Isolation and Characterization of Low Molecular Weight Protein from Safflower Seed (*Carthamus tinctorius* L.)

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The low molecular weight (LMW) protein fraction, often termed the 2S component, was isolated from safflower seed (*Carthamus tinctorius* L.). The LMW protein fraction, even though it sediments as a single peak with an $S_{20,w}$ value of 1.5 S in the analytical ultracentrifuge, appears to be a group of several LMW proteins as indicated by polyacrylamide gel electrophoresis and DEAE-cellulose chromatography. The protein was characterized in terms of ultraviolet and fluorescence spectra, extinction coefficient, sedimentation coefficient, carbohydrate and phosphorus contents, several enzyme activities, and secondary structure. The protein has nearly 30% α-helix and 47% β-sheet in its secondary structure. Amino acid analysis of the protein indicates that the protein is rich in glutamic acid or glutamine and arginine. A chromophore is present along with the protein. The optical properties of the protein change upon dialysis due to dissociation of the chromophore.

Safflower seed (*Carthamus tinctorius* L.) contains 35–40% oil, 15–20% protein, and 35–45% hull fraction (Betschart et al., 1975). Utilization of safflower seed proteins and protein isolates for food and feed has been attempted, but complete utilization is inhibited by color and bitter principles (Betschart et al., 1975; Betschart and Saunders, 1978; Lyon et al., 1979). However, safflower seed proteins are of good nutritional quality if color and bitter principles are removed and if they are supplemented with lysine (Betschart and Saunders, 1978). Latha and Prakash (1984) studied the total proteins from safflower seed and reported their physicochemical properties. The total proteins consist of mainly two fractions: (i) high molecular weight (HMW, 65%); (ii) low molecular weight (LMW, 25%) (Latha and Prakash, 1984). Latha and Prakash (1986) isolated and characterized the HMW protein fraction from safflower seed. In this paper, we report the isolation and characterization of the LMW protein fraction.

MATERIALS AND METHODS

Safflower seeds (variety, A-1) were obtained from Karnataka State Seed Corp. Ltd., Bangalore, India. Defatted safflower flour containing less than 1% fat was prepared as reported previously (Latha and Prakash, 1984).

Isolation of LMW Protein. A 20-g portion of 60-mesh defatted safflower meal was extracted in a rotary shaker twice with 200 mL of 0.01 M phosphate buffer, pH 7.0, containing 1 M NaCl (phosphate-saline buffer) for 1 h. The slurry was spun at 4000*g* for 20 min at 27 °C. To the supernatant, containing the total proteins, was added solid ammonium sulfate (Analar grade) in stages to give 10%, 20%, 30%, and 60% ammonium sulfate saturation. After each saturation the extract was stirred for 2 h at 27 °C and spun at 4000*g* for 20 min to separate the supernatant from the precipitate. The supernatant of 30% ammonium sulfate saturation was made to 60% saturation. The precipitate thus obtained was collected, dissolved in phosphate–saline buffer, and dialyzed against 1000 mL of the same buffer for 2 h and then against 1000 mL of 0.02 M phosphate, pH 7.5, with three changes of the buffer. After a total dialysis time of 20 h, the protein solution was spun at 4000*g* for 20 min at 27 °C and the supernatant was lyophilized.

Protein Concentration. This was determined by measuring the absorbance of the protein solution using a Perkin-Elmer 124 double-beam spectrophotometer and correlating it to the nitrogen value as determined by micro Kjeldahl method (AOAC, 1984). Based on this correlation, a value of $E^{1\%,1cm}_{280nm} = 16.0$ was obtained for freshly prepared lyophilized LMW protein. However, the $E^{1\%,1cm}_{280nm}$ value of the LMW protein fraction appears to vary with the time of dialysis due to the associated chromophore in the protein. To accurately determine the concentration of protein after dialysis, an empirical procedure was followed to correct for change in absorbance during dialysis. The absorbance spectrum of the LMW protein fraction has well-defined peaks around 330 nm (due to associated chromophore) and 265–270 nm (protein and associated chromophore). The freshly prepared protein solution had a A_{280nm}/A_{325nm} ratio of 1.02, and the dialyzed protein (2 × 1000 mL, 16 h) had a ratio of 2.58, respectively. Hence, a constant dialysis volume and time were used for routine preparation of protein solution from the lyophilized sample, and protein concentration was determined accordingly.

