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CHARACTERISTICS OF FRACTION 1 PROTEIN
ISOLATED FROM ALFALFA (MEDICAGO SATIVA) LEAVES

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J. Robert Brunner
Major professor

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CHARACTERISTICS OF FRACTION I PROTEIN ISOLATED FROM ALFALFA (*MEDICAGO SATIVA*) LEAVES

By

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ABSTRACT

CHARACTERISTICS OF FRACTION I PROTEIN ISOLATED FROM ALFALFA (MEDICAGO SATIVA) LEAVES

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Fraction I protein was extracted from alfalfa (Medicago sativa) leaves and purified by ammonium sulfate fractionation, DEAE-cellulose, and Sephadex G-200 gel chromatography. The final produce possessed ribulose-1,5-diphosphate carboxylase (E.C. 4.1.1.39.) activity and had a specific activity of 1.24 units/mg protein. Lyophilization resulted in complete loss of activity. An approximate molecular weight of 573,000 daltons was determined from a $S_{20,w}$ of 18.7 and $D_{20,w}$ of 2.97.

Mercaptoethanol (ME) and 5 M urea were not effective dissociating agents in polyacrylamide gel electrophoresis (PAGE) while pH 11-PAGE did resolve at least 5 subunit components from the protein. Sodium dodecyl sulfate (SDS)-PAGE revealed three subunits with molecular weights of 52,000, 46,000 and 12,500.

Chemical analyses of this protein indicated the presence of 16.4% protein nitrogen and 1.85% hexose and an absence of hexosamine, sialic acid and nucleic acids. The protein contained 99 sulfhydryl groups per mole (573,000 daltons) with 62 of these occurring in disulfide bonds. Amino acid composition of alfalfa Fraction I protein revealed a slightly acidic and hydrophilic nature for the protein.

To my mother and father

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INTRODUCTION

Leaf protein has been of interest to researchers for a number of years. The major protein in leaves is located in the stroma of chloroplast and is designated Fraction I protein as of 1947. This protein accounts for up to 50% of the total soluble protein in leaf extracts and may be the most abundant protein in nature. Recently, it has been considered to be the greatest potential source of dietary protein.

Fraction I protein purified from several plants, i.e., tobacco, spinach, cabbage, has been examined. Its schlieren pattern in analytical ultracentrigutation is a single, symmetrical boundary of 18S. Molecular weights ranging from 480,000 to 590,000 have been reported. Fraction I protein has the enzyme property of ribulose-1,5-diphosphate carboxylase-oxygenase (E.C. 4.1.1.39.) which is involved in primary photosynthesis and photorespiration. It has been argued that it would be desirable to drop the name Fraction I protein in favor of RuDP carboxylase. Recently, it has been demonstrated that RuDP carboxylase/oxygenase in some bacteria possesses different properties from that found in chlorophyll _a_ containing organism. Thus, the term Fraction I protein is used generally to describe an unusually abundant, high molecular weight protein found in O_2-evolving photosynthetic cells. However, the catalysis of carboxylation is an important property of

Fraction I protein. Also, the content of Fraction I protein in leaves may perform a rate-limiting role in photosynthesis and photorespiration.

Isolated Fraction I protein contains no lipid material, whereas the presence of a carbohydrate moiety and disulfide bonds have not been reported consistently. The protein is homogeneous by PAGE and sedimentation analysis. Evidences obtained by physical and chemical studies indicate that Fraction I protein contains large and small subunits of greatly differing molecular weight. Combining the techniques of X-ray diffraction, electron microscopy, crystallization, and molecular weight estimation, the protein obtained from tobacco leaves consists of 8 large subunits and 8 small subunits which form a two-layered, four-fold axis. square-shaped molecule. The large subunit is encoded in the chloroplast genomes and is synthesized by chloroplast ribosomes while the small subunit is encoded in the nuclear genomes and synthesized by cytoplasmic ribosomes. The catalytic site of RuDP carboxylase-oxygenase is located in the large subunits, whereas the small subunits are believed to contain the regulatory site of the enzyme.

