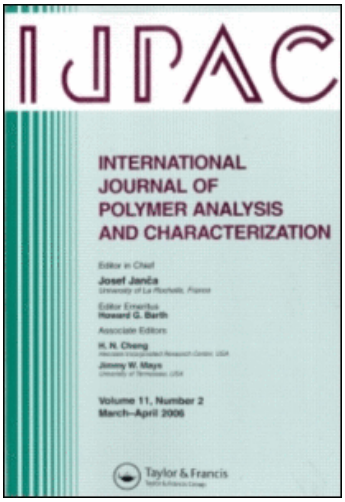

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Improved SDS-PAGE Molecular Weight Determination of a Succinylated Limed Ossein Gelatin

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Solutions of succinylated gelatin are administered intravenously to patients as a substitute for blood plasma, and the weight-average molecular weight (M_w) of the succinylated gelatin is a key parameter that governs the intravascular persistence and its clinical effectiveness as a plasma substitute. However, existing methods for determining M_w values and its distribution for succinylated gelatin yield different answers, as their calibration procedures vary. In this study, SEC/MALLS (size exclusion chromatography with multiangle laser light scattering), a technique that is independent of external calibrants, was used to verify the suitability of two potential SDS-PAGE (sodium dodecyl sulfate-

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polyacrylamide gel electrophoresis) calibration standards (i.e., globular proteins and cyanogen bromide cleaved collagen peptides). It was concluded that both globular proteins and collagen CNBr peptides were unsuitable as calibrants for molecular weight determination of succinylated gelatin by SDS-PAGE. The effect of both chemical composition and structure of proteins on separation by SDS-PAGE suggests that more suitable calibration standards would be those derived from the parent succinylated gelatin.

Keywords: Succinylated gelatin; Molecular weight; Calibration; SDS-PAGE; Size exclusion chromatography-multiangle laser light scattering

Gelatin is partially hydrolyzed collagen, a fibrous protein that gives mechanical and tensile strength to many biological structures. Type I collagen, which is present in bone, is a triple helix of two $\alpha 1$ chains and one $\alpha 2$ chain (Figure 1) that are of identical size (95 kDa) but differ slightly in their amino acid composition[1,2]. Limed ossein gelatin is manufactured from demineralized bone (ossein) by a lime soak followed by extraction into hot water at progressively higher temperatures (50°–100°C)[3]. These processes result in random bond breakage, and the resulting gelatin consists of unhydrolyzed α chains and their oligomers, as well as a broad spectrum of smaller molecular fragments. Hence gelatin is highly polydisperse and contains molecular species ranging from a few thousand to over a million Da[4,5].

As a versatile biopolymer with the useful property of forming thermally reversible gels, gelatin is widely used in the food, pharmaceutical, and photographic industries. The intravenous administration of gelatin as a plasma substitute, however, proved feasible only when its gel-forming properties had been destroyed, and resulted in the development of derivatized gelatins such as succinylated gelatin.

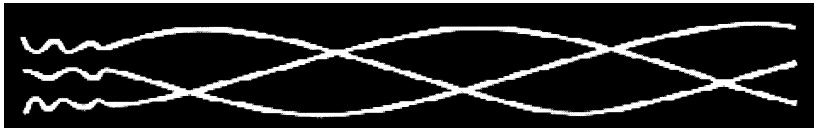

FIGURE 1 A schematic diagram of a collagen triple-helix molecule. Collagen is composed of three chains of equal molecular weight (95 kDa), but that differ in amino acid composition.

The succinylation of gelatin is carried out under hot alkaline conditions, causing further fragmentation of the gelatin polypeptides and the substitution of the positively charged $-NH_3^+$ in lysine residues with the carboxyl ($-COO^-$) groups of succinic acid[6]. As a result, succinylated gelatin has a lower weight-average molecular weight (M_w)and isoionic point than the parent gelatin[7]. The high temperatures used in its manufacture result in the fragmentation of high-molecular-weight components, especially the α chains and higher oligomers, and also induces the racemization of L-aspartic acid residues to the D form. Both of these processes reduce helix formation and markedly decrease gelling power[8]. As a result, the viscosity and gel-point of solutions of succinylated gelatin are reduced compared to the parent gelatin, making it suitable for intravenous administration as a plasma substitute[9,10].