Ion-Exchange Chromatography: DEAE-cellulose (0.8 mequiv/g capacity) after regeneration by the procedure of Peterson (1970) was equilibrated with 0.02 M phosphate buffer, pH 7.5, and packed into a 2.5 × 30 cm column. A 330-mg portion of protein fraction in the above buffer was loaded on the column, and elution was initially carried out with 0.02 M phosphate buffer, pH 7.5 (250 mL). Further elution was carried out with a linear gradient of 0–0.4 M NaCl in the same buffer. Fractions of 4 mL were collected, and absorbance was monitored at 280 and 325 nm. NaCl was determined in the fractions by Volhard's method (Vogel, 1961).

Polyacrylamide Gel Electrophoresis: Polyacrylamide gel electrophoresis (PAGE) was carried out with a Shandon PAGE unit in Tris-glycine buffer, 0.025 M, pH 8.3. A 7.5% acrylamide gel with 0.25% methylene bisacrylamide as the cross-linking agent was used. Gels were prerun in the same buffer. Protein (100–250 μg) in 30% sucrose was loaded and electrophoresis carried out at 3 mA/gel for 70 min, at which time the indicator dye, bromophenol blue, had almost moved to the bottom of the gel. Gels were stained in 0.1% coomassie blue R-250 and destained by diffusion first in methanol–acetic acid–H_2O (3:1:6, v/v/v) and then in isopropyl alcohol–acetic acid–water (1.25:1:7.75, v/v/v).

SDS–Polyacrylamide Gel Electrophoresis: SDS-PAGE was carried out in cylindrical gel tubes on a Shandon PAGE unit following the procedure of Weber and Osborn (1969) in 0.02 M sodium phosphate buffer, pH 7.5. A 10% acrylamide gel with 0.27% methylene bisacrylamide as the cross-linking agent was used. The gels were

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prerun, and nearly 100 μg of protein was loaded to each tube. Standard proteins (lysozyme, pepsin, ovalbumin, bovine serum albumin, chymotrypsin, lactoglobulin) were used as molecular weight markers. The gels were stained and destained as mentioned above, relative mobilities measured, and molecular weights calculated.

Analytical Ultracentrifugation: Experiments were carried out at 27 °C at 59780 rpm with a 12-mm Kel F cell centerpiece in a Spinco Model E analytical ultracentrifuge fitted with a phase plate Schlieren optics and RTIC unit. A 1% protein solution was used. Photographs were taken automatically at regular time intervals, and $S_{20,w}$ values were calculated by standard procedure (Schachman, 1959).

Fluorescence Spectra: This was measured in an Aminco Bowman spectrofluorimeter at 26 °C. A protein solution of 0.03 mg/mL was excited at 285 nm and fluorescence monitored after 10 s when fluorescence intensity was constant.

Viscosity: Intrinsic viscosity of the protein was determined with an Ostwald viscometer having a flow time of 180 s with water and at various protein concentrations up to 2.8% (w/v) protein. The value extrapolated to zero protein concentration was taken as intrinsic viscosity of the protein.

Carbohydrate Estimation: Carbohydrate content of the protein fraction was determined by the phenol–sulfuric acid procedure (Montogomery, 1961).

Phosphorus Estimation: Phosphorus content of the protein fraction was estimated by the procedure of Bartlett (1959).

Enzyme Activities: Proteolytic activity was determined by using 1% casein as substrate in 50 mM citrate–phosphate buffer, pH 5.7, by the method of Chrispeels and Boulter (1975). Lipase activity was determined in 0.1 M phosphate buffer, pH 7.6, with triacetin as substrate (Fiore and Nord, 1949), and α-amylase activity was determined at neutral pH (Robyt and French, 1967).

Amino Acid Analysis: Amino acid analysis was carried out in a LKB 4150 amino acid analyzer following hydrolysis of the protein in 6 N HCl at 110 °C for 24 h (Spackman et al., 1958). Tryptophan content of the protein was determined by spectrophotometric method of Spande and Witkop (1967).

Circular Dichroism (CD): CD spectra of proteins were determined at 25 °C in 0.02 M phosphate buffer, pH 7.5, on a Jasco J-20C spectropolarimeter in the region 350–250 nm (near UV) and 260–200 nm (far UV). In the near-UV region, protein solutions of 0.7–3.5 mg/mL were employed in a 1-cm path length cell, and in the far-UV region protein solutions of 0.15–0.6 mg/mL were used in a 1-mm path length cell.

