Isolation and Characterization of the 10S Fraction of Poppy Seed Proteins

H. Srinivas* and M. S. Narasinga Rao

The high molecular weight protein fraction was isolated to homogeneity by slow dialysis of 1 M NaCl extract of poppy seed meal. The $S_{20,w}$ value of the protein was 10.1S and molecular weight 215000. The intrinsic viscosity was 3.5 mL/g, suggesting a compact globular structure for the protein. SDS–PAGE gave six nonidentical subunits. The protein had an absorption maximum at 278 nm and fluorescence emission maximum at 325 nm. It was rich in aspartic and glutamic acids, arginine, and methionine. It also contained 1.15% carbohydrate and very little of phosphorus (0.01%). The CD spectrum showed that the secondary structure of 10S protein consisted of mainly β-structure and aperiodic structure with little α-helix. The protein was highly resistant to hydrolysis by proteolytic enzymes.

Poppy seed (*Papavar somniferum* L.) contains around 23% protein and 50% oil. The sedimentation velocity pattern of poppy seed proteins from different varieties consisted of four peaks having $S_{20,w}$ values ranging between 0.8 and 1.3, 6.1 and 6.6, 9.1 and 10.3, and 14.1 and 15.1 (Srinivas and Narasinga Rao, 1981). The 10S protein forms 62% of the total proteins and hence is the major fraction. There is practically no data available in the literature on this protein fraction. In this paper, the results of isolation and characterization of the 10S protein fraction are described.

MATERIALS AND METHODS

Materials. Poppy seed of the variety Dhawla chotta was obtained from the Government Opium Factory, Neemuch, Madhya Pradesh, India. Defatted poppy seed meal (80-mesh BSS) was prepared by the method reported earlier (Srinivas and Narasinga Rao, 1981). The protein content of the meal was 50.0%. Sepharose 6B and DEAE-Sephadex A 50 from Pharmacia Fine Chemicals; bovine serum albumin, ovalbumin, pepsin, papain, chymotrypsin, trypsin, lysozyme, *N*-bromosuccinimide, tris(hydroxymethyl)aminomethane, and coomassie brilliant blue R 250 from Sigma Chemical Co.; acrylamide, bis(acrylamide), *N,N,N′,N′*-tetramethylethylenediamine (TEMED) and β-alanine from Koch-Light Co.; ammonium persulfate and alkali-soluble casein from E. Merck; and β-mercaptoethanol from Fluka were used. The other chemicals used were of analytical reagent grade.

Methods. *Isolation of the 10S Protein Fraction.* The protein was isolated by slow dialysis of 1 M NaCl extract of the defatted meal. About 2 g of poppy seed meal was extracted while stirring with 20 mL of 1 M NaCl solution for 1 h at room temperature (~28 °C). The suspension was centrifuged at 6000 rpm for 20 min. The clear supernatant was dialyzed against distilled water for 18 h. The precipitate was collected by centrifugation, dispersed again in 30 mL of 1 M NaCl solution, and centrifuged at 10000 rpm for 20 min. The clear supernatant was again dialyzed against distilled water when precipitation occurred. It was centrifuged, and the precipitate thus obtained was washed with distilled water 2–3 times dissolved in 1 M NaCl solution, andcentrifuged at 10000 rpm for 20 min. For various measurements, where either the pH or salt concentration was to be maintained constant, the clear supernatant was dialyzed against 0.5 M NaCl solution or 0.025 M tris(glycine) buffer of pH 8.3 containing 0.5 M NaCl.

Protein Technology Discipline, Central Food Technological Research Institute, Mysore 570013, India.

Gel Electrophoresis. Electrophoresis was performed according to the method of Davis (1964) using 7.5% gels at 3 mA/tube until the dye front reached the bottom of the tube. For the anionic system, 0.025 M tris(glycine) buffer of pH 8.3 and, for the cationic system, 0.05 M β-alanine–acetic acid buffer of pH 4.5 were used. Approximately 100 μg of protein was applied to the gel. The gels were stained with 0.05% coomassie brilliant blue R 250 overnight and then destained in an isopropyl alcohol–acetic acid–water mixture (5:10:85).