As the M_w of succinylated gelatin governs the intravascular persistence and its clinical effectiveness as a plasma substitute, accurate determinations of molecular weight, and its distribution, are essential for controlling both the manufacturing process and the clinical performance of this material. There is presently no consensus on which method or protocol should be used to determine the molecular weight or polydispersity of succinylated gelatins. As a result, different laboratories, using different techniques, may report widely different values.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a separation method that has been used to identify and quantify discrete molecular weight fragments (e.g., the α chains) of gelatin samples.[11] In other protein applications, SDS-PAGE is calibrated using globular proteins; however, in the case of limed ossein gelatin, an internal calibration is provided by the large numbers of intact α chains (95 kDa) that survive the manufacturing process. Unfortunately, in succinylated gelatin the manufacturing process is designed to cause sufficient molecular fragmentation so that few, if any, α chains survive. Calibration therefore re-emerges as an essential prerequirement for molecular weight determinations using SDS-PAGE.

Size exclusion chromatography with multiangle laser light scattering (SEC/MALLS) theoretically provides an "absolute" method of molecular weight determination and has recently been applied to succinylated gelatins[12]. However, this technique requires major capital investment and considerable technical expertise, and the results are calculated using a series of equations and instrument calibrations. Furthermore, SDS-PAGE can often provide a higher resolution of molecular weight distribution than SEC separations for a polydisperse system such as succinylated gelatin.

The work described in this article, therefore, aims to improve SDS-PAGE molecular weight determinations of succinylated gelatin. It addresses the usefulness of globular proteins and evaluates cyanogen

bromide (CNBr) cleaved collagen peptides as potential molecular weight calibrators. CNBr collagen fragments, which are relatively monodisperse, can be produced with the added advantage of being chemically and structurally more similar to gelatin than globular proteins. The results obtained are compared with molecular weight determinations using SEC/MALLS.

MATERIALS AND METHODS

Materials

Limed ossein gelatin and succinylated limed ossein gelatin were obtained from Croda Colloids Ltd. (Luton, U.K.) and purified rat tail tendon collagen was donated by T. Sims (Collagen Research Group, University of Bristol, U.K.). Globular protein standards for SDS-PAGE (catalog no. 1610318) were purchased from Bio-Rad (U.K.). The moisture content of the gelatin samples was determined by loss of weight, on drying at 105°C for 18 h in a thermostated oven[13]. All chemicals were purchased from Sigma-Aldrich (U.K.) and deionized water, obtained from an Elga Elect system 25/50/80 (USF Elga, U.K.), was used throughout unless otherwise stated. SDS sample buffer (2x) was freshly prepared and consisted of 2 mL glycerol, 4 mL of 10% w/v SDS, 2.5 mL of 0.5 M pH 6.8 Tris-HCl buffer, 1.15 mL water and 350 μL of filtered 0.5% w/v bromophenol blue dye solution. Protogel® (Flowgen, U.K.) solution was used to prepare the hand-cast polyacrylamide gels.

SDS-PAGE: Theory

Polyacrylamide gels are formed by the copolymerization of acrylamide monomers with a cross-linking agent, such as bisacrylamide, to form a 3-dimensional gel matrix. The major factors that affect separation are the size, shape and charge of the protein samples and the pore size of the gel matrix. In the presence of SDS, the intrinsic charge of proteins is effectively masked, resulting in a constant net negative charge density, thus ensuring that separation is dependent only on molecular weight and occurs solely as a result of molecular sieving through the gel matrix[14, 15].

In SDS-PAGE, the mobility of proteins is usually proportional to the logarithm of their molecular weight. Consequently, a calibration plot of log molecular weight against distance migrated in the gel, based on protein standards of known molecular weight, enables the mass of other proteins to be deduced.

SDS-PAGE: Experimental

1% w/v (moisture-corrected weight) solutions of gelatin, succinylated gelatin and collagen were prepared as follows. Gelatin was presoaked in 50 mM pH 7.0 Tris-HC1 buffer for 30 min followed by heating in a 40°C water bath for 20 min. The soaking step was omitted for succinylated gelatin as it did not require pre-swelling to aid dissolution. Collagen was dissolved by incubating at 60°C for 25 min, the higher temperature being required to promote the denaturation of the triple-helix structure. Equal volumes (200 μL) of the 1% w/v sample solutions and SDS sample buffer (2*x*) were mixed in Eppendorf tubes and incubated at 50°C for 20 min, and centrifuged (MSE Micro Centaur, U.K.) at 13,000 rpm for 5 min to remove insoluble particles prior to loading onto the gel.