RESULTS AND DISCUSSION

LMW protein was analyzed by analytical ultracentrifugation, PAGE, and ion-exchange chromatography. Figure 1 (inset) shows the sedimentation velocity pattern of the protein at two concentrations. The pattern is a diffused single peak with a sedimentation coefficient of 1.5 S. The diffused peak possibly indicates microheterogeneity in the sample. This was also indicated by ion-exchange chromatography (DEAE-cellulose) where three major and two minor peaks were observed (Figure 1). The associated chromophore that eluted with the protein was monitored at 325 nm and is also shown in Figure 1. PAGE in buffer only showed several fast-moving bands as can be seen in the densitometer scan of the gel (Figure 2). The densitometer scan shows two major bands (constituting nearly 80% of protein) and four other bands. In SDS-PAGE, the LMW protein showed two bands of molecular weights

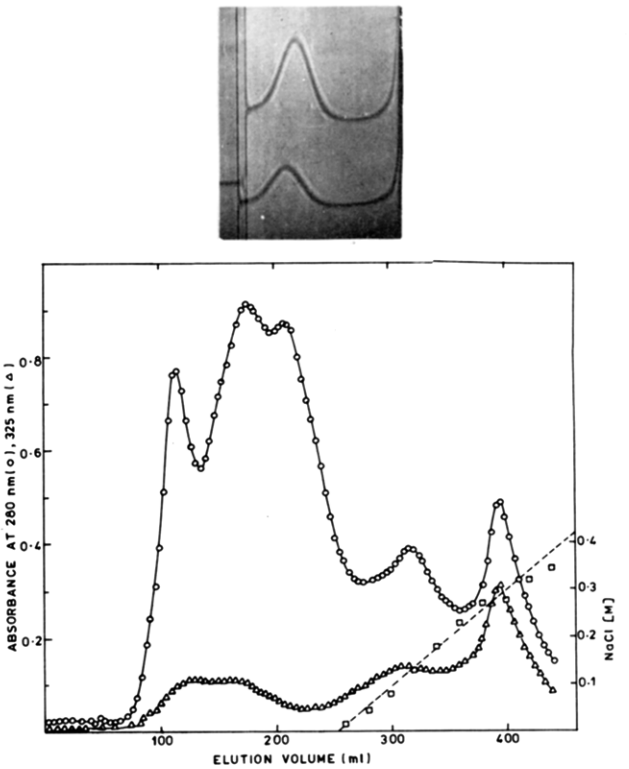

Figure 1. DEAE-cellulose ion-exchange chromatography of LMW safflower seed protein in 0.02 M phosphate buffer, pH 7.5. Inset: Sedimentation velocity pattern of safflower seed LMW protein fraction extracted and dialyzed in 0.01 M phosphate buffer, pH 7.0, containing 1 M NaCl. Protein concentrations are 1.2% (w/v) (upper) and 0.60% (w/v) (lower).

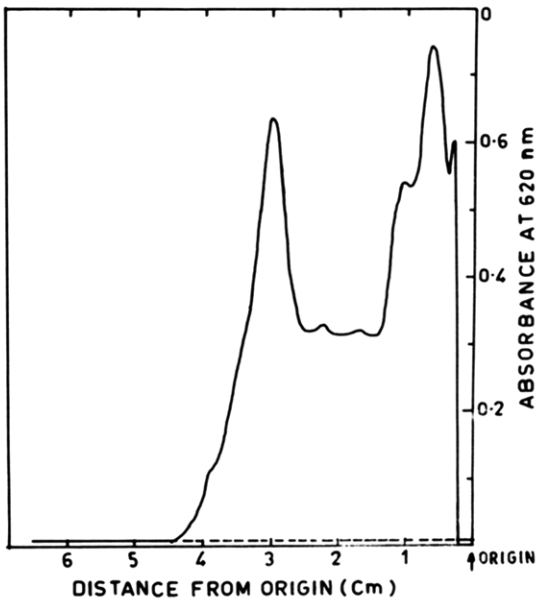

Figure 2. Densitometer scan of polyacrylamide gel electrophoresis pattern of LMW safflower protein in 0.025 M Tris-glycine buffer, pH 8.3. Origin is indicated, and scanning is from right to left.

18500 ± 800 and 13800 ● 800 (Figure 3). These results indicate that LMW protein is a group of proteins of close molecular weights, which can only sediment as a diffused single peak. The criteria for sedimenting species to be separated in an analytical ultracentrifuge and the theoretical principles underlying it are discussed by Prakash and Timasheff (1986). LMW proteins also conform to those criteria.

Chemical and physical properties of the isolated protein were characterized and are summarized in Table I.