Leaf extracts are always characterized by this dominant protein component. Thus, from several points of view, Fraction I protein is extra-ordinarily interesting. Undoubtedly, the characteristics of Fraction I protein would influence the properties of unfractionated alfalfa leaf extract and alfalfa protein concentrate (APC).

Since alfalfa leaf protein is being considered as a source of dietary protein and studies on alfalfa Fraction I protein are

inadequately reported, a study of alfalfa Fraction I protein was undertaken. The objective of the study was to characterize the chemical and physical properties of the principal component (Fraction I protein) of the alfalfa leaf protein.

LITERATURE REVIEW

Leaf Protein

In a vigorously growing leaf, protein is located predominantly in the chloroplast. The remainder of the protein is in a heterogeneous group of organelles, e.g. mitochondria, microsomes, nuclei, etc., or associated with the cytoplasm of the cell (Pirie, 1959, 1971). Most of the leaf protein consists of enzymes. Variation in their concentration and nature relates to physiological changes in the leaf, age, adversity or changes in nutrition and environment (Hanson, 1972).

Leaf proteins can be divided into two fractions, I and II, on the basis of their solubilities in saturated to 0.38 saturation ammonium sulfate solution (Wildman and Bonner, 1948). Singer et al. (1951) and Kawashima & Wildman (1970) further characterized Fraction I protein as homogeneous and as the major protein in the leaf, amounting to about 50% of the total protein. Fraction II is heterogeneous and comprises the rest of the leaf protein.

Cytoplasmic and chloroplastic fractions are defined by their response to heat. The cytoplasmic fraction, which contains varying amounts of organelle proteins, precipitates from solution at a higher temperature than the chloroplastic fraction. The composition of the cytoplasmic fraction is not constant but varies with the physiological state of the tissue and the methods used to remove chloroplast (Pirie,

1971). Lexander et al. (1970) coagulated the cytoplasmic protein at 80 C, following the removal of the chloroplast protein, which was coagulated at 53 C, at different pH values between 4.5 and 6.0. They observed that at higher value of pH yields of cytoplasmic protein were increased. Although this terminology is useful in monitoring the isolation process, it does not describe adequately the nature of the protein in the isolates.

There are no general rules governing the preparation of extracts from leaves (Pirie, 1959). Disintegration of cells is a necessary first step to free the protein contained within the cells and subsequent fractionation is also essential before the individual enzyme or enzyme complexes can be studied. Pirie (1959) and Stahmann (1963) cited a number of factors which make plant protein particularly unstable and difficult to work with, including vacuole acid, polyphenol oxidase, phytic acid and tannins, carbohydrates, proteolytic enzymes, and non-specific proteins aggregations.

The vacuole acid of many leaf cells may lower the pH during comminution sufficiently to denature some proteins. This acid can be neutralized by infiltering with ammonia before maceration of tissue (Coles and Waygood, 1957), adding ammonia (Edwards et al., 1977; Knuckles et al., 1970), or buffering with suitable buffer during extraction (Betschart, 1971; Hood, 1973; Hood and Brunner, 1975, 1976).

Polyphenol oxidases and their substrates are liberated during leaf maceration. In presence of oxygen, quinones are formed rapidly which condense or combine with proteins to produce a characteristic brown or dark colors. In this process protein may be denatured or

altered. To counter this problem, dithionite or metabisulfite can be added to inhibit enzyme activity and the substrates, tannins and phenols, can be removed during cellular disruption. Addition of specific reagents which reduce the quinone to phenol-like compound can also be used (Anderson and Rowan, 1967; Anderson, 1968). Many investigators have used ascorbate to reduce the quinone to phenol precursors to maintain protein stability during isolation. However, the results were variable since only temporary protection is offered by the ascorbate which is gradually oxidized and exhausted (Anderson, 1968). Hood (1973) used the combination of ascorbate and metabisulfite, an enzyme poison, and obtained a high yeild of protein extracted from alfalfa leaves. Insoluble polyvinyl pyrolidene (PVP) has been employed to remove the phenolic materials (Loomis and Battaile, 1966). Lan and Shaw (1970) found greater protein recoveries and higher enzymatic activities in extracts made with the Dowex I-X8 anion exchange resin, a phenolic absorbant under this condition.

