THE SOLUBILITY AND CRYSTALLIZATION OF TOMATO LEAF FRACTION I PROTEIN

by

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APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

Don P. Bourque — Dec 13, 1978

DON P. BOURQUE
Associate Professor of Nutrition
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Date

To my many friends in the Navigators who have made such a significant contribution to my spiritual and personal development.

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ABSTRACT

The solubility and crystallization of tomato leaf fraction I protein were examined. Solubility in $(NH_4)_2SO_4$ with two different buffers and at cold and room temperatures, at various pH and temperature, at low NaCl concentration and varying concentrations of $NaHCO_3$ and three divalent metal ions, Mg^{++}, Ca^{++}, and Mn^{++}, were studied. Tomato fraction I protein was slightly less soluble in $(NH_4)_2SO_4$ than tobacco fraction I protein. When 0.2 M PO_4 was used as a buffering agent instead of 50 mM Tris-HCl/MES, tomato fraction I protein was significantly less soluble in $(NH_4)_2SO_4$. Compared to that of tobacco, little difference was noted in thermal precipitation, in isoelectric point, and in solubility (after dialysis to a salt free buffer) of the tomato fraction I protein. The tomato fraction I protein was significantly more soluble than that of tobacco at various concentrations of bicarbonate and divalent metal ions.

The crystallization of tomato fraction I protein was attempted by two methods: free interface diffusion and micro diffusion cells. Both methods yielded crystals of a "football" shape. Although the two methods approach equilibrium differently, final equilibrium conditions for optimum crystallization were similar: 15-20% saturation $(NH_4)_2SO_4$,

pH 5.5 to 6.5 in 0.2 M K-PO_4 buffers and protein concentrations of 5 to 15 mg/ml.

CHAPTER I

HISTORY AND BACKGROUND INFORMATION

Since man became an omnivore after Noah's flood he has been making use of plant leaf proteins using herbivores to process them into meat. Today men are attempting to use leaf protein more directly as food.

Leaf Protein as a Food Source

A concise review on leaf protein concentrates has been written by Pirie (1975), a leader in the field who has recently retired. As he notes, interest in leaf protein goes back to 1773 when Rouelle wrote a review on the subject. But it was not till 1925 that Ereky suggested that leaf protein should be used as a human food source. Slade re-stated this concept in 1937 and research toward this end developed in the 40's in Pirie's laboratory. Support for the use of leaf protein concentrates came as England faced the possibility of a blockade in World War II and some large scale equipment was tested for suitability for extracting juice from leaves. After the war interest waned and then was revived for four to five years after the lend-lease program ended. Later Dr. Pirie developed small self-contained units that were placed in India to be operated by a single village unit which extracted juice from locally

grown vegetation. These are no longer in use, however (Bickoff, 1975).

With the present concern about a world food shortage interest is returning to leaf protein concentrates as a source of dietary protein. Protein concentrates have been made from 20-30 species of plants (Gerloff, Lima, and Stahmann, 1965), but only with alfalfa and tobacco has progress been made toward commercial applications. Many of the species used are now considered weeds, some of which, like water weeds, grow in untillable land. Tapping leaf protein has great potential in increasing food production per acre of land. One source of leaves that could relatively easily be developed is by-product leaves. Pirie has estimated that 60,000 tons of protein are wasted in England in destroyed potato leaves (Pirie, 1975). Other crops that could be used are sugar beets, peas, and tomatoes.

Although protein concentrates have been made from many sources, alfalfa has been studied most thoroughly in the U. S. because of its high yield of protein per acre. The Western Regional Research Laboratory of the U.S.D.A. has set up a pilot plant which produces three leaf protein products. The first is the solids left after grinding and pressing the leaves. The second is from a heat treatment of the juices and the third is the result of using a high temperature heat treatment. The first two are green and are only fit for animal use. The third, however, is a white

bland powder and is suitable for human use (Edwards et al., 1975).

Another crop that has been studied is tobacco. The Beltsville Maryland Agricultural Research Center has developed a process which has commercial potential in which fraction I protein, the major leaf protein, is crystallized. The tobacco still can be used for commercial smoking and chewing. The removal of the protein from the leaf solids makes smoking less hazardous.

Fraction I Protein

Identification

In 1947 Wildman and Bonner isolated two protein fractions from spinach leaves by ammonium sulfate precipitation. The precipitated protein, called fraction I, was found to be homogeneous by electrophoresis while the remaining fraction, fraction II, consisted of a mixture of proteins. Since then the fraction I protein has been shown to be ribulose 1, 5-bisphosphate carboxylase (RuBPCase) E. C. 4.1.1.39) (Racusen and Foote, 1965; Van Noort and Wildman, 1964; Weissbach, Horecker, and Hurwitz, 1956). This enzyme catalyzes one of the key reactions of life: photosynthetic incorporation of carbon dioxide into sugar. The sugar substrate used is ribulose 1, 5-bisphosphate (RuBP) which, after carboxylation, is split to produce two moles of 3-phosphoglycerate. Magnesium is required for

the reaction:

$$\begin{array}{l} CH_2OPO_3^{=} \\ C{=}O \\ CHOH \\ CHOH \\ CH_2OPO_3^{=} \end{array} + HCO_3^{-} \text{ (or } CO_2) \xrightarrow{Mg^{++}} 2 \begin{array}{l} COOH \\ CHOH \\ CH_2OPP_3^{=} \end{array}$$

In addition the enzyme has an oxygenase function (Andrews, Lorimer, and Tolbert, 1973). In this role RuBP is oxidized by molecular oxygen producing phosphoglycolate and 3-phosphoglycerate:

$$\begin{array}{l} CH_2OPO_3^{=} \\ C{=}O \\ CHOH \\ CHOH \\ CH_2OPO_3^{=} \end{array} + O_2 \xrightarrow[CO_2]{Mg^{++}} \begin{array}{l} COOH \\ CHOH \\ CH_2OPO_3^{=} \end{array} + \begin{array}{l} CH_2OPO_3^{=} \\ COOH \end{array}$$

Both Mg^{++} and CO_2 are required for the reaction to occur in vitro. The CO_2 is required first in a rate limiting reaction (Badger and Lorimer, 1976).

The balance between these two reactions is very important to chloroplast metabolism where they occur. It is thought that both reactions share the same catalytic site. If RuBP is carboxylated the carbon is retained by the chloroplast and may end up as stored starch. If, however, it is oxidized, carbon can leave the chloroplast in the form of glycolate which is eventually lost as CO_2. Different

intermediates in the Calvin cycle can preferentially stimulate either the oxygenase or carboxylase activities (Ryan and Tolbert, 1975). The balance is influenced by light and by changes in pH, Mg^{++} concentration, phosphate intermediates, adenylate energy charge, and the reducing environment (Jensen and Bahr, 1977). It has been shown in tomato that the carboxylase activity decreases and the oxygenase activity increases as the tomato fruit ripens (Bravdo et al., 1977).

Characteristics

The enzyme has a molecular weight of 560,000 daltons (Trown, 1965; Paulson and Lane, 1966). It is composed of two subunits (Rutner and Lane, 1967), the large with a molecular weight of 55,000 daltons and the small with a molecular weight of 15,300 daltons (Gray and Kekwick, 1974). The large subunit is the location of the catalytic site (Sugiyama and Akazawa, 1970) which is believed to be the same for both the carboxylase and oxygenase activities. It is similar in all species that have been examined thus far in amino acid composition (Kawashima and Wildman, 1971) and in antigenic sites (Gray and Kekwick, 1974). The small subunit is thought to be regulatory and is different for each species. The protein consists of 8 each of the large and small subunits (Baker et al., 1975).

Fraction I Protein in Plants

Fraction I protein is located in the stroma of chloroplasts of all C_3 plants. In C_4 plants it is only located in the bundle sheath cell chloroplasts with phosphoenolpyruvate carboxylase, which gives C_4 plants their distinction, found only in mesophyll cells (Huber, Hall, and Edwards, 1976). The large subunit is coded for by chloroplast genes (Chan and Wildman, 1972; Singh and Wildman, 1973) while the small is coded for by nuclear genes (Kawashima and Wildman, 1972). Using isoelectrofocusing of fraction I protein much genetic information has been obtained for the genus Nicotiana (for review, Kung, 1976). Mutant RuBPCase has been found in a study of sixty mutant tomato plants (Anderson, Wildner, and Criddle, 1970), 4 of which showed altered RuBPCase. Two had higher and two lower activity and each set had another mutation on the same chromosome. The mutant proteins also varied in kinetics and electrophoretic mobility.

Its presence in green plants makes fraction I protein probably the most abundant protein in the world. A large percentage of the total protein in leaves is RuBPCase, about 16% of spinach leaf homogenate (Paulson and Lane, 1966). Table 1 lists various species and the approximate amount of fraction I protein in them. It should be noted, however, that the amount of RuBPCase is not constant during the plant life cycle. In tobacco (Nicotiana tobacum)

Table 1. Protein Content in Various Leaves

Plant	Protein in mg/gm fresh weight		
	RuBPCase	Other soluble	Total leaf homogenate
Rumex	3.7	14.4	27.1
Spinach	3.0	11.7	22.9
Maize	1.4	14.9	27.0
Dark-grown beans	3.7	22.5	34.4
Light-grown beans	4.3	11.0	29.3

From Goldthwaite and Bogorad (1971).

the amount can vary from 17% of the cytoplasmic protein in young leaves to 55% just before blooming and drop down again to 25% (Dorner, Kahn, and Wildman, 1957).

Nutritional Value

Studies have been conducted using leave concentrates as a supplemental protein source in animal and human diets. The alfalfa protein concentrate is very promising as a supplement for poultry feed (de Fremery et al., 1975) and also in diets for rats, swine, and rabbits (Cheeke, 1975). Human feeding experiments have been conducted in Jamaica, India, and Nigeria with generally favorable results. Experiments were done with malnourished children in which

weight and height gains and improved alertness were noted (Bickoff, 1975; de Fremery et al., 1975).