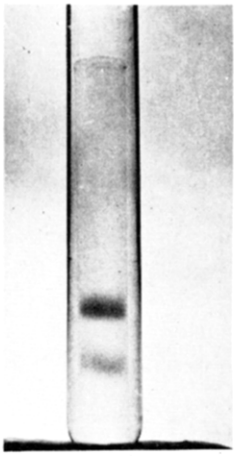

Figure 3. SDS-PAGE pattern of LMW protein.

Table I. Physicochemical Properties of Safflower Seed LMW Protein

parameter	value
sedimentation coeff, $S_{20,w}$	1.5
abs coeff, $E^{1\%,1cm}_{280nm}$ (fresh protein solution)	16.0 ± 0.3
intrinsic visc, dL/g	0.065 ± 0.002
abs max, nm	267, 280 (sh), 330
fluoresc excitation max, nm	280
fluoresc emission max, nm	325
carbohydrate content, % (w/w)	1.61 ● 0.05
phosphorus content, % (w/w)	0.05
proteolytic act., Kunitz units	14.2
lipase act., μequiv of fatty acids released/h per mg protein units	41.3

Freshly prepared protein has absorption maxima at 267 and 330 nm with a shoulder at 280 nm (Table I; Figure 4). It was reported earlier (Latha and Prakash, 1984) that the associated chromophore from defatted safflower meal has absorption maxima at 270 and 320 nm. On the basis of the data, the 330-nm peak in the protein absorption spectrum can be attributed solely to associated chromophore. The peak at 267 nm is due to the associated chromophore, and the shoulder at 280 nm is due to protein (Latha and Prakash, 1984). Because of the associated chromophore, which absorbs in the region of the protein spectrum, the usual protein absorption peak around 280 nm is modified and is seen only as a shoulder in the absorption spectrum (Figure 4). Similarly, chlorogenic acid in sunflower seed also coisolates with the protein and modifies the protein absorption spectrum (Prakash and Narasinga Rao, 1986). On dialysis of the freshly prepared LMW protein solution from safflower seed for 16 h, the intensities of the 330- and 267-nm peaks diminish, indicating that the associated chromophore is dialyzable (see Figure 4). The fluorescence spectrum of the protein fraction (inset, Figure 4) is characterized by an excitation maximum at 280 nm and an emission maximum at 325 nm, indicating the predominance of tryptophanyl residues, possibly embedded in the nonpolar environment of the protein (Shifrin et al., 1971). Similar fluorescence spectral behavior is exhibited by oilseed protein fractions from groundnut, sesame seed, sunflower seed, and safflower seed (Prakash and Narasinga Rao, 1986). The intrinsic viscosity of 0.065 dL/g (Table I) for LMW safflower protein indicates that it is not globular in shape, as are high molecular weight proteins from other oilseeds (Prakash and Narasinga Rao, 1986).

LMW protein has 1.6% carbohydrate (Table I), and this value is in the range of carbohydrate content of other LMW proteins from various oilseeds (Gururaja Rao, 1980; Madhusudhan and Singh 1985; Srinivas, 1985; Prakash and

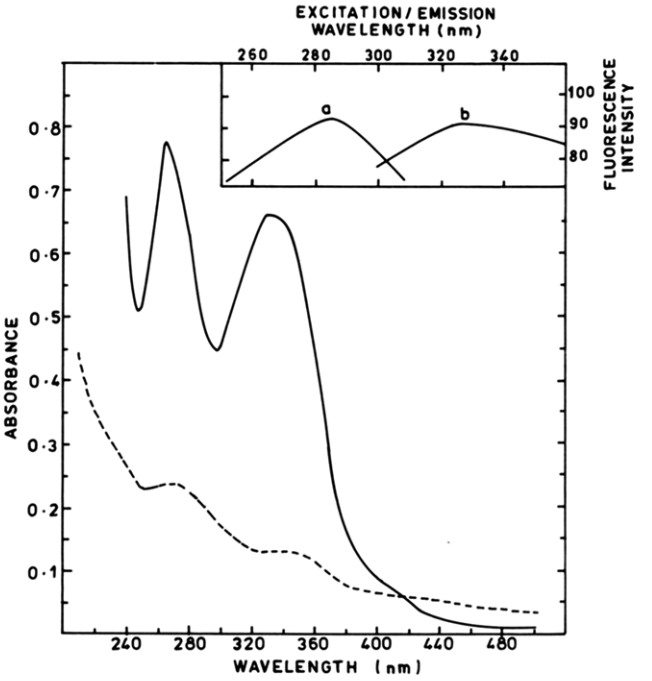

Figure 4. Ultraviolet absorption spectra of freshly prepared solution of LMW protein fraction (0.39 mg/mL, —) and dialyzed LMW protein (0.54 mg/mL, - - -) from safflower seed in 0.02 M phosphate buffer, pH 7.5. Inset: Fluorescence excitation (a) and emission (b) spectra of the LMW protein fraction.