Gel Filtration. Sepharose 6B was packed into a column of 1.5 × 100 cm and equilibrated with 0.5 M NaCl solution. About 70 mg of protein in 0.5 M NaCl solution was applied to the column and eluted with the same solvent at a flow rate of 25 mL/h. Fractions (3 mL) were collected in an automatic fraction collector, and the absorbance was measured at 280 nm.

DEAE-Sephadex Chromatography. DEAE-Sephadex A 50 was equilibrated with 0.02 M phosphate buffer of pH 7.8 and packed into a column of 2.5 × 15 cm. About 100 mg of protein in the buffer was applied to the column. Elution was carried out by using a continuous linear gradient of 0–0.8 M NaCl in the buffer. A flow rate of 30–35 mL/h was maintained, and 4-mL fractions were collected. The absorbance of the fraction was read at 280 nm. The concentration of NaCl in the fractions was determined by the titrimetric method (Vogel, 1961).

Sedimentation Velocity Experiments. The ultracentrifugation experiments were performed with a Spinco Model E analytical ultracentrifuge equipped with RTIC (rotor temperature indicating and control) unit and phase plate schlieren optics. The experiment was done at room temperature (~28 °C) at 59780 rpm with 1% protein solution in 0.5 M NaCl. From the photographs taken at different intervals of time, a $S_{20,w}$ value was calculated (Schachman, 1959).

Protein Concentration. The protein concentration of a solution was determined by Kjeldahl nitrogen estimation using 6.25 as the factor to convert nitrogen to protein. This solution was used to prepare a series of solutions of known concentration and their absorbance was measured at 280 nm. The $E^{1\%}_{cm}$ of the protein thus determined was 7.4 for the 10S protein in 0.02 M sodium phosphate buffer of pH 7.8 containing 0.5 M NaCl.

Viscosity Measurements. The measurements were made at 30 ± 0.1 °C with an Ostwald viscometer having a flow time of 176 s with distilled water. Protein solution in the concentration range of 0.5–2.5% in 0.025 M tris(glycine) buffer of pH 8.3 containing 0.5 M NaCl was used. The flow time was measured to within ±0.1 s. From the flow time, the reduced viscosity $\eta_{red} = [(t - t_0)/t_0]/c$ was calculated; here t is the flow time of the protein solution,

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t_0 that of the solvent, and C the concentration of the protein (g/ml). Corrections for the variation of the density of the solutions, which were minor, were not applied. Intrinsic viscosity was obtained from the intercept of the plot of reduced viscosity vs. protein concentration by extrapolation.

Molecular Weight Determination. Molecular weight was determined by the Archibald method as described by Narasinga Rao (1961). From the $S_{20,w}$ and intrinsic viscosity values, the molecular weight of the protein was also estimated (Schachman, 1959). The partial specific volume of the protein was assumed to be 0.75.

Determination of Subunits and Their Molecular Weight. The method of Weber and Osborne (1969) was used. Bovine serum albumin (69000), ovalbumin (44000), pepsin (35000), α-chymotrypsin (21000), and lysozyme (14400) were used as markers. Electrophoresis was carried out using 10% gels for 7 h at 7 mA/tube; about 100 μg of the protein was loaded. After electrophoresis, the gels were washed with isopropyl alcohol–acetic acid–water (5:10:85) to remove the SDS and stained with 0.05% coomassie brilliant blue R 250 for 18 h. The gels were destained with an isopropyl alcohol–acetic acid–water (5:10:85) mixture. The molecular weight of the subunits was read from a plot of electrophoretic mobility vs. the logarithm of molecular weight of proteins.

Phosphorus Estimation. Phosphorus was estimated according to the method of Taussky and Shorr (1953) using an aliquot of 4% protein solution in 0.5 M NaCl.

Carbohydrate Estimation. Carbohydrate was estimated by the method of Dubois et al. (1956) using an aliquot of 2.5% protein solution.

Amino Acid Analysis. About 30 mg of the protein was hydrolyzed in vacuo with 6 N HCl for 24 h at 110 ± 1 °C. After hydrolysis the excess acid was removed under vacuum in a rotary evaporator. The hydrolysate was taken in 10 mL of 0.2 M sodium citrate buffer of pH 2.2. Amino acid analysis was performed with LKB model automatic α-amino acid analyzer. Tryptophan was estimated by the procedure of Spande and Witkop (1967) using *N*-bromosuccinimide.