The samples were separated by SDS-PAGE according to the method of Laemmli[16]. Gels were cast in a Mini-PROTEAN II dual slab cell (Bio-Rad, U.K.) with dimensions 73 × 80 × 1 mm with 5–10 μL sample loaded onto a 7.5 or 5% discontinuous SDS-PAGE gel comprising of a stacking and resolving gel. Electrophoresis was performed in a glycine-based electrode buffer (0.1% w/v SDS, 0.025 M Tris base, 0.192 M glycine, pH 8.3) at a constant current of 20 mA until the bromophenol blue dye had migrated to the bottom of the gel. The separated proteins were visualized using a solution of Coomassie Blue R-250 (0.1% w/v in 50% v/v methanol and 10% v/v glacial acetic acid), which stained and fixed the proteins within the gel. Excess background staining was removed using a destaining solution (15% v/v isopropanol, 10% v/v glacial acetic acid). The gels were then washed and stored in water in a sealed transparent plastic bag. This staining protocol has a reported detection limit of 0.1 μg of protein[17].

Digital images of the stained gels were obtained using a desktop scanner (Sharp JX-330P SO81J) and analyzed using Phoretix® 1D v 4.0 gel analysis software. A Kodak photographic stepwedge (Cat. 1523393, Eastman Kodak, New York) was used to perform intensity calibration to ensure that the measured intensities were within the linear range of the scanner.

Preparation of Cyanogen Bromide Cleaved Collagen Peptides

Cyanogen bromide cleavage was carried out according to a modified protocol of Bornstein and Piez[18]. A solution of 9 mg of rat tail tendon collagen in 3 mL of 0.1 M HCl was incubated for 24 h with 9.8 mg of cyanogen bromide (CNBr) at 25°C. The reaction was quenched by the addition of 10 mL of water. The mixture was lyophilized, and the cleavage products were recovered after a second cycle of reconstitution with 5 mL of water and re-lyophilization. The molecular weight of the CNBr collagen peptides was obtained by matrix-assisted laser desorption

ionization/time-of-flight mass spectrometry (MALDI-TOF) analysis (carried out by Kratos Ltd., U.K.). The CNBr peptides were dissolved in 50% v/v SDS sample buffer (1 mg/mL) prior to loading onto the gel for SDS-PAGE analysis.

SEC/MALLS: Theory

SEC/MALLS does not rely on external calibration standards and is therefore regarded as an "absolute" method of molecular weight determination. The SEC component fractionates a polydisperse sample according to decreasing hydrodynamic volume, and the molecular weights obtained from each fractionated "slice," M_i, are calculated according to the equation

$$\frac{Kc}{R_\theta} = \frac{1}{P(\theta)}\left(\frac{1}{M_w} + 2A_2c\right)$$

where K is the polymer constant for a particular scattering system, c is the sample (fraction) concentration, R_θ is the excess Rayleigh factor, M_w is the weight-average molecular weight, $P(\theta)$ is a function that contains information about particle dimensions, and A_2 is the second virial coefficient, which can be taken as zero due to the extremely low concentration of the individual sample fractions ($c \approx 1 \times 10^{-5}$ m)[19,21]. If the slices are assumed to be approximately monodisperse, then the weight and number averages over the whole distribution can be found using the usual equations:

$$M_n = \frac{\sum ci}{\sum(ci/Mi)} \qquad \text{Number-average molecular weight}$$

$$M_w = \frac{\sum ciMi}{\sum ci} \qquad \text{Weight-average molecular weight}$$

SEC/MALLS: Experimental

The phosphate/chloride "Paley" elution buffer (0.0128 M $Na_2HPO_4.12H_2O$; 0.0115 M KH_2PO_4; 0.05 M NaCl, ionic strength 0.1 M, pH 6.8)[22] was prepared using HPLC-grade water (Purite®, Thame, U.K.) and then filtered through a 0.45-μm nylon-membrane filter (Whatman, 47 mm). A succinylated gelatin sample solution (4 mg/mL) was prepared using the elution buffer and then filtered through a 0.45-μm syringe filter (Whatman, 13 mm, PVDF). 100 μL aliquots of sample were applied to the SEC columns (TSK G5000 PW, TSK G4000 PW protected by a similarly packed guard column; Anachem Ltd., Luton, U.K.) via a

Rheodyne injection valve (Rheodyne 7125) and were eluted at a flow rate of 0.8 mL/min. Light scattering measurements were undertaken using a Wyatt Technology (Santa Barbara, California) Dawn F multiangle laser light scattering photometer, and protein concentration was determined using an Optilab 903 differential interferometric refractometer. The analysis was performed in triplicate at ambient temperature (20–22°C).

The specific refractive index increment (dn/dc) of succinylated gelatin was measured using an Optilab 903 RI detector at a wavelength of 633 nm in the off-line position using six different concentrations ranging from 0.1 to 0.6 mg/mL.