An alkaline pH is generally maintained during extraction and isolation to avoid the protein hydrolysis by proteolytic enzymes (Stahmann, 1963). Also, the phenol-protein complex formation is inhibited at pH 7.5 to 8.0 (Loomis and Battaile, 1966). Therefore, many investigators used a weak alkaline solution to buffer the gel filtration or dialysis procedures during the isolation of leaf protein (Trown, 1976; Pon, 1967; Hood and Brunner, 1975, 1976).

Stahmann (1963) believed that the aggregation of leaf protein may be caused by non-specific interaction of the various proteins. Addition of sucrose or polyglycols to the extraction media reduced

this phenomenon in cabbage leaf protein (Heitefuss et al., 1959). The association-dissociation of the various leaf proteins is thought to involve sulfhydryl interaction (Stahmann, 1963). An improved resolution of the protein system was achieved by addition of cysteine, a protein interaction minimizing reagent, to the extracting media (Stahmann, 1963). An excess amount of this reducing agent might result in the cleavage of disulfide bonds of the native protein (Hood, 1973).

For studies on the isolation and identification of protein components in extract, most investigators have utilized the methods of chromatography, gel filtration, gel electrophoresis, ultracentrifugation, or immuno-chemical precipitation. The older physical-chemical methods of centrifugation and free-boundary electrophoresis do not have the resolving power or specificity of these newer methods.

Fraction I Protein

Since Wildman and Bonner (1947) used the terms, "Fraction I and II" to express the leaf proteins, Singer et al. (1951) studied the protein components of leaf protein by using sedimentation analysis and designated the homogeneity of the 18S material from tobacco leaves as Fraction I protein and remaining proteinaceous components (4S) were designated as Fraction II protein. The schlieren pattern of sedimentation velocity assays of leaf extracts showed that 10% of the total area of the pattern was composed of the faster sedimenting 70S and 80S ribosomes of leaves, 40% was in the form of 4 to 6S protein and about 50% of the area of the pattern represented an 18S component (Kawashima and Wildman, 1970). Ellis (1973) employed gel electrophoresis and gel

spectrophotometric scanning to characterize leaf protein. He found the soluble protein pattern dominated by one component which accounted for up to 50% of the total soluble protein in leaf extracts. He labeled this major soluble chloroplast protein as Fraction I protein. It was characterized to be similar to the 18S protein by sedimentation analysis.

Pon (1967) observed that $S_{20,w}$ values of Fraction I protein from spinach leaves were 18 and 26, the latter being the dimer of the former, while values of 18.57 and 18.3 were obtained by Trown (1965) and Kawashima and Wildman (1971b). Ridley et al. (1967) obtained an 18.3S for Fraction I protein from the spinach beet leaves with a molecular weight of 585,000 daltons. Pon (1967) believed that spinach Fraction I protein consisted of one species of molecules of 475,000 daltons in presence of another molecule of 545,000 daltons. Trown (1965) used low temperature and shorter times in sedimentation equilibrium studies and obtained a homogeneous molecular weight of 515,000. Shorter times, higher speeds, and higher temperature were employed in the Kieras and Haselkorn's (1968) determination which agreed very closely with Trown's result. Kawashima and Wildman (1970) summarized the $S_{20,w}$ of Fraction I protein from different species of plants, ranging from 16.2 to 19.5, close to 18.5 for a pure specimen. The molecular weight varied from 4.8 to 5.9 X 10^5 as evaluated by sedimentation equilibrium. The heterogeneous demonstrated by Pon's result was attributed to protein denaturation during long periods of centrifugation. Most investigators believe that Fraction I protein, especially from tobacco leaves, is an 18S molecule possessing a

molecular weight of 515,000 - 560,000 daltons.