When fraction I protein is compared to the FAO recommendations for humans it is a complete protein with an amino acid score of 100. Table 2 shows the essential amino acid composition of various proteins. In a study (Wildman, Kwanyuen and Ershoff, 1977) using recrystallized tobacco fraction I protein, twenty young rats were divided into two groups. One was fed a normal diet with casein as the protein source and the other with twice crystallized tobacco fraction I protein. The protein was at a 10% level with sucrose as a source of carbohydrates. After a four week period, the average protein efficiency ratio (PER) for each group was calculated. At each interval the PER was higher for the fraction I protein fed rats with the final value of 3.01 as compared to 2.83 for the casein fed group.

This study raises the potential for the use of fraction I protein as stated in the following abstract (Wildman et al., 1977, p. 165):

> It is anticipated that the crystalline protein may have medical applications such as reducing the frequency of hemodialysis for patients suffering renal failure, maintaining adequate protein nutrition of patients with pylorospasms and other disorders of the gastro-intestinal tract, and as a more adequate milk replacer for infants and children allergic to cow's and goat's milk. The absence of taste together with superior nutritional quality would indicate F-I-P [fraction I protein] to have

Table 2. Essential Amino Acid Content of Various Protein Sources

Amino acid	Gram amino acid per 16 gram nitrogen						
					Fraction I		
	1973 FAO standard	Hen's egg	Cow's milk	Alfalfa white protein	Tobacco	Spinach	Spinach beet
Lysine	5.5	6.4	7.8	6.5	6.0	6.0	6.1
Histidine	--	2.4	2.7	2.9	3.3	3.7	4.9
Threonine	4.0	5.1	4.6	5.8	5.7	5.9	5.9
Methionine + Cystine	3.5	5.5	3.1	3.7	2.0[a]	4.4	3.8
Valine	5.0	7.3	6.9	7.2	5.8	5.8	7.4
Isoleucine	4.0	6.6	6.4	5.4	3.4	4.0	4.6
Leucine	7.0	8.8	9.9	9.4	9.4	9.4	8.9
Phenylalanine + Tyrosine	6.0	10.0	10.0	11.7	12.2	12.9	11.6
Tryptophan	1.0	1.6	1.4	2.4	--	4.6	2.2

[a]Methionine only, Cystine not available.

From Food and Agriculture Organization (1973), Edwards et al. (1975), Kawashima and Wildman (1970), and Williams et al. (1974).

advantages over current protein hydrolysates employed in medically supervised reducing diets.

Crystallization of Fraction I Protein

Previous Work

In June, 1972, Dr. S. G. Wildman's laboratory at UCLA published a simple procedure for the crystallization of tobacco fraction I protein (Chan et al., 1972). This procedure involved grinding leaves in a high salt buffer, centrifugation, passing through a Sephadex G-25 column, concentration, incubation in Mg^{++} and HCO_3^-, and dialysis against a salt free buffer. Crystals would form overnight. Subsequent work at UCLA produced two other tobacco crystal forms which were suitable for X-ray diffraction methods (Baker, Won Suh, and Eisenberg, 1977).

This method was then simplified by Lowe (1977). In his procedure the tobacco leaves are ground in high salt buffer, heated to 40°C for 10 minutes, centrifuged, and then desalted on a Sephadex G-50 column. Then Mg^{++} and HCO_3^- were added and crystals formed after 1-3 days at 4°C.

Attempts to crystallize other species have failed until very recently. Sargit Johal has obtained crystals of spinach, potato, soybean, and alfalfa fraction I in Dr. Bourque's laboratory at The University of Arizona. Polyethylene glycol is used as a precipitant in vapor diffusion boxes to induce crystallization (Johal and Bourque, 1978).

It is possible that nature routinely crystallizes fraction I protein in vivo as a storage mechanism. Several workers have observed crystals in chloroplasts of dark-grown leaves or leaves which were stored in darkness or low light overnight (Sprey, 1976). Sprey has obtained evidence that these crystals are fraction I protein. A fraction rich in the crystals gave two peaks on SDS gels with mobilities of subunits of RuBPCase, has an elution pattern on Sephadex G-100 the same as RuBPCase, and has the same antigenic properties as the purified enzyme.

Crystal Formation

Little is understood about the forces which cause proteins to crystallize. McPherson (1976) has written an excellent review on the subject. To form any crystal a minimization of free energy is required. For small ionic solids and compounds the forces involved are well known. However the complexity and size of protein molecules makes it difficult to understand the interactions involved.

Regardless of the size of the molecule, however, the minimum free energy occurs when they are arranged in a crystal. Most often this occurs when molecules are fully solvated. If this is approached too rapidly the molecules will aggregate and precipitate out of solution. If done slowly the molecules may be able to arrange themselves into a crystal lattice. Therefore the approach most commonly

used is to slowly approach a solubility minimum with little supersaturation. Many factors influence the solubility of proteins and a protein can have many different solubility minima. The nature of the electrolyte and its concentration, the protein concentration, pH, and temperature are major variables which are manipulated. The more that is known about the solubility of the protein being investigated the easier it is to predict which condition will be most probable for crystal formation.

Two techniques that have been successful in inducing protein to crystallize have been micro diffusion cells (Zeppezauer, Eklund, and Zeppezauer, 1968) and free interface diffusion (Salemme, 1972). With the former method capillary tubes approximately 2.5 mm inside diameter are sealed at one end with dialysis membrane. The protein solution is placed inside the tube and the other end is sealed with nonpermeable material. The capillaries are then placed vertically or horizontally in a precipitant solution, which can be left constant or varied as desired. The precipitant slowly diffuses through the dialysis membrane until the protein solution is at the same concentration of precipitant as the outside solution.

The free interface diffusion method approaches equilibrium differently. A protein solution is carefully placed above the precipitant. The final concentration of each is therefore dependent not only on the starting

concentrations of each but on the ratios of the volumes of protein and precipitant solution. The initial interface will be supersaturated and form seed crystals which will grow as the two solutions slowly mix. Variations in the initial concentrations, volume of each solution, and ratio of the volumes will influence the possibility of crystallization in addition to other factors previously noted.

Goals of This Investigation

Choice of Tomatoes as a Source of Fraction I Protein

As noted above, by-product leaves are a good potential source of protein. This thesis examines tomato fraction I protein. Tomatoes are members of the Solanaceae, the nightshade, family which also includes the potato, tobacco, petunia, and some poisonous plants such as belladonna and mandrake. Tomatoes probably originate from Mexico. They were brought to Europe in the early sixteenth century where they were grown in Italy and France (Morrison, 1938). The first references to tomatoes in North America were in 1710 by William Salmon and 1782 by Thomas Jefferson (Rick, 1978). However it was not till 1830-1840 that they became popular in the U. S. (Morrison, 1938). In spite of an early association of the tomato with poisonous plants, the tomato fruit has been widely accepted as a menu item. Since 1920 the per capita consumption has risen 300% (Rick,

1978). In the U. S., tomatoes are the leading processed vegetable in per capita consumption. The average American eats 54 pounds of processed vegetables per year, 23 of which are tomatoes (Gould, 1974). It is second only to sweet corn in commercial acreage of vegetable crops. The raw product value of tomatoes was $914.1 million in 1977 (Rick, 1978).

Although tomatoes are reasonably resistant to heat and drought, the flower will drop off in hot drying winds and the fruit will not increase in size with temperatures above 95°F. Optimum temperature for growth is 65-85°F and they grow well in many soil types (Gould, 1974). California dominates in tomato production with 76% of U. S. production and is a major source in the world production (Rick, 1978). Most of the tomatoes produced in Arizona are in the Parker area. Last year producers received $1,739,000 for the 20,000 tons produced (Korzan, 1977).

Specific Goals

The goal of this investigation is to determine solubility characteristics and investigate possible approaches to crystallizing tomato fraction I protein. These data can then be used to help develop strategies to use tomato leaf protein as a human food source. The solubility was determined under a number of conditions. Solubility in $(NH_4)_2SO_4$ was determined at varying concentrations of the salt, temperature, and using differing buffering agents.

Solubility was determined at varying temperatures and pH; at low ionic concentration; and at various low concentrations of $NaHCO_3$, $CaCl_2$, $NaCl_2$, and $MgCl_2$. Crystallization studies were done using micro diffusion cell and free interface diffusion methods with $(NH_4)_2SO_4$ as the precipitant. Various protein and salt concentrations, temperatures, and pH combinations were examined.

CHAPTER II

MATERIALS AND METHODS

Procedures for purification of protein and solubility experiments were adapted from those of Bahr, Bourque, and Smith (1977). This allows direct comparison of results with those obtained with tobacco, spinach, and maize.

Extraction and Purification of Fraction I Protein

Tomato (Lycopersicon esculentum L. var Floradel) plants were grown at The University of Arizona's Agricultural Biochemistry greenhouse. Some preliminary experiments were done with spinach (Spinacia oleraceae) leaves which were obtained commercially or at times grown under artificial light in a growth chamber. Tomato and spinach leaves were deribbed, washed with tap water, partially dried, and stored in a cold room.

The leaves were ground in a Waring blender using one ml of extraction buffer per gram of leaves. Generally about 200 gm of leaves were used. Two different extraction buffers were employed. Buffer A was 50 mM Tris(hydroxymethyl)aminomethane (Tris-HCl), 1.0 M NaCl, 80 mM 2-mercaptoethanol, 2 mM $MgCl_2$, and 1 mM (ethylenedinitrilo)-tetraacetic acid (EDTA) at pH 7.4 or better at pH 8.0. Some

problems were encountered due to phenolic oxidation (Jones and Lyttleton, 1972) so a buffer, buffer J, was used which was 100 mM Tris-HCl, 200 mM NaCl, 0.1% (w/v) Bovine Serum Albumin (BSA), 10% (w/v) Dowex 1x-10, 10 mM $Na_2S_2O_5$, and one mM KCN at pH 8.5. The Dowex was prepared by washing with 1 M NaOH and distilled H_2O. The $Na_2S_2O_5$ and KCN were added to stock solutions just before use. This buffer was very effective but fresh buffer A was also effective if care was taken to work rapidly using cold solutions and blender jars.