RESULTS

SDS-PAGE

Globular Protein Markers

Figure 2 compares denatured collagen, gelatin, and typical denatured globular protein markers separated in neighboring lanes using a 5% resolving gel and a 2.5% stacking gel. Denatured collagen shows a typical pattern of discrete monodisperse bands arising from its constituent α1 and α2 polypeptides (95 kDa), their dimers (β), and higher oligomers. Gelatin is much more polydisperse but there are two strong bands that correspond to α1 and α2 peptides[23], the migration of which are displaced by 6 to 9% from that for corresponding bands in collagen as calculated from the R_f values in Table I. By comparison, the 127 kDa protein marker had migrated further in relation to the 95 kDa α bands of collagen and gelatin, and consequently these globular proteins are poor markers for the molecular weight determination of gelatin.

Cyanogen Bromide Cleaved Collagen Peptides

Figure 3 shows parallel SDS-PAGE separations of gelatin, succinylated gelatin, and CNBr collagen peptides on a 7.5% resolving gel and a 5% stacking gel. It can be seen that succinylated gelatin contains fragments that are polydisperse with respect to molecular weight, but without any discrete bands. In particular, the α1 and α2 bands present in gelatin were not apparent. The CNBr cleaved collagen peptides form a series of distinct monodisperse fragments. These peptides plus the α1 band of gelatin were used as markers to enable the molecular weight distribution of the succinylated gelatin to be divided into six discrete bands labeled 1 to 6. The amount of protein present within each region (1 to 6 in Figure 3) was determined using digital densitometry and the results expressed as a percentage of the total protein present (Table II). The boundaries of the regions 2 to 6 were shifted up by approximately 6% to account for the different migration displayed by the α1 chains of collagen in relation to

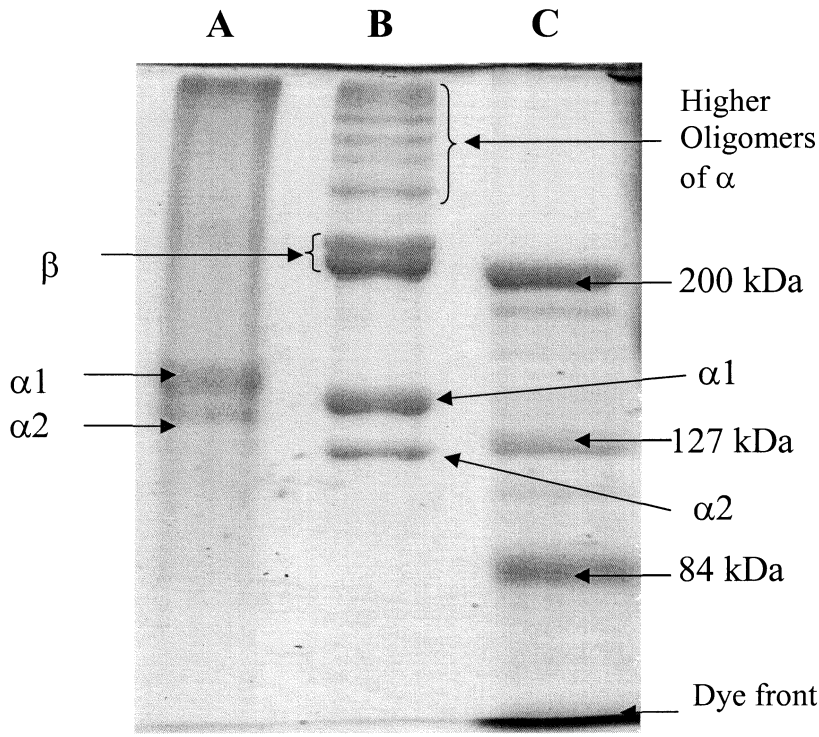

FIGURE 2 SDS-PAGE separation of gelatin (A), rat tail tendon collagen (B), and globular protein markers (C) on a 5% resolving gel and a 2.5% stacking gel.

gelatin (Table I). A further correction factor of 15% was applied to all the regions (including those already corrected by 6%) to account for the effect of succinoylation. The 15% displacement was calculated based on the mobility difference of succinylated and unsuccinylated calf collagen shown by Furthmayr and Timpl[24].

TABLE I Calculated R_f^a values for the α1 and α2 chains of gelatin and collagen.

Sample	α1	α2
Gelatin	0.47	0.53
Collagen	0.50	0.58

[a] R_f = distance migrated by band/distance travelled by dye front. See Figure 2.