Most investigators thought that Fraction I protein functioned as the enzyme, ribulose-1,5-diphosphate carboxylase-oxygenase (RuDP carboxylase) or carboxydismutase (E.C. 4.1.1.39.), in the photosynthetic, CO_2 assimilation reaction (Mendiola and Akazawa, 1964; Thornber et al., 1966; Trown, 1965; Pon, 1967; Sugiyama et al., 1968; Kieras and Haselkorn, 1968; Kawashima and Wildman, 1970, 1971b; Kung, 1976; Hood, 1973; Hood and Brunner, 1976). On the basis of its molecular weight and its behavior toward ammonium sulfate precipitation, RuDP carboxylase of New Zealand spinach leaves was shown to be associated with Fraction I protein (Mayaudon, 1957). Trown (1965) concluded that Fraction I protein was a crude carboxydismutase. Thornber et al. (1966) also arrived at the conclusion that RuDP carboxylase activity of spinach beet leaf was inseparable from Fraction I protein. Ridley et al. (1967) believed that Fraction I protein and the enzyme were identical. But Anderson et al. (1968) isolated a high molecular weight RuDP carboxylase, significantly different from Fraction I protein, from the green and blue-green algae and the purple sulfur photosynthetic bacterium. Consequently, it is believed that the retention of the Fraction I protein nomenclature is useful for designating a particular high molecular weight protein found wherever chlorophyll a is present. Undoubtedly this protein fraction is associated with the photosynthetic apparatus in higher green plants.

The carboxydismutase activity in Fraction I protein from various plants varied from 0.21 to 1.9 units/mg protein, depending upon the methods of purification and condition of the enzymatic reaction (Pon,

1967; Trown, 1965; Andrews et al., 1973; Paulsen and Lane, 1966; Rice and Pon, 1978). Kawashima and Wildman (1970) published an excellent review on the properties of RuDP carboxylase activity of Fraction I protein. Lorimer et al. (1973, 1976) and Whitman and Tabita (1976, 1978a,b) reported the details of the inhibition and kinetics of this enzyme. In this thesis the author has elected not to discuss the inhibition and kinetics aspects of the enzyme.

Recent workers found that RuDP carboxylase (Fraction I protein) could also act as an oxygenase, adding oxygen to ribulose-1,5-diphosphate to give 2-phosphoglycollate and 3-phosphoglycerate (Andrews et al., 1973; Badger and Andrews, 1974; Lorimer et al., 1977; Ellis, 1973; Paech et al., 1977; Kung, 1976). Andrews et al. (1973) believed that the carboxylase was less stable than the oxygenase in Fraction I protein isolated from both soybean and spinach leaves. Ellis (1973) concluded that RuDP oxygenase was one of enzyme activities of Fraction I protein and suggested that the content of Fraction I protein in leaf was rate-limiting to photosynthesis and photorespiration.

The electron micrographs of Fraction I protein revealed particles with a diameter of 10-20 nm, some displaying electron dense centers (Trown, 1965; Ridley et al., 1967; Sugiyama and Akazawa, 1967; Steer et al., 1968). Haselkorn et al. (1965) reported that Fraction I protein preparation from Chinese cabbage leaves showed uniform cubical particles 12 nm along each edge, often with a central depression. Kawashima and Wildman (1971a) observed that crystals of this protein were composed of 12 faces, in the form of parallelograms united to form a complicated quaternary structure.

When examined by electron microscopy, Haselkorn et al. (1965) suggested that cabbage leaf Fraction I protein, purported RuDP carboxylase, was composed of 24 identical subunits. During later investigation, evidences for non-identical subunits having distinct molecular weight and amino acid composition were presented. The molecular weight of the large subunit ranged from 50,000 to 60,000 daltons in various species of plants while the small subunit ranged from 12,000 to 18,000 daltons (Rutner and Lane, 1967; Sugiyama et al., 1971; Sugiyama and Akazawa, 1967, 1970; Kawashima and Wildman, 1970; Moon and Thompson, 1969; Blair and Ellis, 1973; Rutner, 1970). Large and small subunits from the same species had dissimilar amino acid composition. The amino acid compositions of large subunits from different species were very similar, whereas those of small subunits from different species were quite dissimilar (Kawashima and Wildman, 1971b; Rutner and Lane, 1967; Sugiyama et al., 1971; Kung, 1976).