After blending, the homogenate was squeezed through cheesecloth and miracloth and then centrifuged. Initially, this was done with a Sorvall RC2 with SS 34 or GSA head at 16,000 to 17,000 x g for 30 minutes at 4°C. Better clarification and less clogging of column filters was obtained by using a Beckman L5-75 centrifuge with a type 35 rotor run at 30,000 rpm (70,500 x g) for 1 hour at 4°C.

The supernatant was then passed through one of two Sephadex G-25 (medium) columns (4.5 x 43 cm or 15 x 35 cm). Columns were equilibrated and eluted with 25 mM Tris-HCl, 200 mM NaCl, 0.5 mM EDTA at pH 7.4 with 10 mM 2-mercaptoethanol added in some runs. Elutant giving a precipitate with 10% (w/v) trichloroacetic acid (TCA) was collected.

Solid $(NH_4)_2SO_4$ was added to the sample to 30% saturation (0°C) and the solution was allowed to stand a minimum of 1 hour in the cold. It was then centrifuged in

the Sorvall centrifuge at 12,000 x g. The supernatant was then saturated to 60% $(NH_4)_2SO_4$ and allowed to stand a minimum of 1 hour (frequently overnight) in the cold. Preliminary results showed that the $(NH_4)_2SO_4$ saturation values could be lowered to 20% and 45% respectively which resulted in an increase in yield.

The fraction I protein was pelleted by centrifuging as above. The pellets were suspended in 10 mM Tris-HCl, 200 mM NaCl, 3 mM NaN_3, 1 mM 2-mercaptoethanol, and 0.5 mM EDTA at pH 7.4 or in an experimental buffer if no further purification was done. In either case the solution was dialyzed overnight against the same buffer to remove residual $(NH_4)_2SO_4$.

With the exception of protein used in preliminary crystallization experiments samples were further purified by use of agarose (Bio Gel A-5m). A column (2.5 x 40 cm) in the cold was equilibrated and eluted with the same buffer in which the protein was dissolved. The eluant was monitored by absorbance at 254 nm and collected in 10 to 15 ml fractions. Protein containing fractions were determined by checking for TCA precipitate and often by determining A_{280}/A_{260} ratio. Two peaks consistently formed. The first was sharp on the monitor, green in color, contained little protein, and had a low A_{280}/A_{260} ratio (< 0.8). The second was much broader, contained fraction I protein (as shown by polyacrylamide gels), was faint green in color, and

generally had an A_{280}/A_{260} ratio above 1.5. Figure 1 shows a profile of a typical run. After using a 20-45% $(NH_4)_2SO_4$ fractionation, a second run with agarose yielded a small first peak and a fraction I peak with an A_{280}/A_{260} ratio of 1.75 or above. These samples were then used for experimentation. Figure 2 shows polyacrylamide gels of protein purified in this manner. Little protein other than that of the subunits of fraction I protein is present.

Further purification was done with some samples with a DEAE cellulose (Whatman DE 52) column (2.5 x 38 cm). The column was equilibrated with 10 mM Tris-HCl, 3 mM NaN_3, 1 mM 2-mercaptoethanol, and 0.5 mM EDTA at pH 7.4. After application of a protein sample, 150 ml of buffer was eluted and then a 0-200 mM linear NaCl gradient was applied to elute protein. The column was then washed first with 1 M NaCl buffer then with 1 M HCl followed by 1 M NaOH.

Determination of Fraction I Protein Concentration

Protein purity and concentration were routinely monitored by the absorbance at 280 and 260 nm. Concentration of tomato RuBPCase was estimated by 1 O.D. = 0.6 mg/ml. This relationship is in agreement with determination by protein spot test. The spinach extinction coefficient of $E_{280}^{1\%} = 1.82$ was used (Pon, 1967). The absorbance ratio of A_{280}/A_{260} was routinely used as an indication of purity. Only samples with a ratio above 1.75 were used in experiments.

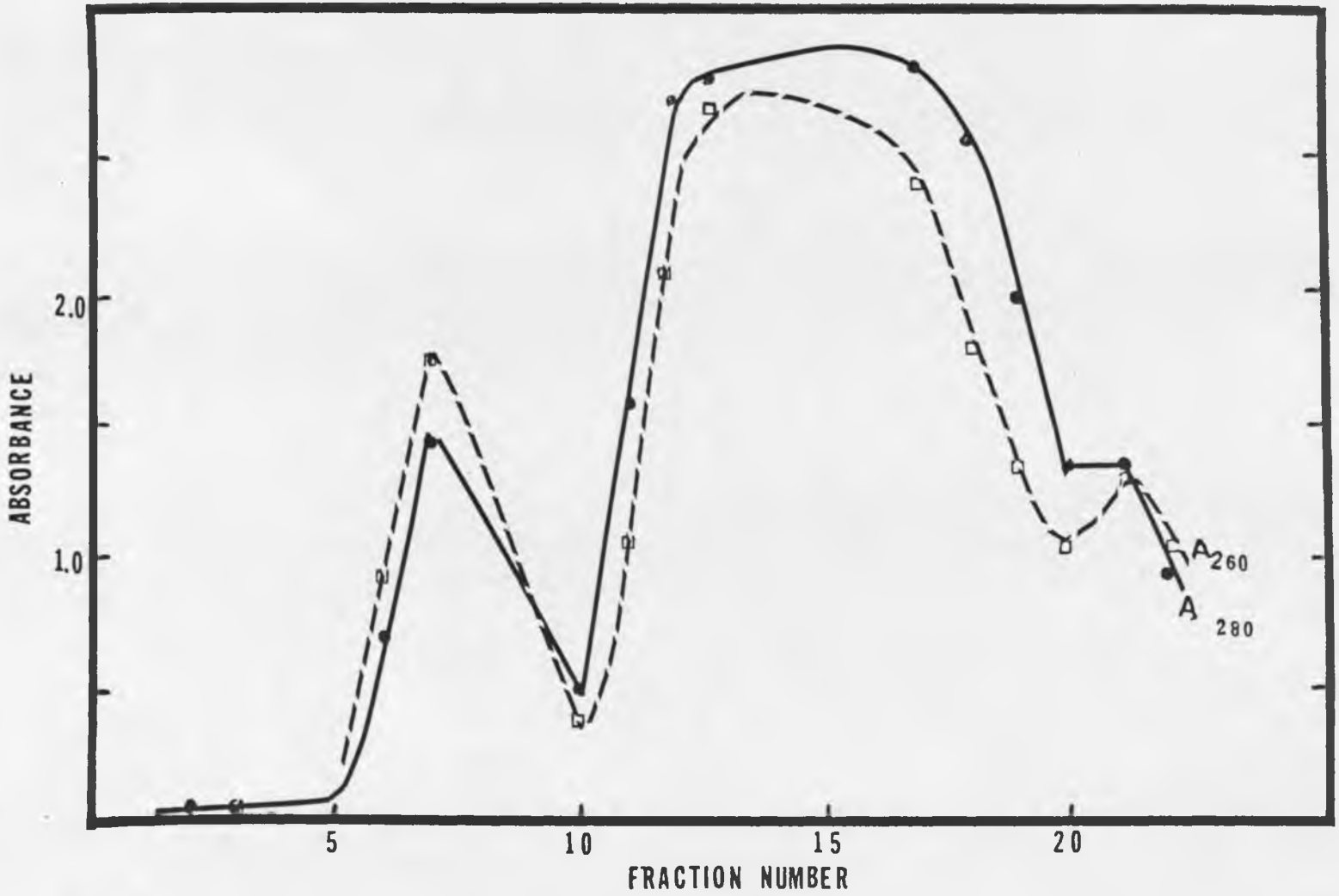

Figure 1. Profile of Agarose Column Eluant of Tomato Fraction I Protein

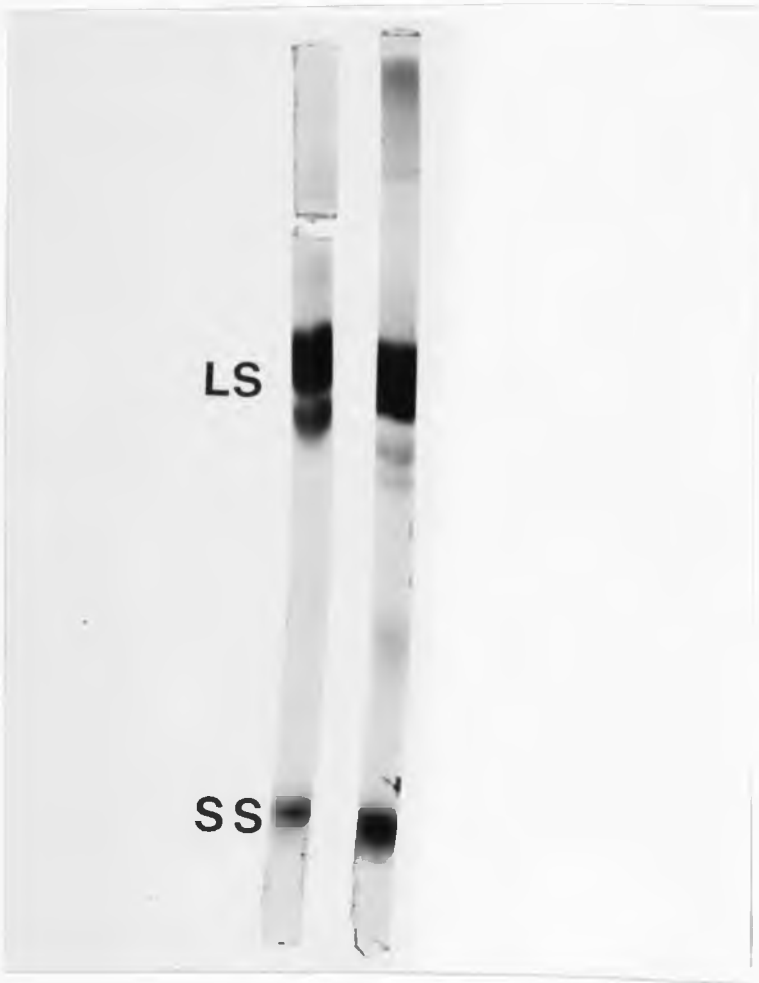

Figure 2. Polyacrylamide Gel of Purified Tomato Fraction I Protein -- A is recrystallized tobacco fraction I protein and B is tomato fraction I protein purified as described in the text. LS is the large subunit and SS is small subunit.