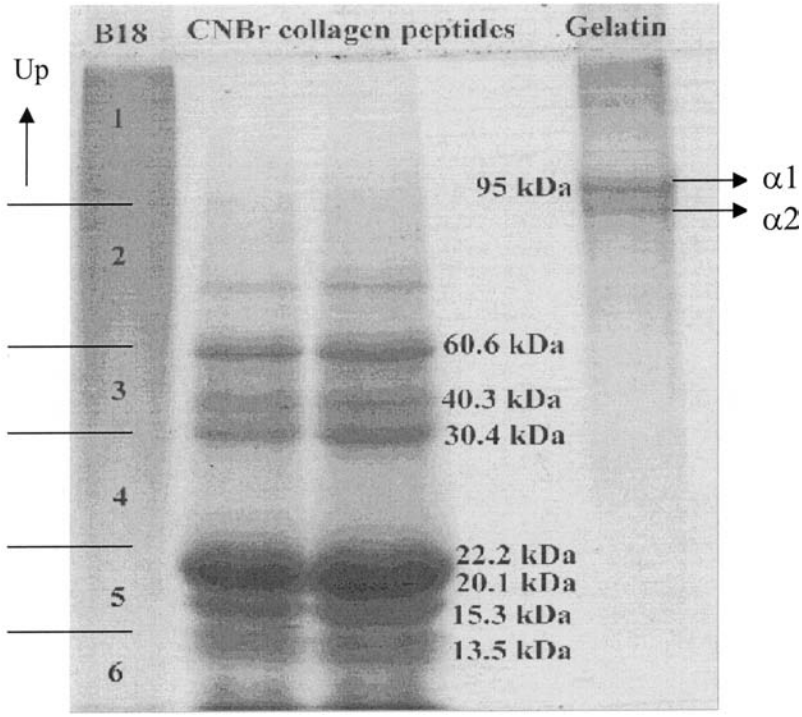

FIGURE 3 SDS-PAGE separation of succinylated gelatin (B18), collagen CNBr peptides and gelatin on a 7.5% resolving gel and a 5% stacking gel. The horizontal lines divide the succinylated gelatin lane into molecular weight regions according to the position of the adjacent markers. The migration correction factors were applied by moving the boundaries of the regions up (indicated by the arrow) by the required amount.

SEC/MALLS

A dn/dc value of 0.177 mL/g was determined for succinylated gelatin and was used in the molecular weight calculations. Figure 4 shows a typical chromatogram of succinylated gelatin separated using SEC/MALLS. The average $M_w \pm 95\%$ confidence interval calculated from three separate chromatography runs, was determined to be 44000 ± 2400 g/mol. The polydispersity (M_w/M_n) of the sample was 2.2, indicating that the sample is highly polydisperse. Figure 5 shows the cumulative molecular weight distribution of the succinylated gelatin sample. This representation enabled quantification of the amount of

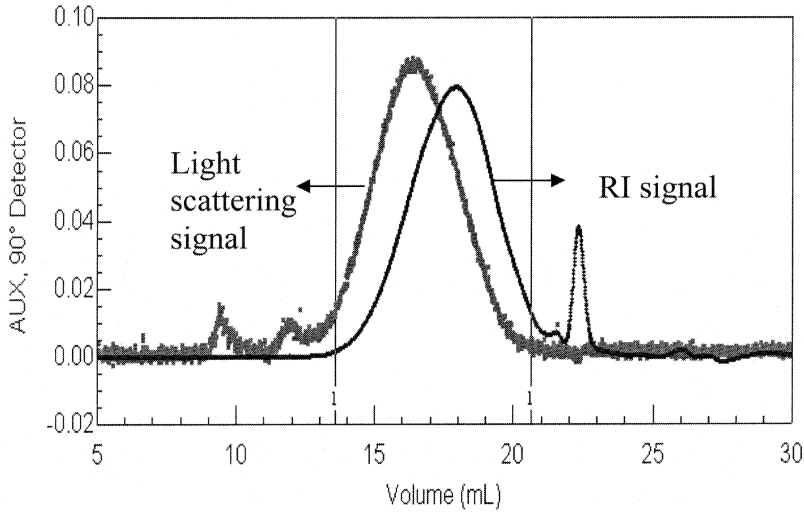

FIGURE 4 A typical SEC/MALLS chromatogram of succinylated gelatin. The response of the 90° light scattering and the AUX refractive index (RI) detectors is shown.

material within particular molecular weight ranges. The results (from SEC/MALLS) calculated for the regions 1 to 6 delineated in Figure 3 are compared with the results from SDS-PAGE in Table II.