Baker et al. (1975, 1977a,b) utilizing the combined information of X-ray diffraction data, electron micrographs, the crystal density and molecular weight, demonstrated that the most likely structure of tobacco Fraction I protein consisted of eight large and eight small subunits, clustered in two layers, perpendicular to a four-fold axis of symmetry. However, it is widely believed that eight of each of the subunits are aggregated in most forms of this enzyme (Baker et al., 1975, 1977a,b; Chen and Sand, 1979; Roy et al., 1978; Kung, 1976). The large subunits were encoded by chloroplast DNA and contain the catalytic site of enzyme (Nishimura and Akazawa, 1973; Sugiyama and Akazawa, 1970). Murai and Akazawa (1972) found that CO_2 was a homotropic

effector in the regulation of RuDP carboxylase. The small subunits are encoded by nuclear DNA and possibly have a regulatory function, although no definitive evidence exists to support this activity (Nishimura and Akazawa, 1973; Rutner, 1970). Kung (1976) concluded that Fraction I protein had a molecular weight of 560,000, consisting of eight large and eight small subunits arranged in two layered structure, each layer consisting of four large and four small subunits. The large subunit with a molecular weight of 55,000 contained the catalytic site of the enzyme, whereas the small subunit, 12,500 daltons, was involved in a regulatory function.

It is uncertain whether Fraction I protein contains carbohydrates as a part of this structure. Ridley et al. (1967) obtained a positive reaction for carbohydrates when the Fraction I protein gel electrophoresis band was analyzed. He found that the protein isolated from spinach beet leaves contained glucose, xylose, and trace amount of galactosamine and galactose. He also reported that the protein might be conjugated with lipid because the protein yielded a positive reaction when treated with Sudan black following electrophoresis. The protein isolated from spinach leaves was found lacking in carbohydrates (Paulsen and Lane, 1967). Trace of lipid were found in highly purified samples but are likely only a contaminant.

Several free sulfhydryl groups have been detected on the protein and have been deemed essential for enzyme activity (Sugiyama et al., 1968; Sugiyama and Akazawa, 1967). Sugiyama and Akazawa (1967) obtained 96 free sulfhydryl groups by using PCMB titration. Kawashima and Wildman (1970) used the molar ratio of cystine to total amino

acids found in spinach beet leaves Fraction I protein (Ridley et al., 1967) and a molecular weight of 515,000 to calculate 84-SH groups. Based on the comparison of their calculated result and those obtained experimentally by Sugiyama and Akazawa (1967), they concluded that disulfide bonds did not exist in Fraction I protein. However, Hood (1973) found 32 disulfide bonds in the major protein component of alfalfa leaf. A review of the literature revealed a lack of agreement in this issue. Contamination of the protein by nucleic acids has been reported (Eggman et al., 1953) but by exercising appropriate precautions during the isolation process, protein preparations free of these materials were obtained (Pon, 1967; Kawashima and Wildman, 1970; Hood, 1973; Sarkar et al., 1975).

Alfalfa

The major amount of protein in alfalfa occurs in the leaves, between 30 and 50% of the protein being present in the chloroplast. Stahmann (1968) recognized that the alfalfa plant presented the greatest potential for exploitation to increase available dietary protein. Recent workers, especially the group in the Western Regional Research Center of the USDA-ARS, are striving to develop an edible alfalfa protein (De Fremery et al., 1973; Knuckles et al., 1975; Edwards et al., 1975; Kinsella, 1970; Spencer et al., 1971; Kuzmicky and Kohler, 1977). It is beyond the scope of this thesis to cover the subjects of these authors.