Polyacrylamide Electrophoresis

The protein purity with respect to other contaminating proteins was determined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. A modified method of Weber and Osborn (1969) was employed. Solutions for 1% SDS and 7.5% acrylamide gels at pH 7.2 were made according to Table 3. Gels were polymerized in glass tubes 5 x 125 mm. Stock solutions were degassed and mixed using 4.50 ml of solution I, 0.30 ml of solution II, 6.67 ml of solution III, 10 µl of N,N,N',tetramethylenediamine (TEMED), and 1.85 ml H_2O. Water was layered on top of the polymerizing gels which would set within 30 minutes. Samples were incubated in sample buffer in a boiling water bath for 2 minutes. Thirty to fifty µg of protein was applied to each gel. A current of 8 mA per tube was applied for about 5 hours until the tracking dye was near the bottom of the tubes. Gels were stained in either 0.25% coomassie blue in 5:5:1 methyl alcohol:H_2O:acetic acid or 0.1% coomassie blue in 50% (w/v) TCA for a minimum of 4 hours. Soaking gels in 7.5% (v/v) acetic acid for one hour before staining improved staining and stain life. Gels were then destained in 5% (v/v) methanol and 7.5% (v/v) acetic acid in a filtered destainer.

Table 3. Stock Solutions for Polyacrylamide Gel Electrophoresis

Solution I	Solution II
22.2 gm Acrylamide	30 mg Ammonium Persulfate
0.6 gm N, N' Methylene Bis-acrylamide	2 ml H_2O
to 100 ml H_2O	prepare fresh
Solution III	**Reservoir Buffer**
23.25 ml 1 M NaH_2PO_4	Dilute Solution III 1:1 with H_2O
145 ml 0.5 M Na_2HPO_4	
10 ml 10% SDS	
to 500 ml H_2O	
Sample Buffer	
1.0% SDS	
0.1 M PO_4 pH 7.2	
0.14 M 2-mercaptoethanol	
10% (v/v) glycerol	
0.002% Bromophenol blue	

Solubility Studies on Tomato Fraction I Protein

Incubation

Before most solubility experiments the sample protein was incubated in 25 mM $MgCl_2$ and 25 mM $NaHCO_3$ or 0.3 mM RuBP for 2 hours at 37°C. These treatments can affect some solubility properties (Bahr et al., 1977).

Common Procedures

In all experiments samples were done in triplicate. Centrifugation was done unless otherwise noted with a Beckman Microfuge B (3000 x g) for 3 to 5 minutes. All experiments were evaluated for protein remaining in solution by optical density at 280 nm.

Determination of Solubility in $(NH_4)_2SO_4$

The solubility of tomato fraction I protein was determined for varying concentrations of $(NH_4)_2SO_4$ at various pH's and temperatures. Experiments were conducted using two different buffering agents. The first was Tris-HCl/MES made with 50 mM Tris-HCl, 50 mM 2-(N-morpholino)-ethanesulfonic acid (MES) and 1 mM EDTA. With this buffer the protein samples were incubated in $MgCl_2$ and $NaHCO_3$. The second buffer was potassium phosphate which contained 200 mM $K\text{-}PO_4$, 3 mM NaN_3, and 0.5 mM EDTA. No incubation was done for protein samples used in this system.

Stock $(NH_4)_2SO_4$ solutions were made by making solutions 75% saturated (using 0° and 25°C tables as appropriate) with the other components the same as in the system used. Test conditions were made by mixing $(NH_4)_2SO_4$ stocks with respective buffer according to Table 4. These were mixed in small centrifuge tubes and 25 µl of protein solution at approximately 30 mg/ml was added and mixed. Samples were allowed to incubate 2 hours at the desired temperature.

Thermal Precipitation Determination

Protein, $NaHCO_3$- and $MgCl_2$-incubated, solution at about 9 mg/ml buffered by 10 mM Tris-HCl, 200 mM NaCl, 1 mM 2-mercaptoethanol, and 0.5 mM EDTA at pH 7.4 were placed in 1.5 ml microfuge tubes. Both tomato and twice recrystallized tobacco were used. Tobacco was prepared by method of Chan et al. (1972). Protein was incubated in a stirring water bath at each desired temperature for 5 minutes. Bahr (n.d.) showed that a sample inside the microfuge tubes reaches equilibrium with the bath within 3 minutes. After an 0.1 ml aliquot was drawn, the tube was heated to the next temperature. Optical density measurements were made with a ten-fold dilution of the aliquots.

Isoelectric Point Determination

To determine the isoelectric point of tomato fraction I protein, 25 µl of $NaHCO_3$ and $MgCl_2$ incubated protein solution at about 40 mg/ml was added to 500 µl of

Table 4. Solutions for the Determination of Tomato Fraction I Protein Solubility in $(NH_4)_2SO_4$

Experiment number	A: 75% Saturated $(NH_4)_2SO_4$ Buffer	B: Buffer	% Saturated $(NH_4)_2SO_4$: A+B	With 25 µl protein	Adjusted[a]
1	0 µl	200 µl	0%	0%	0%
2	16	184	6	5.3	--
3	32	168	12	10.6	--
4	48	152	18	16.0	14.7
5	64	136	24	21.3	19.5
6	80	120	30	26.7	24.5
7	96	104	36	32.0	29.3
8	112	88	42	37.3	34.2
9	128	72	48	42.7	39.2
10	144	56	54	48.0	44.0
11	160	40	60	53.3	48.9
12	176	24	66	58.7	53.8

[a] $(NH_4)_2SO_4$ stock buffers were made according to 0°C table for Tris-HCl/MES buffer system. A factor of 0.917 was applied to correct for 25°C incubations.

200 mM NaCl and 0.5 mM EDTA in small test tubes. The pH of each sample was adjusted by adding 1 M acetic acid and/or 1 M sodium acetate according to Table 5. Sodium acetate was added first and then the acetic acid to make the final concentration 50 mM. Each tube was then mixed with a vibrating mixer and allowed to stand 30 minutes after which the pH of each was read. Tubes were then centrifuged in a table top centrifuge at top speed and A_{280} determined after 1/3 dilution of the supernatant.

Table 5. Solutions for the Adjustment of pH for Determination of Isoelectric Point

Experiment	1 M Acetic Acid	1 M Sodium Acetate	pH
1	25 µl	0 µl	3.0-3.2
2	20	5	4.0
3	17.5	7.5	4.2-4.4
4	15	10	4.4
5	12.5	12.5	4.5-4.6
6	10	15	4.8
7	5	20	5.0-5.2
8	0	25	6.8

Determination of Solubility
at Low Ionic Strength

Three 0.3 ml aliquots of tomato protein, $NaHCO_3$ and $MgCl_2$ incubated, buffered by 10 mM Tris-HCl, 200 mM NaCl, 3 mM NaN_3, 1 mM 2-mercaptoethanol, and 0.5 mM EDTA at pH 7.4 were dialyzed against 100 ml of the same buffer without the NaCl included. At pH 7.0 and below, MES instead of Tris-HCl was used. All buffers were adjusted to pH at the temperature they were used. The protein aliquots were placed in dialysis tubing (prepared by boiling in 10 mM EDTA for 10 minutes) and allowed to stand overnight in the dialysis buffers in a cold room (about 4°C). A_{280} was measured before and after the overnight dialysis.

Determination of Solubility
in Bicarbonate

The solubility of RuBP treated protein buffered by 10 mM Tris-HCl, 3 mM NaN_3, and 0.1 mM EDTA at pH 7.4 was determined in low $NaHCO_3$ concentrations in the presence of 2 mM $MgCl_2$. $NaHCO_3$ solutions were made according to Table 6. Two-tenths ml of test solution was pipetted into microfuge tubes and then 0.1 ml of protein solution at about 10 mg/ml was added and mixed. These were then incubated at room temperature for the desired length of time. The 25 mM $NaHCO_3$ solution was made in excess and used as a blank and to make dilutions for optical density measurements.

Table 6. Preparation of Test Conditions for $NaHCO_3$ and Metal Ion Solubility Experiments

Condition	Buffer	Test ion 0.1 M	Constant ion 0.1 M	Experimental concentration
1	.970 ml	0 µl	30 µl	0 mM
2	.962	7.5	30	0.5
3	.955	15	30	1.0
4	.925	45	30	3.0
5	.880	90	30	6.0
6	.820	150	30	10
7	.595	375	30	25

Determination of Solubility in Divalent Metal Ions

The solubility of tomato fraction I protein was determined in the presence of low concentrations of $MgCl_2$, $MnCl_2$, and $CaCl_2$. The same procedure used in $NaHCO_3$ solubility determination was used with metal ions as test ions and $NaHCO_3$ concentration held constant at 2 mM. Optical density measurements were done with blanks the same as with each test concentration.

Crystallization Attempts

Free Interface Diffusion

The free interface diffusion technique (Salemme, 1972) was adapted using 6 x 44 mm test tubes. One-tenth (2/10 at pH 5.0 to 6.0) ml of 0.60% saturated $(NH_4)_2SO_4$ solution was buffered with either 10 mM Tris-HCl at pH 7.4 or 0.2 M K-PO_4 at pH 5.0 to 7.0, in 0.5 unit increments, with 3 mM NaN_3 and 0.5 mM EDTA. One-tenth ml of protein solution at pH 7.4, 7.0, or 6.5 (which was used for the lower pH conditions) was carefully layered above the precipitant. The protein was buffered in the same way as was the precipitant solution it was used with and ranged in concentration from 5 to 40 mg/ml. Samples were allowed to stand 3-7 weeks at room temperature or in a cold room (4°C).