DISCUSSION

The two equally sized (95 kDa) α chains of collagen and gelatin were resolved by SDS-PAGE with the α2 chain migrating slightly further than the α1 (Figure 2). This difference in mobility may be explained by a difference in the amount of SDS bound by the individual polypeptide chains. Kubo and Takagi[25] showed that 1 g of the α2 chain of collagen maximally bound 1.6 g of SDS whereas the corresponding value for the α1 chain was only 1.4 g. A higher amount of bound SDS would increase the negative charge on the polypeptide, consequently increasing its electrophoretic mobility toward the anode.

The displacement of the gelatin α chains by 6 to 9% with respect to the collagen α chains (Table I) could also be due to differences in SDS-binding. In the conversion of collagen to gelatin, the amide groups of asparagine and glutamine residues are removed by alkaline hydrolysis. This lowers the isoionic point of the gelatin whilst also reducing the

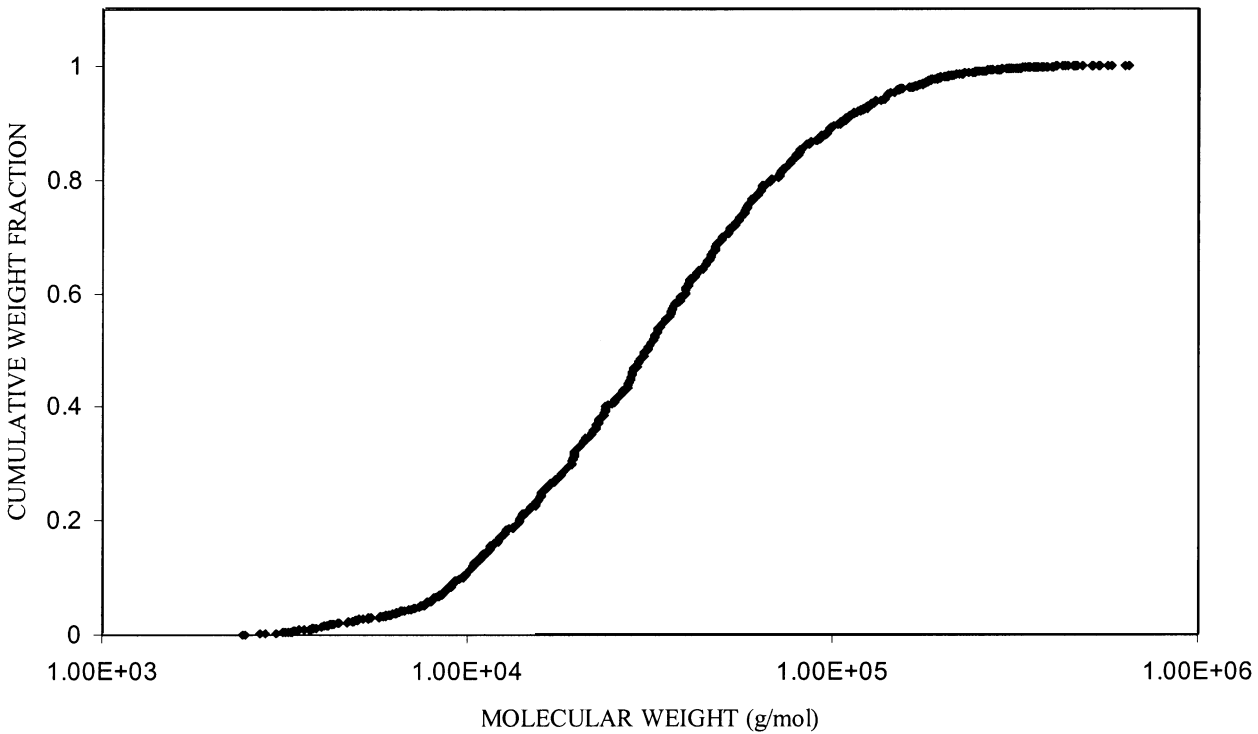

FIGURE 5 Cumulative weight fraction plot for succinylated gelatin. This was derived from the data in Figure 4 using the ASTRA® chromatography software (Wyatt Technology Corporation, California).

TABLE II Comparison of the molecular weight distribution of succinylated limed ossein gelatin obtained using SEC/MALLS and SDS-PAGE. The figures are expressed as a percentage of the total amount of protein and were calculated from Figures 3 and 5.