Osborne et al. (1921) extracted alfalfa protein by grinding frozen tissue in the presence of water. The addition of ethanol to

the fiber-free extract yielded a precipitate consisting of 70% protein. The protein isolated by this procedure was insoluble in water but could be solubilized by heating in weak alkaline solution. In 1923 and 1924, Chibnall and Nolan described the preparation of purified protein from cytoplasm and vacuoles of leaves of the alfalfa plant. They concluded that cytoplasmic protein resembled the precipitate of Osborne's. The isoelectric zone of these protein was between pH 4.0 and 4.6.

Mertz and Matsumoto (1956) reported the first electrophoretic analysis of alfalfa protein mixture. By moving-boundary electrophoresis, they found that 75-80% of the cytoplasmic protein was presented in one boundary which corresponded to the soluble protein obtained from other plant species (Singer et al., 1951; Lyttleton, 1956). This major component was sensitive to the mechanism of sulfur metabolism in the plant since the concentration of the protein was decreased 50% if isolated from sulfur deficient plants (Mertz and Matsumoto, 1956). McArthur et al. (1964) found that a considerable portion of the soluble protein mixture displayed a single, symmetric boundary of 18.2 to 18.7 S. They reported an electrophoretic mobility in phosphate buffer of -7.25 Tiselius Units (T.U.) and suggested that the protein was similar to the Fraction I protein observed by Lyttleton (1956). They also observed no nucleic acids in this protein in contrast to the work of Mertz and Matsumoto (1956). Later, McArthur and Miltmore (1969) isolated and identified an 18S protein from alfalfa, known as Fraction I protein, having a molecular weight of about 500,000.

Pon (1967) performed extensive physical characterization of the enzyme from spinach and reported mobilities of -3.21 to -6.08 T.U. at

pH values from 6.3 to 9.45 in moving-boundary electrophoresis. The major protein component of alfalfa which was isolated by Hood (1973) possessed carboxydismutase activity and had a mobility of -4.4 T.U. in pH 8.0 veronal buffer. Hood's value, -4.4 T.U. agreed closely with the value reported by Pon, -4.57 T.U. observed at pH 8.07, but lower than the -5.37 T.U. in pH 7.0 cacodylate buffer reported by Mertz and Matsumoto (1956).

The zonal electropherograms of soluble alfalfa protein mixture in starch gels showed 16 bands wherein most of proteins were associated with a single band (Kleczkowska, 1969a). Rommann et al. (1971) obtained 13-15 protein zones by polyacrylamide gel electrophoresis according to Davis' method (1964). Fraction I protein in the gel appeared as a large, dark band near the top of the gel (Kleczkowska, 1969a,b; Hood, 1973; Ellis, 1973).

Hood and Brunner (1975, 1976) reported that Fraction G, a half-saturated ammonium sulfate salted-out protein, contained an 18-20S species identified as RuDP carboxylase, as the principal component. Electropherograms of this protein confirmed this conclusion. Hood (1973) isolated the major protein component from Fraction G by employed ion-exchange and gel filtration. This component exhibited positive RuDP carboxylase activity. The major component contained no nucleic acids and displayed a high sedimentation coefficient (25.3). Its molecular weight, 786,800, is higher than previously reported for Fraction I protein. He also found 1.1% hexose, no hexosamine and the existence of disulfide bonds, whereas Kawashima and Wildman (1970) noted that no disulfide bonds were present. In polyacrylamide gel

electrophoresis Hood (1973) observed that Fraction I protein was more completely dissociated by high pH system than by the system presented mercaptoethanol or 5 M urea. Three protein zones were observed by SDS-PAGE according to the method of Weber and Osbone (1969). Tomimatsu (1978) purified the RuDP carboxylase (Fraction I protein) from alfalfa by using ammonium sulfate fractionation, DEAE cellulose and G-200 Sephadex gel chromatography. The final product had a molecular weight of 548,000 or 497,000 based on ultracentrifugation and light scattering, respectfully. He observed that Fraction I protein did not undergo dissociation of the subunit structure during the scattering experiment. He suggested that in solution the molecule exists in a globular configuration rather than as an extended conformation.