Rate of Hydrolysis with Proteolytic Enzymes. The rate of hydrolysis of the protein by trypsin, α-chymotrypsin, and papain was determined according to the procedure described by Gururaj Rao and Narasinga Rao (1981).

Absorption Spectrum. The absorption spectrum of the protein in 0.025 M tris(glycine) buffer of pH 8.3 containing 0.5 M NaCl was recorded in a perkin-Elmer double-beam recording spectrophotometer, Model 124, in the range of 240–330 nm.

Fluorescence Spectrum. Fluorescence emission spectrum was recorded in the range of 300–400 nm after excitation at 280 nm using a Perkin-Elmer fluorescence spectrophotometer, model 203. Protein solution in 0.025 M tris(glycine) buffer of pH 8.3 containing 0.5 M NaCl having an absorbance of 0.1 at 280 nm was used.

Circular Dichroism (CD). CD measurements were made at room temperature (~28 °C) in a Jasco J20 automatic spectropolarimeter equipped with a Xenon arc lamp. The CD spectrum was recorded in the range of 200–260 nm in 1-mm path length cell using 0.03% protein solution in 0.025 M tris(glycine) buffer of pH 8.3 containing 0.5 M NaCl. Mean residue ellipticities $[\theta]_{MRW}$ values were calculated by the standard procedure (Adler et al., 1973) using a value of 115 for mean residue weight (MRW), which was obtained from amino acid composition data.

RESULTS AND DISCUSSION

The isolated protein fraction was tested for homogeneity by gel filtration, ion-exchange chromatography, gel electrophoresis, and ultracentrifugation.

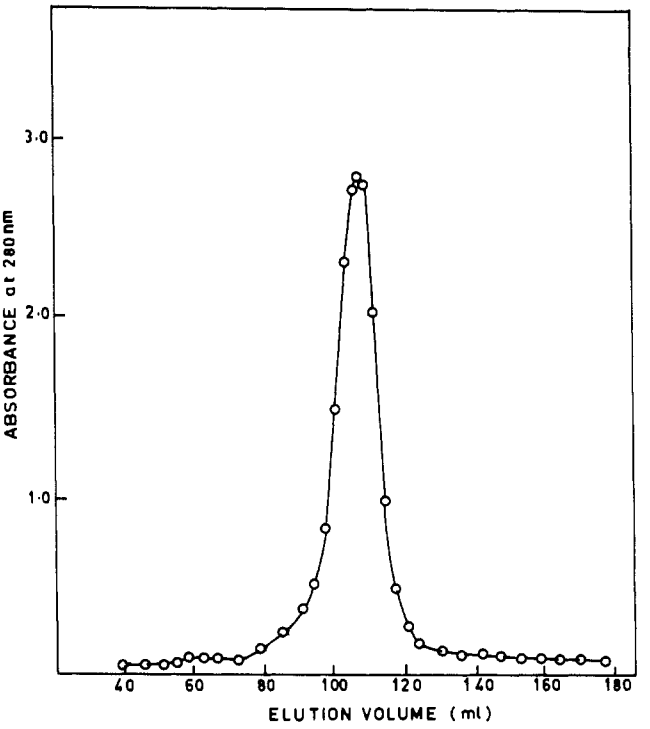

Figure 1. Gel filtration of 10S protein in 0.5 M NaCl solution on Sepharose 6B (1.5 × 100 cm) (recovery from the column: 98% of loaded protein).

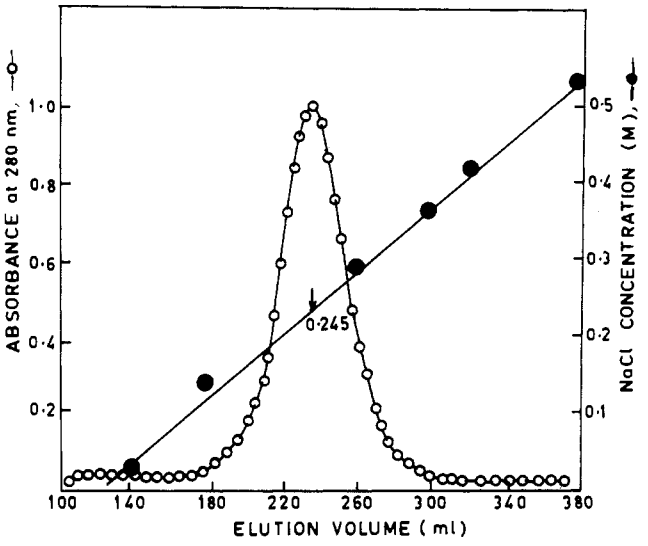

Figure 2. DEAE-Sephadex A 50 chromatographic pattern of 10S protein in 0.02 M phosphate buffer, pH 7.8 (recovery from the column: 97% of loaded protein).