		SDS-PAGE		
MW regions (kDa)	SEC/MALLS	Result	Correction A[a]	Correction B[b]
(1) >95	10.5%	27.9%	27.9%[c]	25.3%
(2) 60.6–95	11.3%	36.6%	34.5%	27.3%
(3) 30.4–60.6	30.5%	13.5%	13.5%	14.5%
(4) 22–30.4	11.3%	14.6%	14.8%	14.6%
(5) 13.5–22	18.8%	5.3%	5.2%	9.0%
(6) <13.5	17.5%	2.0%	4.1%	9.3%

[a]Recalculation of marker band positions (2) to (6) by 6% to correct for mobility differences between α1 bands of collagen and gelatin (Table I).

[b]Further recalculation of marker band positions (1) to (6) by 15% to correct for reported mobility differences on succinylation (Ref.[24]).

[c]Band (1) is not corrected as it is already based on the gelatin α1 marker.

hydrophobicity of its polypeptide chains[5]. The latter may reduce binding of SDS, which would slightly lower their electrophoretic mobilities relative to those displayed by the collagen chains.

By comparison to globular protein molecular weight standards, the apparent molecular weight of both α chains was in excess of 127 kDa (Figure 2), indicating that predictions using such markers would result in an overestimation of the molecular weight of gelatin. This finding confirms the work done previously by other groups[24,26]. A possible explanation is that as the polypeptide chains of gelatin (and collagen) have a more rigid structure than globular protein chains, their migration through the gel matrix is hampered. The greater rigidity was attributed by von Hippel to the high content of proline and hydroxyproline present in collagen and gelatin[27]. Thus, it was concluded that globular proteins are unsuitable as SDS-PAGE calibrants for gelatin. This conclusion may also be applied to succinylated gelatin as it has a similar amino acid composition to gelatin.

Cyanogen bromide cleavage of collagen was carried out to generate molecular weight calibration standards that were structurally more similar to the succinylated gelatin. Cyanogen bromide (CNBr) cleaves peptide bonds adjacent to the amino acid methionine[28] and the low methionine content of collagen (approximately 6 or 7 per 1000 residues[29]) ensures that only a few well-defined peptide fragments are generated. Ideally, CNBr peptides of gelatin should have been used;

however, this would have been extremely difficult due to the highly polydisperse nature of gelatin itself. Collagen and gelatin, do however, share a similar amino acid composition, although they differ in their isoionic point, which is approximately 9 for collagen and 5 for the limed ossein gelatin[30]. In order to compensate for the difference in migration rates observed for gelatin and the collagen peptides, the position of the CNBr peptide markers was corrected by 6% (Table II), based on the differences in mobility of their respective α1 chains (Table I).

The molecular weights of the CNBr peptides were determined by MALDI-TOF analysis (Figure 3), and the values agreed closely to those obtained in a similar study[31]. However, when these peptides were used as SDS-PAGE markers to analyze the molecular weight distribution of succinylated gelatin, the results differed greatly from the SEC/MALLS profile. By SDS-PAGE, it was estimated that 64.5% of the succinylated gelatin sample had a molecular weight greater than 60.6 kDa, whereas a value of only 22.8% was obtained by SEC/MALLS. Despite correcting for mobility differences between collagen and gelatin and the effect of succinylation (Table II), the re-calculated results continued to show large differences in the molecular weight distributions obtained using the two techniques. As the molecular weights determined by SEC/MALLS are independent of external calibration standards, the comparison in Table II showed that CNBr collagen peptides are unsuitable for use as standards for the SDS-PAGE analyses of succinylated gelatin.

In the earlier study, in which the 15% displacement value between succinylated and unsuccinylated calf collagen was calculated, Furthmayr and Timpl[24] stated that the lower mobility of the succinylated collagen could not be accounted for by a difference in electrical charge (i.e., SDS-binding is not a factor). Therefore, we can hypothesize that the presence of succinyl groups on the gelatin polypeptides sterically hindered their migration through the gel matrix. However, other factors may also affect the separation of succinylated gelatin, because even after applying both correction factors, the values from SDS-PAGE failed to correlate with those obtained by SEC/MALLS (Table II). Together, this suggests that any calibration standard that will be suitable for molecular weight determination by SDS-PAGE should be derived from the parent succinylated gelatin to ensure that the same factors affect their separation.

CONCLUSION

In conclusion, gelatin and collagen migrate at different rates in relation to globular protein markers of similar size as a result of their different amino acid composition. Thus, the globular protein standards were unsuitable as SDS-PAGE calibrants for succinylated gelatin.

Furthermore, comparison of the SDS-PAGE results with the molecular weight distribution obtained by SEC/MALLS proved that CNBr collagen peptides are also unsuitable as SDS-PAGE calibrants, despite the application of migration correction factors. It would appear that any suitable SDS-PAGE standard must be derived from the parent succinylated gelatin. Although SEC/MALLS is more expensive for routine use, it has proved useful in assessing the viability of calibration standards with the aim of improving SDS-PAGE as a technique to determine the molecular weight distribution of succinylated gelatin.