Sarkar et al. (1975) extracted Fraction I protein from alfalfa leaves by using sodium sulfate precipitation and Sepharose 6B gel chromatography. They reported a sedimentation coefficient, $S_{20,\,buffer}$, of 17.9S and that two fragments were observed when Fraction I protein was dissociated by SDS. These authors also isolated Fraction II protein which, as most investigators believed, was very heterogeneous (Kawashima and Wildman, 1970; Ellis, 1973; Singer et al., 1951; Jones and Lyttleton, 1972; Hood and Brunner, 1975). Fraction II protein was resolved into high molecular weight, 125,000 (6.8S) and low molecular weight, 40,000 (3.8S) protein groups (Sarkar et al., 1975).

The factors affecting the extractability of alfalfa leaf protein are essentially those pointed out by Stahmann (1963). Kleczkowska (1969b) obtained one more component in starch-gel electrophoresis when he extracted alfalfa protein with 0.1 M K_2HPO_4 or 0.1 M sodium

phosphate buffer, pH 7.0, containing 1 M NaCl than when he extracted with unbuffered distilled water. Betschart (1971) attributed the improved extractability to the presence of the reducing substances, ascorbic acid or mercaptoethanol, in the extraction buffer. He also compared the efficiencies of maceration achieved with a "Micromill" and the laboratory blender to conclude that the extent of maceration effects the amount of protein extracted. Hood (1973) used several comminution and dialysis media to extract the alfalfa leaf protein fractions and found that superior yields and solubility for the soluble protein fractions were achieved with an extraction medium consisting of 0.1 M Tris-HCl buffer, pH 8.0, and containing 10 mM potassium bisulfite and 0.1% of ascorbic acid followed by dialysis against 0.02 M Tris-HCl buffer, pH 8.0. Sarkar et al. (1975) found that metabisulfite is a powerful poisoning agent for o-diphenol oxidase whose activity, if not restricted, results in an increase of insoluble protein.