Table II. Amino Acid Composition of Safflower Seed LMW Protein

amino acid	g/100 g of protein	amino acid	g/100 g of protein
aspartic acid[a]	6.6	methionine	1.5
threonine	2.3	isoleucine	1.8
serine	2.5	leucine	5.1
glutamic acid[a]	38.3	tyrosine	2.8
proline	1.7	phenylalanine	2.8
glycine	7.6	histidine	2.3
alanine	3.2	lysine	4.0
cysteine	1.5	arginine	12.1
valine	4.0	tryptophan[b]	0.2

[a] Values reported include contribution from aspargine and glutamine, respectively. [b] Determined by the method of Spande and Witkop (1967).

Narasinga Rao, 1986). The phosphorus content of the protein fraction is low, indicating little or no contaminatin by nucleic acids. The protein had both proteolytic and lipase activity. However, no α-amylase activity was detectable.

In Table II is shown the amino acid composition of LMW protein. It is rich in glutamic acid or glutamine and arginine. Also it contains moderate amounts of glycine and aspartic acid or aspargine. The amount of glutamic acid + glutamine (38%) in safflower LMW protein is similar to that for LMW protein of poppy seed (Srinivas, 1985), rapeseed, mustard seed (Gururaja Rao, 1980), and sesame seed (Rajendran and Prakash, 1987). CD spectra of freshly prepared and dialyzed LMW protein are shown in Figure 5. In the near-UV region the spectrum shows a single minimum at 268 nm for freshly prepared LMW protein. After dialysis for 16 h, the intensity of the 268-nm CD band is reduced by 46%, indicating contribution from both protein aromatic amino acids and the associated chromophore. The CD minimum of the protein in the near-UV region coincides with the UV absorption maximum of the protein (267 nm). The far-UV CD spectra of the protein showed a typical α-helical pattern, with minima at 222 and

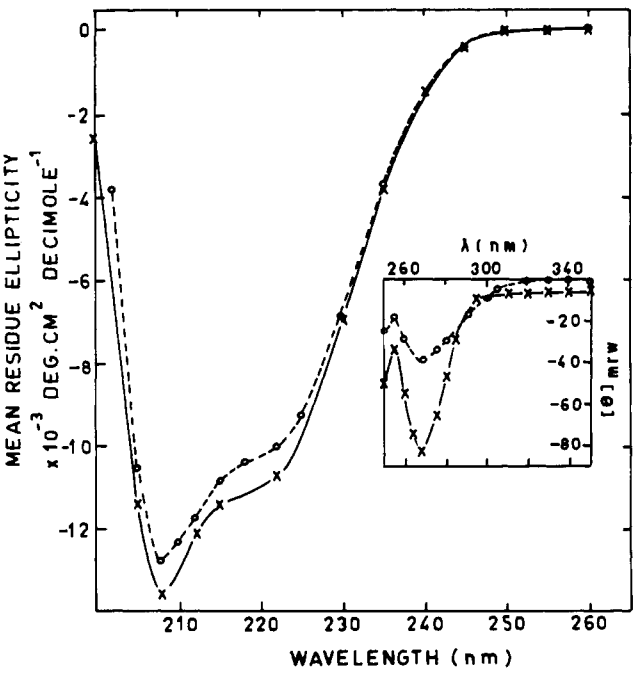

Figure 5. Far-UV and near-UV CD (inset) spectra from 200 to 360 nm in 0.02 M phosphate buffer, pH 7.5, of freshly prepared LMW protein (×) and dialyzed LMW protein (○).