Gel Filtration. The gel filtration pattern of the isolated protein consisted of a single symmetrical peak (Figure 1). Its elution volume coincided with that of the major protein fraction of poppy seed total proteins (Srinivas and Narasinga Rao, 1981).

DEAE-Sephadex A 50 Chromatography. The DEAE-Sephadex chromatographic pattern of the isolated protein consisted of a single symmetrical peak eluting at 0.245 M NaCl (Figure 2). The major protein of poppy seed total proteins also eluted at the same salt concentration.

Gel Electrophoresis. The gel electrophoresis pattern of the isolated protein at pH 8.3 and 4.5 is given in Figure 3. At pH 8.3 a single band with low mobility was observed. There was also a faint band at the origin. At pH 4.5, a

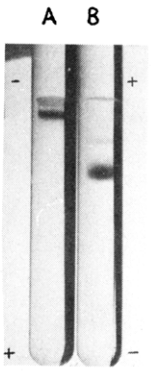

Figure 3. Polyacrylamide gel electrophoresis of 10S protein: (A) 0.025 M tris(glycine) buffer, pH 8.3; (B) 0.05 M β-alanine–acetic acid buffer, pH 4.5.

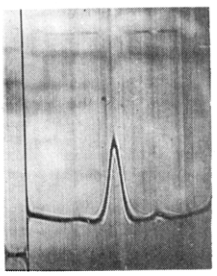

Figure 4. Sedimentation velocity pattern of 10S protein in 0.5 M NaCl solution.

Table I. Amino Acid Composition of Poppy Seed 10S Protein (g/16 g of Nitrogen)[a]

aspartic acid	8.96	isoleucine	3.27
threonine	3.38	leucine	7.03
serine	4.89	tyrosine	3.92
glutamic acid	26.38	phenylalanine	4.33
proline	5.28	histidine	2.74
glycine	4.39	lysine	2.41
alanine	4.33	arginine	9.77
valine	4.43	tryptophan	0.75
methionine	2.38		

[a] Values are the average of two independent determinations.

single band with high mobility and also a slow-moving faint band were observed.

Ultracentrifugation Studies. The sedimentation velocity pattern of the protein consisted of a symmetrical major peak with an $S_{20,w}$ value of 10.1S and a small faster moving component of 14S constituting around 8% of the total (Figure 4).

Thus, the isolated protein appeared to be better than 90% homogeneous by the techniques of ultracentrifugation and gel electrophoresis. However, gel filtration and ion-exchange chromatography did not indicate the presence of any impurities.

Carbohydrate and Phosphorus Content. The carbohydrate content of 10S protein was 1.15%. The protein contained very little phosphorus (0.01%). Thus, it appeared to be essentially a nonconjugated protein.

Amino Acid Composition. The amino acid composition of the 10S protein is given in Table I. The protein is rich is aspartic and glutamic acids, arginine, and methionine. Cystine was not detected; perhaps its proportion was too low. The lysine and tryptophan contents were low. It appears that the protein contained a higher amount of glutamic acid than that of soybean glycinin; sesame α-globulin and linseed 12S protein; methionine compared to glycinin and linseed 12S protein (Badley et al., 1975; Prakash and Nandi, 1978; Madhusudan and Singh, 1985). However, the values of lysine, aspartic acid, and tryptophan were lower compared to helianthinin, glycinin, and linseed 12S protein (Schwenke et al., 1979; Badley et al., 1975; Madhusudhan and Singh, 1985). The physicochemical data are listed in Table II.