Micro Diffusion Cells

The diffusion technique using capillary tubes was adopted (Zeppezauer et al., 1968). Glass capillaries, 2 mm (I.D.) 7 mm (O.D.) x 20 mm, were sealed on one end with dialysis membrane held in place by either a ring of cut tubing or pieces of tubing cut to have "legs" so the capillaries could stand on end. Fifty µl of protein solution, 5 to 30 mg/ml, was pipetted into the capillaries with a micro syringe taking care to avoid air bubbles. The other ends of the capillaries were sealed with parafilm held by a ring of cut tubing. The capillaries were then placed in

precipitant containing 0% to 40% saturated $(NH_4)_2SO_4$ buffered with 10 mM Tris-HCl at pH 7.4 or 0.2 M K-PO_4 at pH's 4.5 to 7.0 with 3 mM NaN_3 and 0.5 mM EDTA in each.

In another experiment, protein solution in a salt buffer was dialyzed against the same buffer without NaCl using a buffer of 10 mM Tris-HCl at pH 8.0 and 7.3 and 10 mM K-PO_4 at pH 6.0. All buffers contained 3 mM NaN_3 and 0.5 mM EDTA and samples were stored in a cold room.

CHAPTER III

EXPERIMENTAL RESULTS

Solubility of Tomato Fraction I Protein

Although the solubility characteristics of tomato fraction I protein were generally the same as those of other species, variations were noted in some conditions tested. Comparisons with tobacco, maize, cotton, and spinach are made with data from Bahr et al. (1977).

Solubility in Ammonium Sulfate

The solubility in ammonium sulfate of a sample of recrystallized tobacco fraction I protein was also measured along with the chromatographically-purified tomato samples for comparison. This was done only with the Tris-HCl/MES buffer system and results were in agreement with data of Bahr (n.d.). The solubility in ammonium sulfate under the various conditions determined is shown in Figure 3. The pH of the buffering agent can cause a significant shift in the amount of $(NH_4)_2SO_4$ needed to cause precipitation. The effect of K-PO_4 is probably dependent on its high concentration (0.2 M) which would lower the ability of H_2O to solvate the protein and thus enhance the effect of the $(NH_4)_2SO_4$. Temperature had little effect with the Tris-HCl/MES system. A small shift in solubility of 2% higher saturation is shown

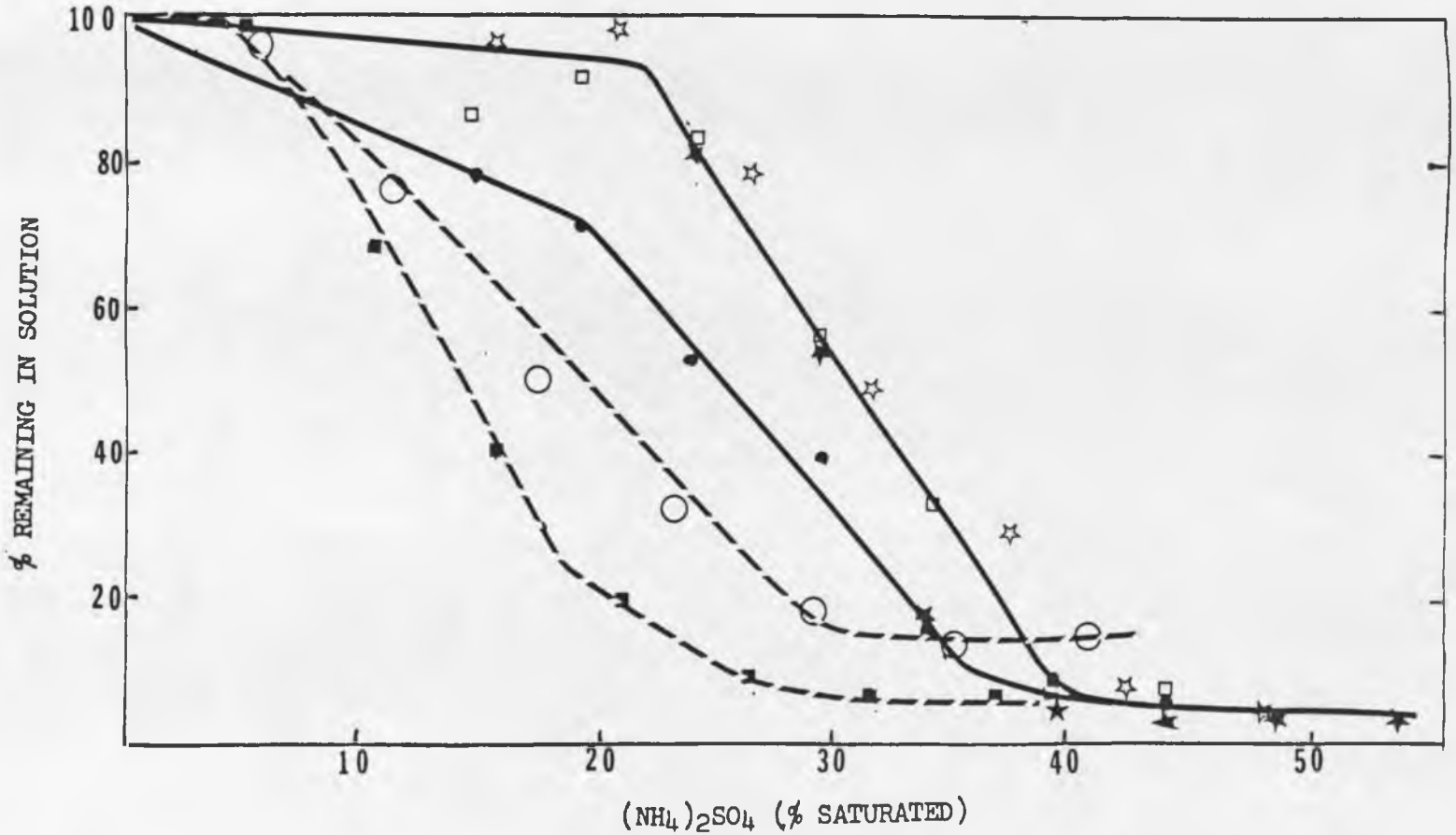

Figure 3. Solubility of Tomato Fraction I Protein in $(NH_4)_2SO_4$ -- Procedure is explained in the text. Concentration at 0% saturation $(NH_4)_2SO_4$ was 4-7 mg/ml with Tris-HCl and 2.1 mg/ml with K-PO_4. Tris-HCl/MES system at 25°C: -●- pH 6.3, -□- pH 7.3, pH 8.3; Tris-HCl/MES at 4°C at pH 7.3 ; K-PO_4 system (0.2 M) at pH 7.3: --■-- 25°C, --O-- 4°C.

at 4°C. With the K-PO_4 system significantly higher ammonium sulfate concentration (by about 6%) was needed to obtain maximum precipitation at 4°C.

The tomato fraction I protein precipitates at lower concentrations of $(NH_4)_2SO_4$ than protein from other species. The concentration required to reduce solubility to 2 mg/ml at pH 7.3 at 25°C is 31%, which compares to values for tobacco of 38%, spinach of 36%, untreated cotton of 44%, and untreated maize of 33% (Bahr et al., 1977).

Thermal Precipitation

The tobacco and tomato samples were determined simultaneously. The temperature varied less than 1°C during incubation. The results are plotted in Figure 4. Little difference can be seen between the tobacco and tomato samples. A temperature of 57°C was required to precipitate 50% of the protein. These values are 10°C lower than that reported for spinach and is similar to that of maize (Bahr et al., 1977).

Isoelectric Point

The isoelectric point appears to be between pH 4.5 and 4.8 as can be seen from Figure 5. This is the same as that for spinach and cotton and slightly higher than that for tobacco and maize which are at pH 4.4 (Bahr et al., 1977).