REFERENCES

[1] Brown, J. C., and Timpl, R. (1995). *Int. Arch. Allergy Immunol.*, **107**, 484.
[2] Burgeson, R. E., and Nimni, M. E. (1992). *Clin. Orthop. Rel. Res.*, **282**, 250.
[3] Hinterwaldner, R. (1977a). In: *The Science and Technology of Gelatin*, eds. A. G. Ward and A. Courts, Academic, London, 295–314.
[4] Hinterwaldner, R. (l977b). In: *The Science and Technology of Gelatin*, eds. A. G. Ward and A. Courts, Academic, London, 315–365.
[5] Veis, A. (1964). *The Macromolecular Chemistry of Gelatin*, Academic, London, 127–22l.
[6] Klotz, I. M. (1967). In: *Methods in Enzymology*, ed. C. H. W. Hirs, Academic, New York, vol. 11, 576–580.
[7] Aitken, A., and Learmonth, M. (1996). In: *The Protein Protocols Handbook*, ed. J. M. Walker, Humana, Totowa, New Jersey, 343–344.
[8] Aoyagi, S., and Ohsawa, T. (1997). *Imaging Sci. J.*, **45**, 249.
[9] Wood, P. D. (1977). In: *The Science and Technology of Gelatin*, eds. A. G. Ward and A. Courts, Academic, London, 414–438.
[10] Tourtellotte, D., and Williams, H. E. (1957). In: *Recent Advances in Gelatin and Glue Research*, ed. G. Stainsby, Pergamon, London, 246–251.
[11] Bruce, M. O. (1985). In: *The Fifth RPS Symposium*, ed. S. J. Band, Royal Photographic Society, Oxford, 287–289.
[12] Loret, C., Chaufer, B., Sebille, B., Candau, S., et al. (1988). *Int. J. Biol. Macromol.*, **10**, 361.
[13] British Standards Institution (1975). *British Standard BS 757*.
[14] Harris, E. L. V., and Angal, S., eds. (1989). *Protein Purification Methods*, Oxford University Press, New York, 18–31.
[15] Hawcroft, D. M. (1996). *Electrophoresis: The Basics,* Oxford University Press, Oxford, 117.
[16] Laemmli, U. K. (1970). *Nature*, **227**, 680.
[17] Hames, B. D., and Rickwood, D., eds. (1990). *Gel Electrophoresis of Proteins*, 2nd ed., Oxford University Press, New York.
[18] Bornstein, P., and Piez, K. A. (1965). *Science*, **148**, 1353.
[19] Wyatt, P. J. (1993). *Anal. Chim. Acta*, **272**, 1.
[20] Wyatt, P. J., and Villalpando, D. N. (1997). *J. Liq. Chromatogr. Rel. Technol.*, **20**, 2169.
[21] van Holde, K. E. (1985). *Physical Biochemistry*, 2nd ed., Prentice-Hall, Englewood Cliffs, New Jersey, 209–234.

[22] Green, A. A. (1933). *J. Am. Chem. Soc*., **55**, 2331.
[23] Aoyagi, S., Ohkubo, N., and Otsubo, Y. (1997). *Imaging Sci. J*., **45**, l78.
[24] Furthmayr, H., and Timpl, R. (1971). *Anal. Biochem*., **41**, 510.
[25] Kubo, K., and Takagi, T. (1984). *Collagen Relat. Res*., **4**, 201.
[26] Cole, C. G. B., and Roberts, J. J. (1996). *J. Soc. Leather Technol. Chem*., **80**, 136.
[27] von Hippel, P. H. (1967). In: *Treatise on Collagen: Chemistry of Collagen*, ed. G. N. Ramachandran, Academic, London, vol. 1, 253–366.
[28] Smith, B. J. (1996). In: *The Protein Protocols Handbook*, ed. J. M. Walker, Humana, Totowa, New Jersey, 369–373.
[29] Eastoe, J. E. (1967). In: *Treatise on Collagen: Chemistry of Collagen*., ed. G. N. Ramachandran, Academic, London, vol. 1, 1–71.
[30] Rose, P. I. (1977). In: *The Theory of the Photographic Process*, 4th ed., ed. T. H. James, Collier Macmillan, London, 51–87.
[31] Aoyagi, S. (1988). In: *The 5th IAG Conference Proceedings*, IAG, Fribourg, 158–173.