Table II. Chemical and Physicochemical Constants of Poppy Seed 10S Protein

sediment coeff	10.15
intrinsic viscos, mL/g	3.5
mol wt	
(a) Archibald method	204 000
(b) sediment velocity and viscos method	227 000
$E_{cm}^{1\%}$ at 280 nm	7.4
carbohydr content, %	1.15
phosphorus content, %	0.01
abs max, nm	277–278
fluoresc emission max, nm	325
no. of subunits	6
secondary struct, %	
(a) α-helix	5
(b) β-pleated struct	20
(c) aperiodic struct	75

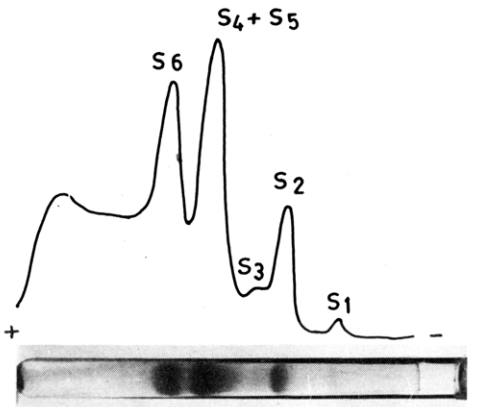

Figure 5. SDS–polyacrylamide gel electrophoresis and densitometric tracing pattern of 10S protein. Molecular weight of subunits: S_1, 62 800; S_2, 56 600; S_3, 38 100; S_4 and S_5, 31 000 and 20 100; S_6, 14 600.

Intrinsic Viscosity. The intrinsic viscosity of the 10S protein in 0.025 M tris(glycine) buffer of pH 8.3 containing 0.5 M NaCl was 3.5 mL/g. Globular proteins generally have intrinsic viscosities in the Yange of 3.0 – 4.0 mL/g (Tanford, 1961). Thus, the 10S protein of poppy seed appeared to be compact and globular in shape.

Molecular Weight of the Protein. The molecular weight of the protein by the Archibald method was 204 000 Da. From a combination of sedimentation coefficient and viscosity, a value of 227 000 Da was obtained. The molecular weight by the two method agreed reasonably well. If an average of these values is taken, the molecular weight of the 10S protein of poppy seed is 215 000 Da.

Since the molecular weight of the 10S protein was high, it was likely to be an oligomeric protein with subunit structure. SDS–PAGE was carried out to determine the number and molecular weight of the subunits. The protein consisted of at least six nonidentical subunits having molecular weights of 62 800, 56 600, 38 100, 31 000, 20 100, and 14 600 (Figure 5). Only the bands corresponding to molecular weights of 56 600, 20 100, and 14 600 were intense and prominent. Since, in SDS–PAGE experiments, the bands were not of equal intensity and stoichiometry of the subunits could not be established, no attempt was made to determine the molecular weight of the protein from these experiments.

Absorption and Fluorescence Spectrum. The absorption spectrum of the protein showed an absorption maximum at 277–278 nm and minimum at 254 nm. The