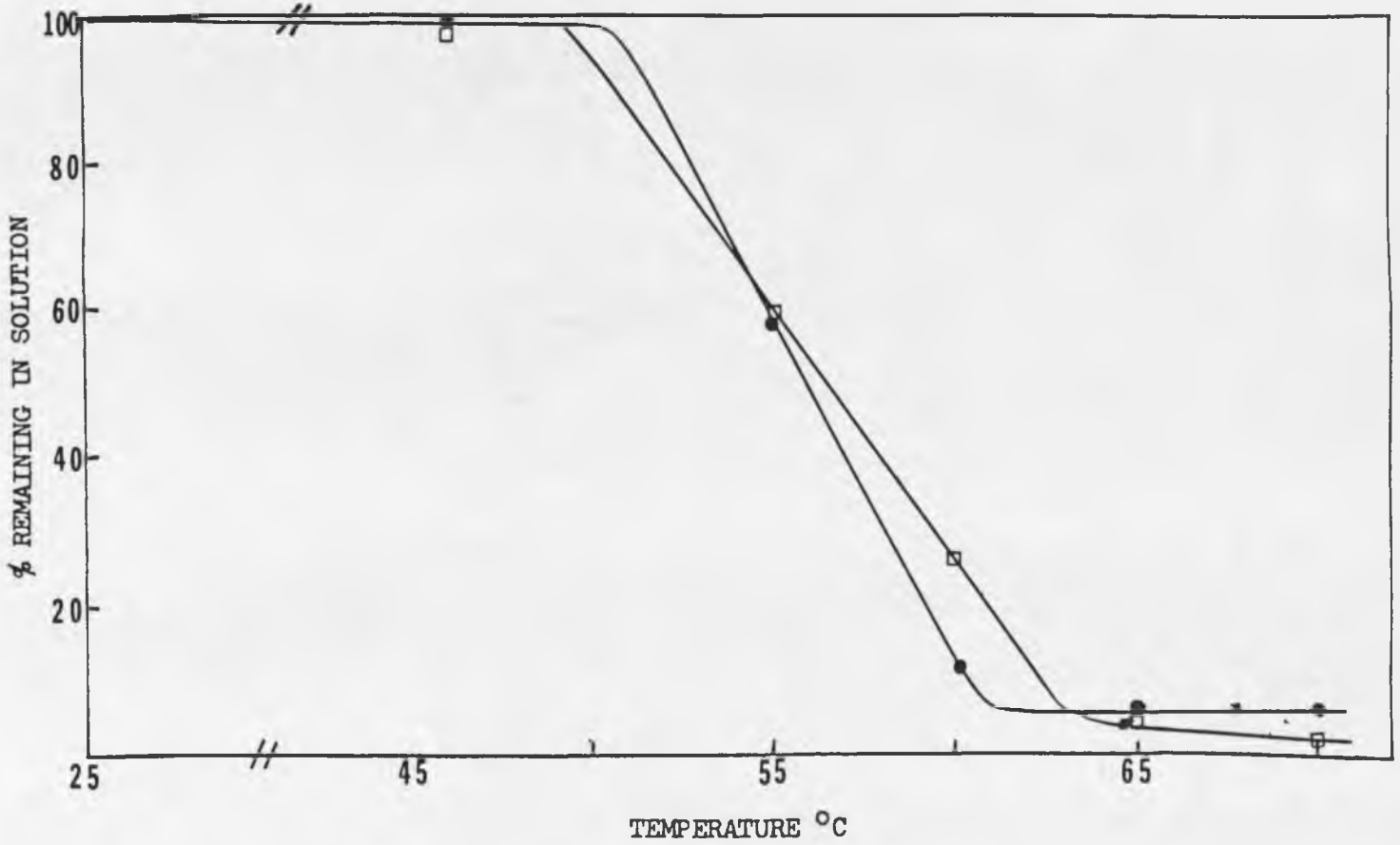

Figure 4. Thermal Solubility of Tomato and Tobacco Fraction I Protein -- Protein concentration at 25°C was 8-9 mg/ml and was used as reference. ● Tobacco, □ Tomato.

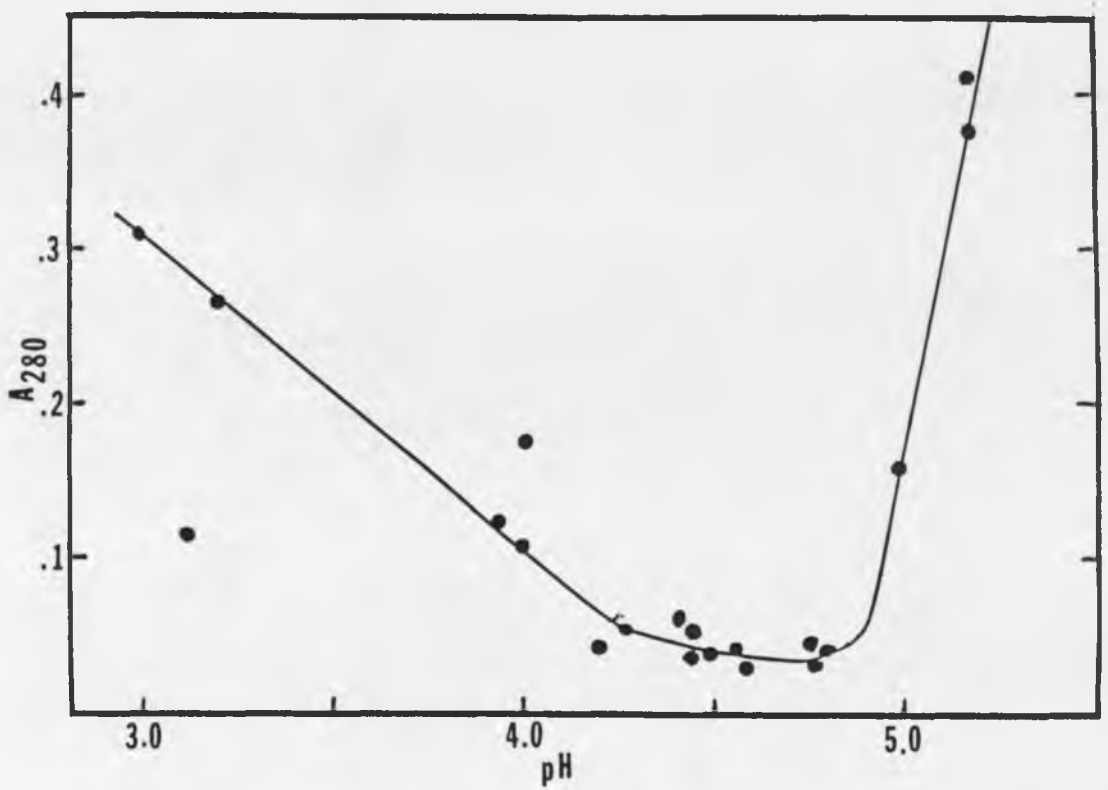

Figure 5. Isoelectric Point of Tomato Fraction I Protein -- Procedure described in the text. A_{280} is shown at 1/3 dilution of the supernatant.

Solubility in Low Salt Concentrations

The solubility of tomato fraction I protein after dialysis to low NaCl concentration (about 2 mm) is shown in Figure 6. Precipitates were examined for possible crystal formation; however, none was observed. Temperature did not affect the solubility. Tomato fraction I protein precipitates in low salt at about 1 pH unit higher than spinach, cotton, or maize. Although precipitation is complete at the same pH, 6.5, as tobacco fraction I protein, tomato remains in solution to lower pH values, especially at 4°C, than tobacco which starts precipitation between pH 9.3 at 4°C (Bahr et al., 1977).

Effect of Bicarbonate on Solubility

The effect of bicarbonate on the solubility of tomato fraction I protein was determined without incubation in RuBP. However, inconsistent results were obtained. Incubation in 10 mm 2-mercaptoethanol significantly lowered the solubility of tomato fraction I protein under the experimental condition as shown in Table 7. This effect was gradually lost with time and after one week had little influence.

Procedure is the same as that for the bicarbonate solubility experiment as explained in the text. Incubation was for four hours. Results are A_{280} at 1/5 dilution. No

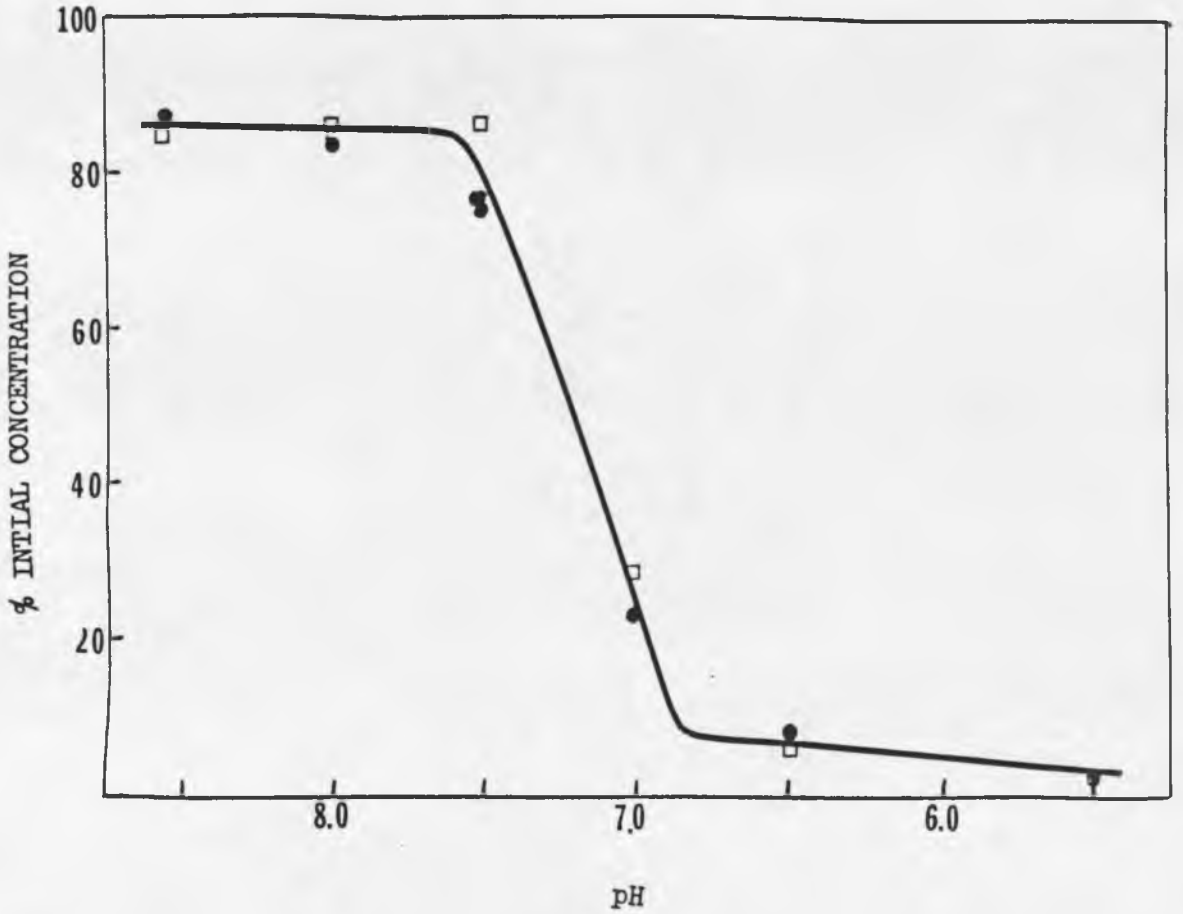

Figure 6. Solubility of Tomato Fraction I Protein at Low NaCl Concentration at Various pH's -- Procedure described in the text. Initial concentration before dialysis was approximately 10 mg/ml. ● at 4°C (two experiments), □ at 37°C.