208 nm. The far-UV CD spectra of freshly prepared and dialyzed LMW protein were similar, and the molar residue ellipticity values differed by only 5–6%, indicating that the associated chromophore had very little effect on the protein conformation. The average α-helical content of LMW protein was 30% by the methods of Chen and Yang (1971) and Greenfield and Fasman (1969). The β-structure content calculated by the method of Sarkar and Doty (1966) was 47%; the remainder of the structure (23%) was aperiodic. These α-helical and β-structure contents of safflower seed LMW proteins are similar to values for LMW proteins from poppy seed (Srinivas, 1985), sunflower seed (Schwenke et al., 1973), linseed (Madhusudhan and Singh, 1985), rapeseed and mustard seed (Gururaja Rao, 1980), and sesame seed (Rajendran and Prakash, 1987). The CD minimum at 208 nm is more intense than that at 222 nm. Hence, LMW safflower seed proteins appear to belong to an α + β class of proteins, containing separate α-helix and β-sheet rich regions, as described by Manavalan and Johnson (1983).

In conclusion, the LMW proteins from safflower seed appear similar to LMW proteins from various other oilseeds with respect to sedimentation coefficient, microheterogeneity, optical properties, amino acid composition, and secondary structure.

Registry No. Proteinase, 9001-92-7; lipase, 9001-62-1.

LITERATURE CITED

AOAC *Official Methods of Analysis*, 14th ed.: Association of Official Analytical Chemists: Arlington, VA, 1984.
Bartlett, G. R. *J. Biol. Chem.* **1959**, *234*(3), 466.
Betschart, A. A.; Saunders, R. M. *J. Food Sci.* **1978**, *43*(3), 964.
Betschart, A. A.; Lyon, C. K.; Kohler, G. O. In *Food Protein Sources*; University: Cambridge, U.K. 1975; Vol. 4, p 79.
Chen, Y. H.; Yang, J. T. *Biochem. Biophys. Res. Commun.* **1971**, *44*, 1285.
Chrispeels, M. J.; Boulter, D. *Plant Physiol.* **1975**, *558*, 1031.
Fiore, J. V.; Nord, F. F. *Arch. Biochem.* **1949**, *23*, 473.
Greenfield, N.; Fasman, G. D. *Biochemistry* **1969**, *8*, 4108.
Gururaja Rao, A. Ph.D. Thesis, University of Mysore, Mysore, India, 1980.
Latha, T. S.; Prakash, V. *J. Agric. Food Chem.* **1984**, *32*, 1412.
Latha, T. S.; Prakash, V. *Die Nahrung Food* **1986**, *30*(8), 833.
Lyon, C. K.; Gumbmann, M. R.; Betschart, A. A.; Robbins, D. J.; Saunders, R. M. *J. Am. Oil Chem. Soc.* **1979**, *56*, 560.
Madhusudhan, K. T.; Singh, N. *Phytochemistry* **1985**, *24*(11), 2507.
Manavalan, P.; Johnson, W. C., Jr. *Nature (London)* **1983**, *305*, 831.
Montogomery, R. *Biochem. Biophys. Acta* **1961**, *48*, 591.
Peterson, E. A. *Laboratory Techniques in Biochemistry and Molecular Biology*; Amsterdam, 1970; Vol. 2, p. 223.
Prakash, V.; Narasinga Rao, M. S. *CRC Crit. Rev. Biochem.* **1986**, *20*, 264.
Prakash, V.; Timasheff, S. N. *Methods Enzymol.* **1986**, *130*, 3.
Rajendran, S.; Prakash, V. *J. Agric. Food Chem.* **1987**, in press.
Robyt, J. F.; French, D. *Arch. Biochem. Biophys.* **1967**, *122*, 8.
Sarkar, P. K.; Doty, P. *Proc. Natl. Acad. Sci.* U.S.A. **1966**, *55*, 981.
Schachman, H. K. *Ultracentrifugation in Biochemistry*; Academic: New York, 1959.
Schwenke, K. D.; Raab, B.; Ublig, J.; Tkocz, H.; Behlke, J.; Boeltger, M.; Freimuth, U. *Die Nahrung* **1973**, *17*, 791.
Shifrin, S.; Luborsky, S. W.; Grochowski, B. J. *J. Biol. Chem.* **1971**, *246*, 7708.
Spackman, D. H.; Stein, W. H.; Moore, S. *Anal. Chem.* **1958**, *30*, 1190.
Spande, T. F.; Witkop, B. *Methods Enzymol.* **1967**, *9*, 498.
Srinivas, H. Ph.D. Thesis, University of Mysore, Mysore, India, 1985.
Vogel, A. I. *A Text Book of Quantitative Inorganic Analysis Including Elementary Instrumental Analysis*; The English Language Book Society and Longmons, Green and Co. Ltd.: London, 1961; p 266.
Weber, K.; Osborn, M. *J. Biol. Chem.* **1969**, *244*, 4406.

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