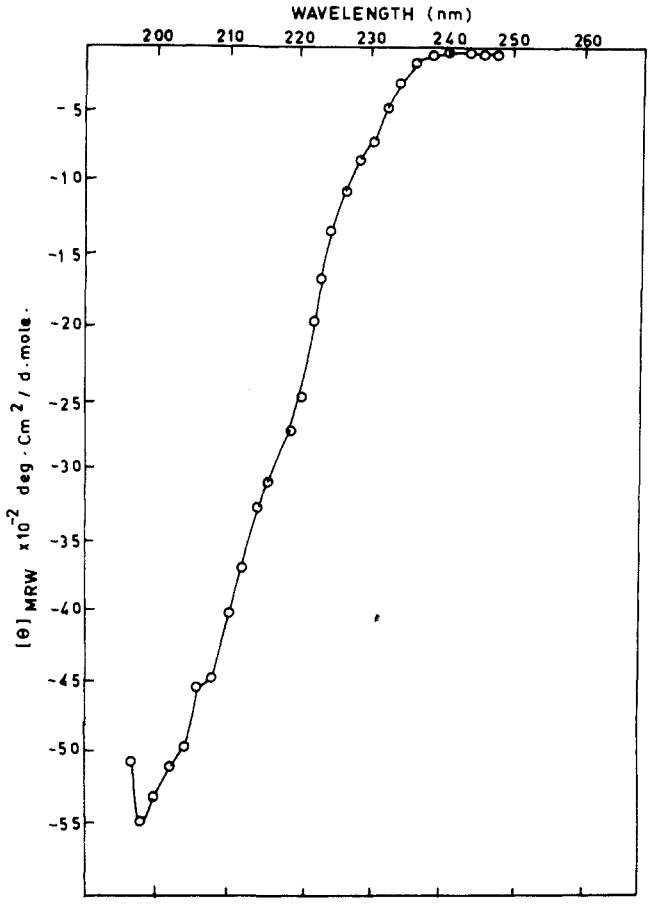

Figure 6. Far-UV-CD spectrum of 10S protein in 0.025 tris-(glycine) buffer of pH 8.3 containing 0.5 M NaCl.

ratio of the absorbance at 280 nm/260 nm was 1.63. This was compatible with the observation that the protein did not contain any phosphorus (Layne, 1957). The maximum fluorescence intensity of the protein occurred at 325 nm. The fluorescence emission of protein containing tryptophan and tyrosine residues is more characteristic of tryptophan residues, and the contribution of tyrosine is not predominant (Teale, 1960). The tryptophan content of the protein was 0.75%.

Far-UV-CD Spectrum. The far-UV-CD spectrum is shown in Figure 6. The protein exhibited a trough at 208 nm with molar residue ellipticity value of $-5512°\cdot cm^2/dmol$. Since the spectrum did not show any fine structure, the protein possibly consisted of β-structure and aperiodic structure. Using the ellipticity values at 208 and 222 nm for 100% α-helix (Greenfield and Fasman, 1969) and at 218 nm for 100% β-structure (Sarkar and Doty, 1966), the proportion of α-helix and β-structure was found to be 5% and 20%, respectively, the rest being the aperiodic structure. These values are comparable to those of oligomeric proteins of other oilseed proteins (Mohan Reddy et al., 1982; Rahman and Narasinga Rao, 1981; Gururaj Rao and Narasinga Rao, 1981).

Rate of Hydrolysis by Proteolytic Enzymes. Figure 7 shows the hydrolysis of the protein by trypsin. For comparison, data on the hydrolysis of casein are also included. Both the rate and extent of hydrolysis of the protein was much lower than that of casein. Similar results were obtained when the protein was hydrolyzed by chymotrypsin or papain. Thus, the protein was resistant to hydrolysis by these proteolytic enzymes. Since poppy seed is not reported to contain trypsin inhibitor (Srinivas and Narasinga Rao, 1981) or protein inhibitors (Sathyanarayana et al., 1956), it appears that the 10S protein of poppy

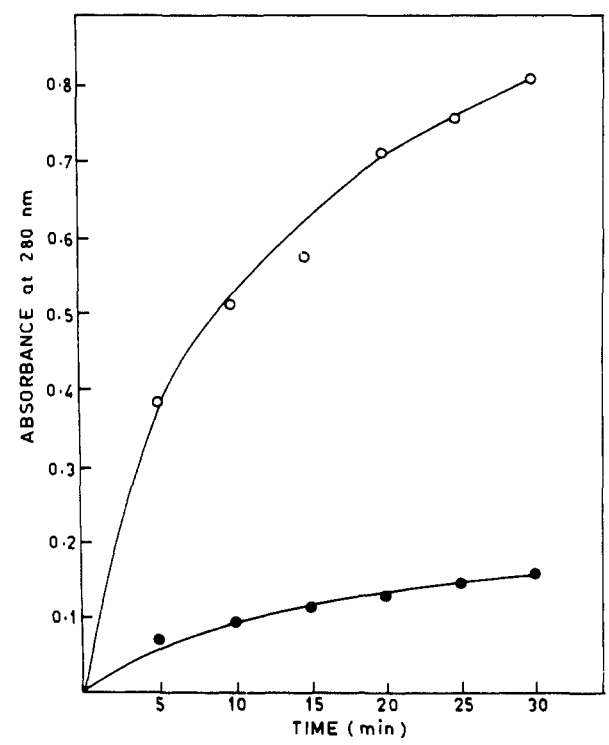

Figure 7. Rate of hydrolysis by trypsin in 0.1 M phosphate buffer, pH 7.6: ●, 10S protein; ○ casein.

seed possesses a very compact stable conformation. Similar observations have been reported in the case of other vegetable proteins: 12S protein of rapeseed/mustard seed (Gururaj Rao and Narasinga Rao, 1981) and the 11S protein of sunflower seed (Sripad, 1984).

