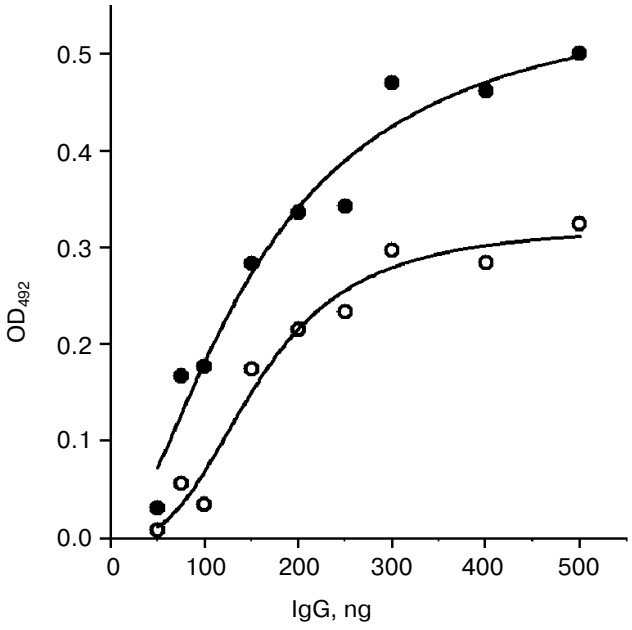

Fig. 4. ELISA showing the cross-reactivity of native (•) and succinylated (o) IgG with urease. Each point represents the average of three independent determinations.

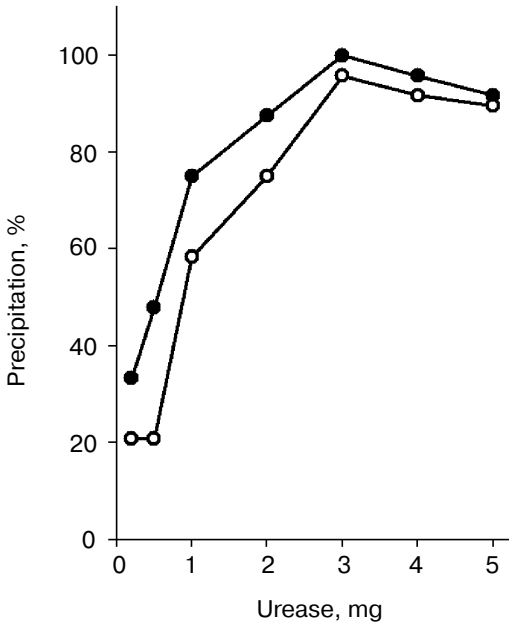

Fig. 5. Quantitative precipitin titration showing the cross-reactivity of native (•) and succinylated (o) IgG with urease. The reaction was performed in 10 mM sodium phosphate buffer, pH 7.0. Protein content, 0.5 mg. Each point represents the average of three independent determinations.

tion shows less affinity for urease compared to the native IgG preparation. The result of quantitative precipitin titration of the native and succinylated IgG with urease is shown in Fig. 5. Both preparations cross-react with urease and insoluble antigen–antibody complexes are formed. However, precipitation in the case of succinylated IgG was less as compared to native IgG when the amount of urease taken for the precipitin reaction was varied from 0.2-5.0 mg. Immunological studies revealed that succinylation of IgG caused a decrease in its cross-reactivity against urease. This decrease may be due to the conformational changes that occurs in IgG upon succinylation and/or due to the disruption of the lysine residues in the antigen-binding site of IgG upon succinylation, which may be involved in binding the antigen. Chemical modification of proteins by succinic anhydride has the limitation that it randomly modifies the surface exposed and the partially buried amino groups. Therefore, more novel site-specific approaches, for example, site-directed mutagenesis can clearly prove whether or not the lysine residues of IgG are important for antigen binding.

It is remarkable that the succinylated IgG retains its ability to bind to the specific antigen although it has undergone a substantial change in conformation upon modification. This implies that IgG is able to bind to its antigen even when its conformation is partially modified. Such behavior has also been observed for F_{ab} fragments, which exhibited positive immunoprecipitation in spite of its partially modified conformation [9]. Since both IgG and F_{ab} consist of the antigen-binding site, this implies that the partial modification in the antigen-binding site is permissible in order to bind to the antigen.

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REFERENCES

1. Van Oss, C. J., and Absolom, D. R. (1984) in *Molecular Immunology* (Atassi, M. Z., van Oss, C. J., and Absolom, D. R., eds.) Marcel Dekker Inc., New York, pp. 337-360.
2. Davies, D. R., and Cohen, G. H. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 7-12.
3. Lamminmaki, U., and Kankare, J. A. (2001) *J. Biol. Chem.*, **276**, 36687-36694.
4. Glazer, A. N. (1976) in *The Proteins* (Neurath, H., ed.) Academic Press, New York, pp. 1-103.
5. Shiao, D. D., Lumry, R., and Rajender, S. (1972) *Eur. J. Biochem.*, **29**, 377-385.
6. Butler, P. J. G., and Hartley, B. S. (1972) *Meth. Enzymol.*, **25**, 191-199.
7. Di Prisco, G., dU'dine, R., Pepe, M., and Scalengh, F. (1970) in *Chemical Reactivity and Biological Role of Functional Groups in Enzymes* (Smellie, R. M. S., ed.) Academic Press, New York, pp. 107-146.
8. Qasim, M. A., and Salahuddin, A. (1978) *Biochim. Biophys. Acta*, **536**, 50-63.
9. Nakagawa, Y., Liaw, W. V., Hunt, A. H., and Jirgensons, B. (1974) *Immunochemistry*, **11**, 483-488.
10. Ram, J. S., and Maurer, P. H. (1957) *Arch. Biochem. Biophys.*, **72**, 119-126.
11. Ara, J., and Ali, R. (1995) *Biochem. Mol. Biol. Int.*, **35**, 213-222.
12. Larson, R. S., Menard, V., Jacobs, H., and Kim, S. W. (2001) *Bioconj. Chem.*, **12**, 861-869.
13. Klotz, I. M. (1967) *Meth. Enzymol.*, **11**, 576-580.
14. Abdel, L. M. S., and Guilbaunt, G. G. (1990) *J. Biotechnol.*, **14**, 53-62.
15. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
16. Srivastava, P. K., Kayastha, A. M., and Srinivasan (2001) *Biotechnol. Appl. Biochem.*, **34**, 55-62.
17. Yamaski, Y., Sumimoto, K., Nishikawa, M., Yamashita, F., Yamaoka, K., Hashida, M., and Takakura, Y. (2002) *J. Pharmacol. Exp. Ther.*, **301**, 467-477.
18. Habeeb, A. F. (1966) *Analyt. Biochem.*, **14**, 328-336.
19. Heidelberger, M., and Kendall, F. E. (1935) *J. Exp. Med.*, **61**, 563-591.
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265-275.
21. Habeeb, A. F., Cassidy, H. G., and Singer, S. J. (1958) *Biochim. Biophys. Acta*, **29**, 587-593.
22. Kidwai, S. A., Ansari, A. A., and Salahuddin, A. (1976) *Biochem. J.*, **155**, 171-180.
23. Buijis, J., Norde, W., and Lichtenbett, J. W. T. (1996) *Langmuir*, **12**, 1605-1613.
24. Vermeer, A. W. P., Bremer, M. G. E. G., and Norde, W. (1998) *Biochim. Biophys. Acta*, **1425**, 1-12.
25. Habeeb, A. F. (1967) *Arch. Biochem. Biophys.*, **121**, 652-664.