Table 7. Effect of $NaHCO_3$ and $MgCl_2$ on the Solubility of RuBP Treated Tomato Fraction I Protein

Condition		Treatment		
2 mM Mg^{++}	3 mM HCO_3^-	RuBP	Untreated	2-mercaptoethanol
-	-	1.089	1.172	1.073
+	-	.666	.726	.236
+	+	1.004	.989	.729
-	+	1.118	1.150	1.102

precipitate was observed in samples with an absorbance greater than 1.050.

Figure 7 shows the solubility of RuBP treated protein in the presence of 2 m $MgCl_2$ after three different incubation periods. The difference in maximum solubility of the 1 hour incubation is due to the use of a different protein preparation for that experiment. Tomato fraction I protein is significantly more soluble than tobacco which almost completely comes out of solution at 0.5 to 6 mM $NaHCO_3$ and rises to only 1 mg/ml at 25 mM $NaHCO_3$ (Bahr et al., 1977).

The effect of the presence of $MgCl_2$ was determined by incubating RuBP treated tomato fraction I protein for 4 hours with various combinations in the presence of 3 mM

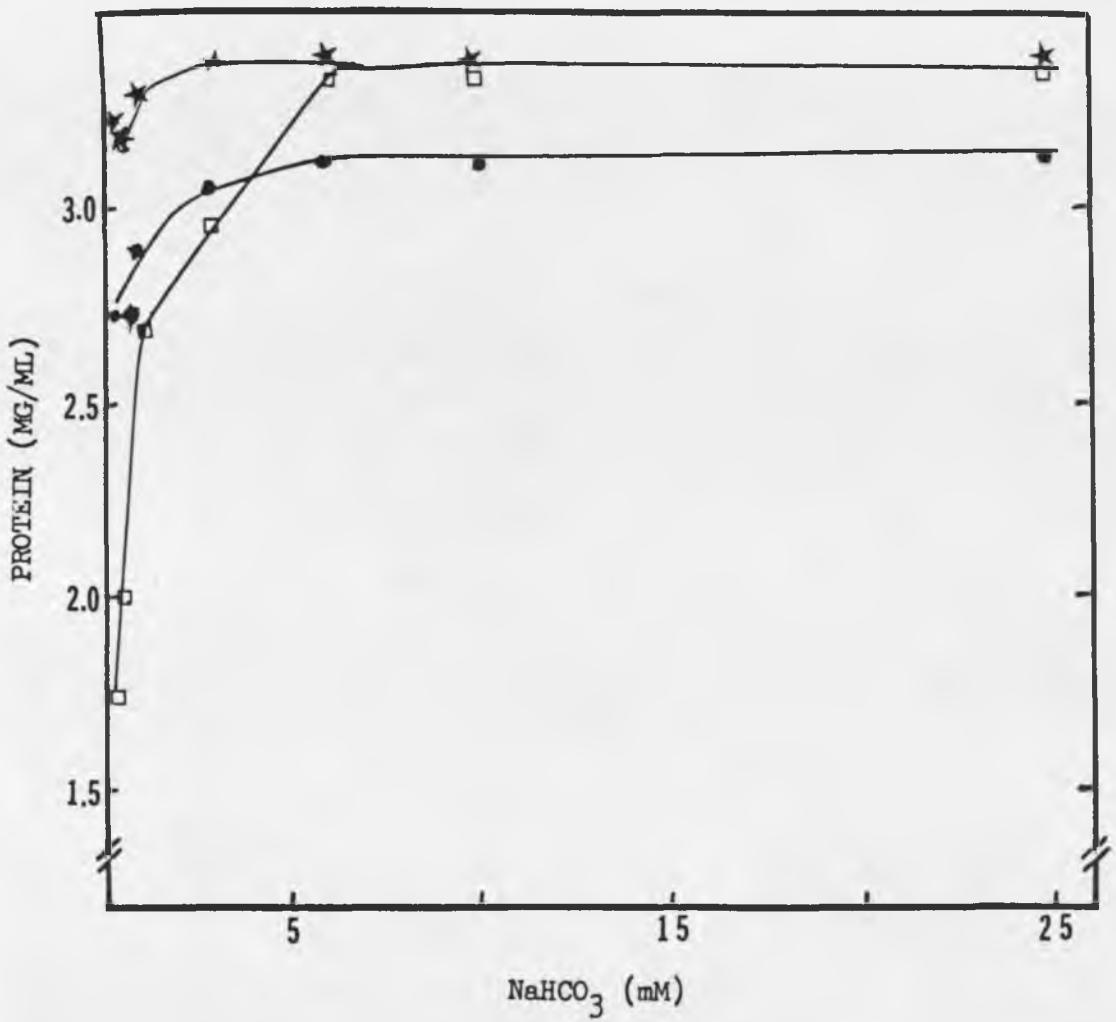

Figure 7. Solubility of Tomato Fraction I Protein in $NaHCO_3$ -- Procedure described in the text. Protein concentration estimated from A_{280}. ★, 0.5 hour incubation; ●, 1 hour incubation; □, 6.75 hour incubation.

$NaHCO_3$ and 2 mM $MgCl_2$. As can be seen from Table 7, both samples incubated in the absence of $MgCl_2$, one without $NaHCO_3$ and one with $NaHCO_3$, the protein completely remained in solution. However in the presence of $MgCl_2$ precipitation occurred. Sixty per cent of the protein remained in solution with no $NaHCO_3$ present and 90% remained in the presence of $NaHCO_3$. This indicates the precipitation of tomato fraction I protein is caused by the $MgCl_2$ and not by the bicarbonate which enhances the solubility of the protein.

Effect of Metal Ions on Solubility

The effect of Mn^{++}, Ca^{++}, and Mg^{++} on the solubility of tomato fraction I protein in the presence of 2 mM $NaHCO_3$ is shown in Figure 8. As with bicarbonate, tomato fraction I protein is more soluble than tobacco fraction I protein. Tobacco is insoluble at 0.5 to 3 mM of the three metals and then increases to full solubility at 10 mM Mg^{++} and Ca^{++} (Bahr et al., 1977). The difference with tomato fraction I protein is partially due to the effect of the $NaHCO_3$.

Crystallization of Tomato Fraction I Protein

Both methods yielded crystals. Crystallization was not quantitative under any condition examined although some were nearly complete. Crystals were smaller (about one-half the size) than those generally obtained with tobacco using

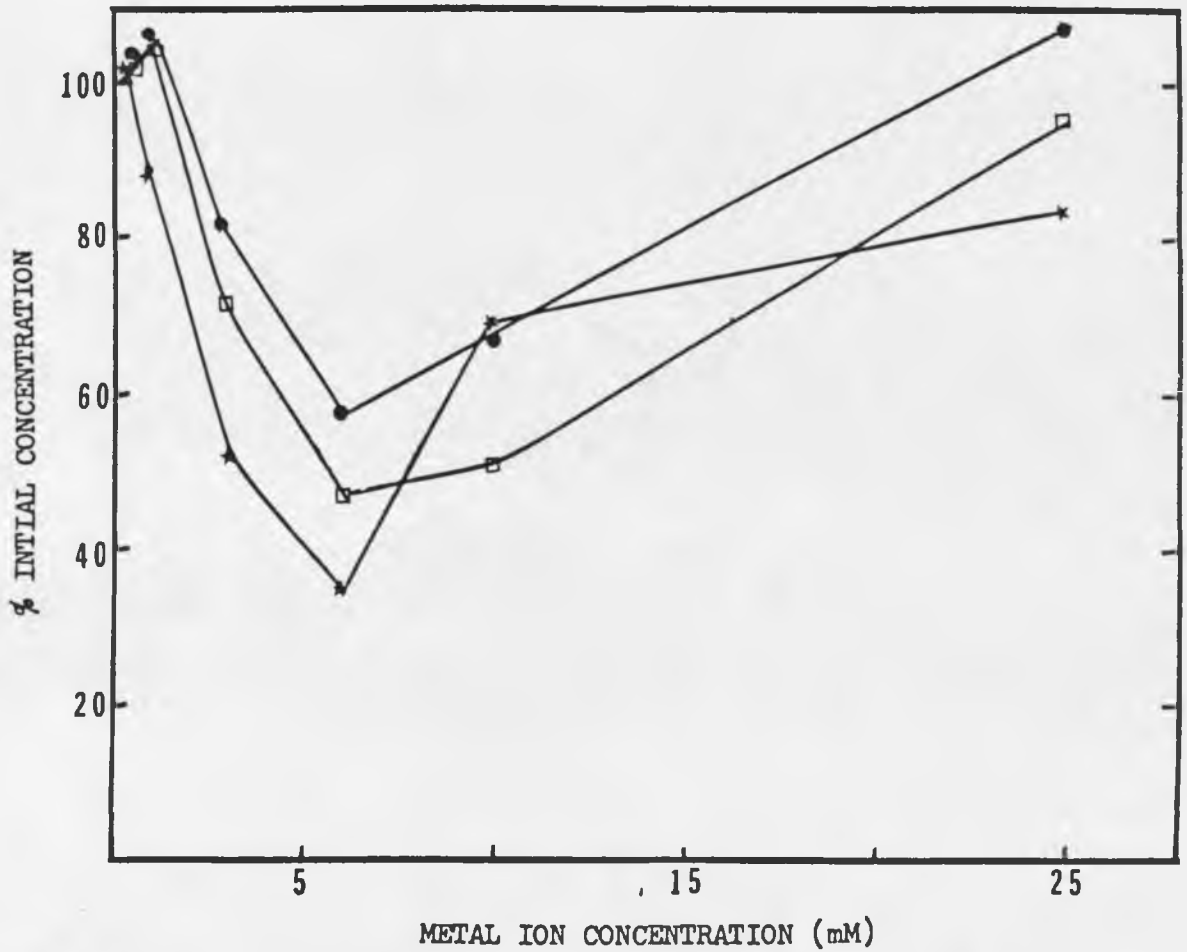

Figure 8. Solubility of Tomato Fraction I Protein in Divalent Metal Ions -- Procedure described in the text. Initial protein concentration estimated to 3.1-3.4 mg/ml. $MnCl_2$, ● $MgCl_2$, □ $CaCl_2$.

the method of Chan et al. (1972). Both methods yielded the same structure and were repeated to insure reproducibility.