The physicochemical properties of the major high molecular weight protein fraction from different oilseeds such as groundnut (Jayarama Shetty and Narasinga Rao, 1974, 1976), soybean (Koshiyama, 1972; Peng et al., 1984), mustard/rapeseed (Gururaj Rao and Narasinga Rao, 1981), sesame (Prakash and Nandi, 1978; Prakash et al., 1980), and sunflower (Rahma and Narasinga Rao, 1981) have been reported. These proteins have $S_{20,w}$ values of 11–12S and are rich in glutamic acid and low in lysine. The intrinsic viscosity of these proteins ranges between 3 and 5 mL/g and the molecular weight between 240 000 and 350 000 Dal. The fluorescence emission maximum of these proteins occurs at 315–325 nm. They all have predominantly β-structure and aperiodic structure with little α-helix and are resistant to hydrolysis by proteolytic enzymes.

The values for the various physicochemical properties of the high molecular weight protein fraction of poppy seed fall within the range of values reported for the high molecular weight protein fraction of various oilseeds. However, in one respect the 10S protein of poppy seed behaved differently from the high molecular weight proteins of other oilseeds. The protein could be crystallized easily by slow dialysis of 1 M NaCl extract of poppy seed meal whereas the majority of other oilseed protein could not be. It is not obvious why this protein crystallyzes so readily although it has similar amino acid composition and physicochemical properties as of other proteins.

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Compartmental Model Describing the Foliar Behavior of Tridiphane on Giant Foxtail

Philip J. McCall,* Lester E. Stafford, and Patrick D. Gavit

A compartmental model of the foliar behavior of the postemergence grass herbicide tridiphane has been developed with use of data collected in an environmentally controlled ecosystem. Rate constants for penetration, desorption, volatility, and metabolism have been determined for the chemical applied to giant foxtail as a function of temperature, spray variables, and spray adjuvants. Temperature had the most dramatic effect on the rate constants, which increased 2–5-fold with each 10 °C rise in temperature. Crop oil concentrate (COC) adjuvant significantly increased the rate of foliar penetration with no effect on the volatility rate. Spray variables such as drop size and spray volume showed smaller effects. The model was tested under the varying environmental conditions in a field experiment and found to reasonably predict the behavior of tridiphane.

INTRODUCTION

Understanding the behavior of a pesticide following a foliar application is a complex problem. In the field a chemical is subjected to a variety of environmental conditions that affect the processes that act on the system: volatilization, penetration into the plant, metabolism, translocation. In addition, additives to the spray solution and spray variables (volume, droplet size, pressure) can affect the behavior of a chemical. Spray variables or wetting agents in the spray can affect distribution and coverage of the chemical on the plant, which may increase overall penetration of the chemical into the plant. Certain additives can directly increase penetration of chemicals through the cuticle of the leaf. These variables, plus the environmental effects on the rate processes, can enhance or restrict the net amount of chemical entering a plant and subsequently affect the biological efficacy of the material. Therefore, methods that attempt to quantitate the expected behavior of pesticides in plant systems can be useful in gaining insight into identifying the most important processes and application variables controlling chemical transport and transformations in plants.

Because of the variability encountered in the field, it is very difficult to conduct a well-controlled experiment to determine the effect of individual parameters on the foliar fate of a chemical. Therefore, a laboratory ecosystem has been designed after a system described by Nash and Beall (1977). This provides a standardized system for evaluation of chemical behavior in a controlled environment where environmental variables, formulation properties, and plant properties can be studied.

Compartmental computer models have been proposed to describe foliar uptake and movement of chemicals in plants (Bridges and Farrinton, 1974). Volatility of chemicals from plant surfaces has also been studied (Hartley, 1969; Nash et al., 1977). However, a realistic environmental model must consider collectively all the processes acting on the chemical. The modeling approach we have chosen is to describe as simply as possible the overall behavior of a chemical following a foliar application that will allow prediction of behavior in the field. We have not chosen at this stage to consider various plant substrate

Agricultural Products Department, The Dow Chemical Company, Midland, Michigan 48640.

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