Free Interface Diffusion Method

Initial experiments were conducted with partially purified protein (through the 30-60% saturated $(NH_4)_2SO_4$ step) and were successful in yielding crystals in a habitat of 45% to 60% initial saturation $(NH_4)_2SO_4$. The range of $(NH_4)_2SO_4$ concentrations was expanded in the subsequent trial with protein purified through two passes on an agarose column. Best conditions for crystallization were at 22.5 and 30% initial saturation $(NH_4)_2SO_4$ with an initial protein concentration of 8 to 16 mg/ml. Final equilibrium concentrations would then be 15 to 20% saturation $(NH_4)_2SO_4$ and 5.3 to 2.7 mg/ml protein. Optimum conditions were from the samples at initial pH 5.0, 5.5, and 6.0 which would have higher final pH due to the protein being added at pH 6.5. Samples which were stored in the cold had more complete crystallization. No crystallization occurred at protein concentrations of 30 and 40 mg/ml. Crystals were a "football" shape as shown in Figure 9.

Micro Diffusion Cells

Crystals obtained by use of micro diffusion cells were the same football shape as those obtained by free interface diffusion. These football shaped crystals were obtained over a range of conditions with the optimum at pH

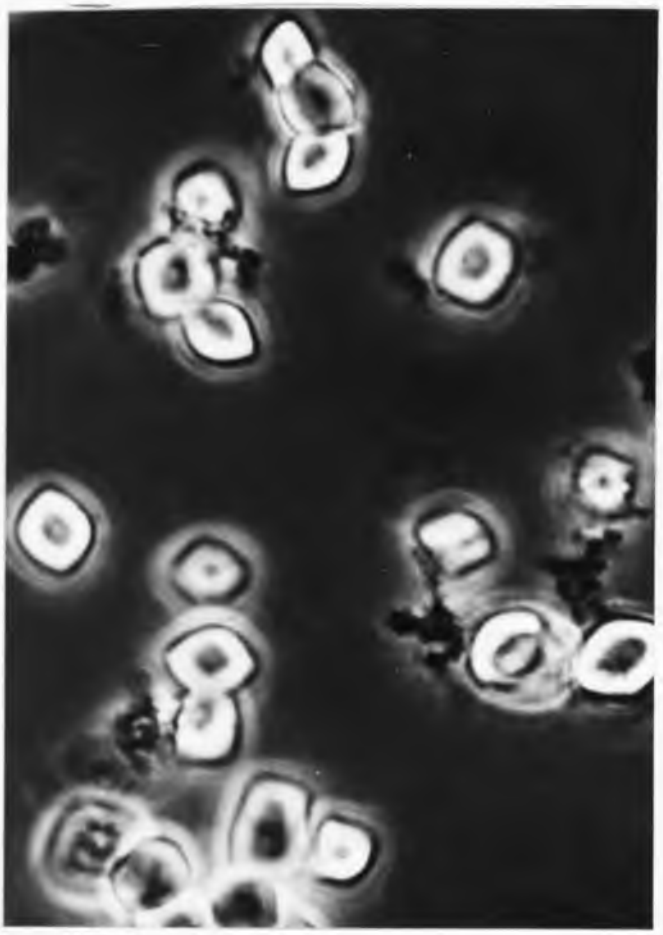

Figure 9. Tomato Fraction I Protein Crystals -- Crystals were obtained by the free interface diffusion method under conditions described in the text.

6.0 and 6.5 at 20 to 35% saturation $(NH_4)_2SO_4$. Optimum protein concentrations were at 10 and 20 mg/ml and samples stored in the cold had more complete crystallization. Star shaped crystals mixed with the football crystals and uncrystallized precipitate were observed in many samples as pH 6.5 and 7.0 at 30 and 35% saturation $(NH_4)_2SO_4$. These are very similar in appearance to crystals obtained by dialysis of tomato fraction I protein against a salt free buffer at pH 6.8.

Identity of Crystals

The crystals were pooled from five samples and the suspension layered over 10 mM K-PO_4 buffer at pH 6.5, 40% saturated $(NH_4)_2SO_4$. This was centrifuged with a table top centrifuge for 5 minutes and the supernatant discarded. The pellet was dissolved in 10 mM Tris-HCl, 200 mM NaCl, 3 mM NaN_3, and 0.5 mM EDTA at pH 7.4 and an aliquot was run on SDS polyacrylamide gel. The gel, Figure 10, clearly indicates fraction I protein the predominant component, if not the only protein of the crystals and precipitate. Only the expected large and small subunits are seen as major components on the gels.

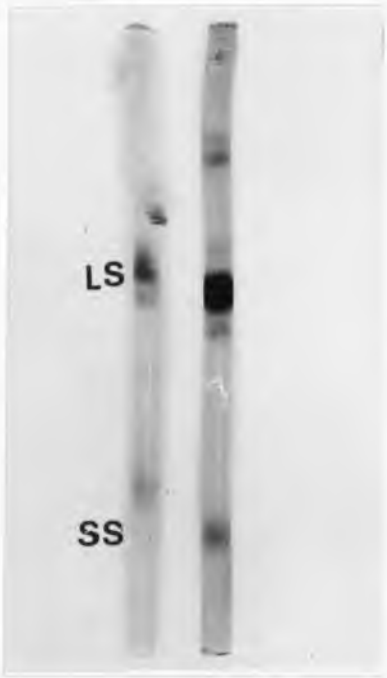

Figure 10. SDS Polyacryamide Gel of Crystallized Protein -- Protein was obtained from tomato fraction I protein crystallized by free interface diffusion as described in the text. LS is large subunit and SS is small subunit. A, recrystallized tobacco; B, crystallized tomato.

CHAPTER IV

DISCUSSION

Solubility of Tomato Fraction I Protein

The solubility properties of tomato fraction I protein are generally similar to other species studied. Some of the differences observed could significantly affect purification schemes or crystallization attempts.

$(NH_4)_2SO_4$ fractionation is a relatively easy and common method for purification of protein. A 20% to 45% saturation fraction of tomato fraction I protein yields a product with little contamination from other proteins. Unfortunately, other non-protein impurities also precipitate under these conditions, but it still remains an effective purification step for fraction I protein.

The difference in solubility when Tris-HCl/MES and K-PO_4 buffers are used illustrates the effect different electrolytes have on the solubility of a protein and on potential crystallization conditions. About one-half as much $(NH_4)_2SO_4$ is needed to cause precipitation of tomato fraction I protein if 0.2 M PO_4 is present than if 50 mM Tris-HCl/MES is used.

In the event procedures and equipment are set up to process leaves from different species, only a small

adjustment would be necessary to obtain maximum yield of purified tomato fraction I protein using an $(NH_4)_2SO_4$ purification step. Should a heating step be included, as has been done with alfalfa (Edwards et al., 1975) and tobacco (Lowe, 1977), tomato fraction I protein could be processed without making any adjustments from a tobacco process.

Since the thermal precipitation and low salt solubility of tomato and tobacco fraction I protein are very similar, potentially tomato fraction I protein could be crystallized by a slightly modified Lowe procedure. Crystals from tomato fraction I protein have been obtained by Philip (1977) using the dialysis to a salt free buffer technique.

A significant difference between tobacco and tomato fraction I protein was noted in the bicarbonate and divalent metal ion solubility experiments. Some workers are investigating the use of low concentrations of $MgCl_2$ to selectively precipitate tobacco fraction I protein from leaf extracts. This approach would not appear to be feasible with tomato fraction I protein. However, it is possible metal ions could be used with other precipitants such as polyethylene glycol which could produce selective precipitation or crystallization.

Crystallization of Tomato Fraction I Protein

Until recently, only fraction I protein from tobacco has been crystallized. Subsequent to the successful attempts described in this thesis, fraction I protein has been crystallized from a number of agriculturally important plants and improved methods of crystallizing tomato fraction I protein have been found (Johal and Bourque, 1978).

When using impure leaf hornopentates, crystallization with $(NH_4)_2SO_4$ as the precipitant has the disadvantage that crystallization is either not complete or impurities also come out of solution as precipitate. This problem is minimal at low concentrations of $(NH_4)_2SO_4$, but reduced yield thus occurs.

Although, as noted previously dialysis to a salt free buffer has yielded tomato fraction I crystals, the attempts using micro diffusion cells and examination of precipitates from low salt solubility experiments did not reveal any sign of crystallization. This may be due to the presence of 2-mercaptoethanol or some other factor in the buffer solution. However, in view of the solubility characteristics of tomato fraction I protein and the sometimes successful crystallizations, further investigation of this approach could prove to be very fruitful.

Future of Tomato Fraction I Protein

Since tomato fraction I protein is very similar in solubility properties to tobacco fraction I protein, its future for use as a human protein food source is bright since more and more attention is being given to developing tobacco fraction I protein as a food source. Technology that is developed to process pure fraction I protein from tobacco leaves could easily be adapted for tomato leaves. Tomato has an advantage over tobacco and many other species since tomato fruit is picked while the leaves are still green. In many other plants, the leaves die before the fruit ripens or in the case of tobacco, the leaves need to be mature and are beyond peak protein yield before harvesting for smoking purposes. This is an important consideration since it is not economically feasible to grow plants just for the protein content of their leaves. It would be possible to get 2 or 3 products from tomato acreage: the fruit, protein from the leaves, and animal fodder or mulch for the soil from the residue. The results would be greater economic return to the producer and more food for animals and man to ease growing pressure on food supplies.

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