EXPERIMENTAL

Materials and Chemicals

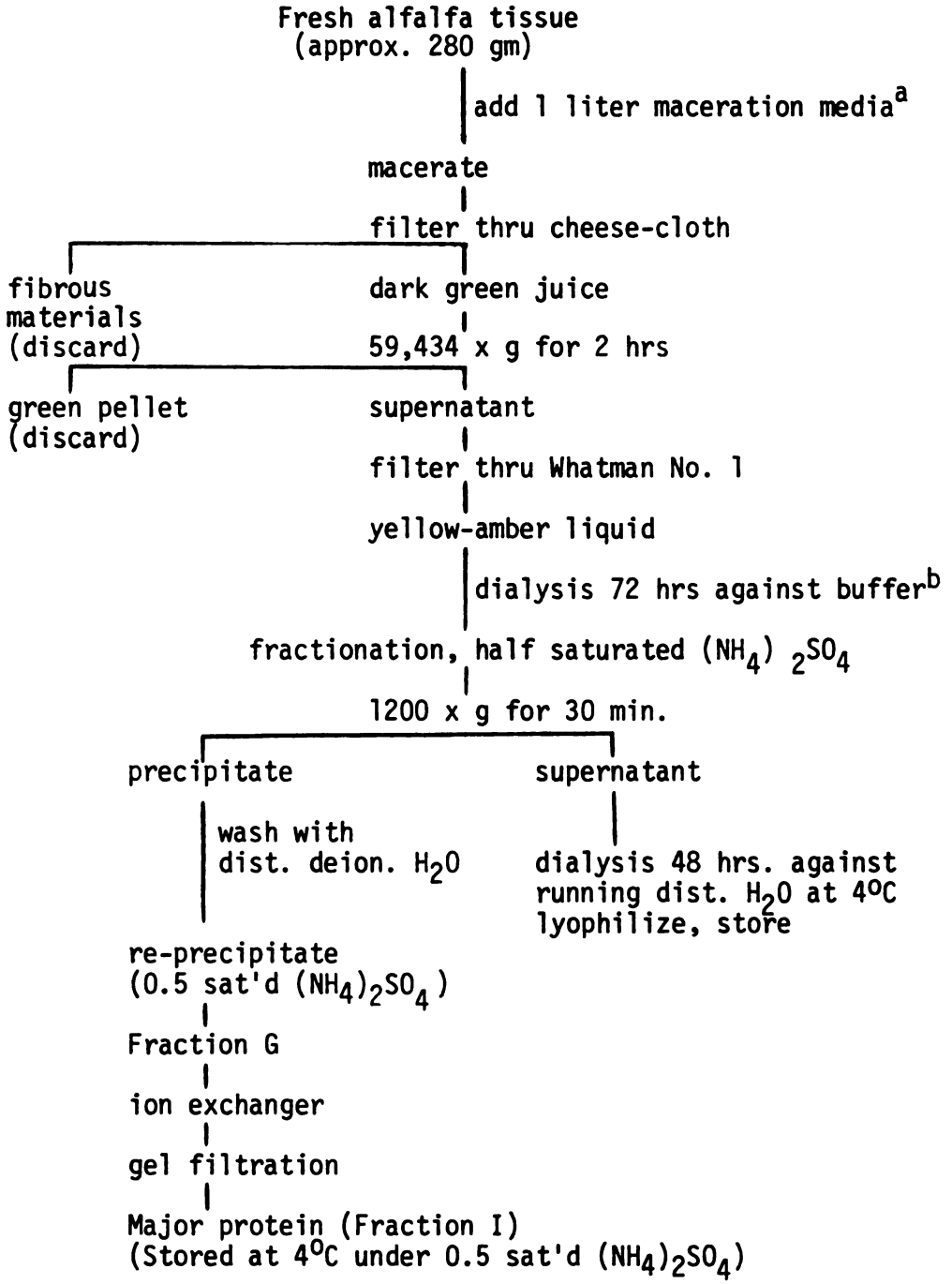
Two sources of fresh alfalfa, Medicago sativa, were used in this study. Plants were grown in the field during summer and fall and potted for green house growth during winter and spring. These plants were maintained by the Department of Crop and Soil Science at Michigan State University. No attempt was made to prepare protein extract from a specific variety of plants. The plants were cut at the stage of first bud or one-tenth bloom. Leaf collection with small amount of leaf stalk and extraction procedures were conducted at room temperature within one hr after harvest.

The chemicals used in this study and their source are listed in the Appendix, Table A1. All were reagent grade unless otherwise specified. Distilled or distilled deionized water was used in the preparation of all buffers and solutions.

Preparative Procedure

Preparation of Alfalfa Protein Fractions

Figure 1 outlines the procedure employed to extract alfalfa leaf protein. The extraction medium, 0.1 M Tris-HCl buffer, pH 8.0, containing 10 mM potassium metabisulfite, 0.1% ascorbic acid and 0.04% sodium azide, was prepared 10-15 min prior to grinding the leaf



[a]0.1 M Tris-HCl buffer, pH 8.0, containing 10 mM $K_2S_2O_5$, 0.1% ascorbic acid and 0.04% NaN_3.

[b]0.02M pH 8.0 Tris-HCl buffer containing 0.05% $K_2S_2O_5$.

Figure 1. Outline for the preparation and isolation of alfalfa leaf protein.

tissues. The macerated material was filtered through a 4-folds of cheesecloth and clarified by centrifugation at maximum speed of 59,434 X g for 2 hr on a Beckman-Spinco Model L preparative ultracentrifuge at 4 C. The amber-colored supernatant was filtered through a Whatman No. 1 filter paper to remove floats. The filtrate was pooled and dialyzed against 0.02 M Tris-HCl buffer, pH 8.0, containing 0.05% potassium metabisulfite at 4 C for 3 days. Ammonium sulfate was added to the dialyzate to half-saturation and centrifuged in 250 ml bottles with the International Centrifuge at 1,200 X g for 20 min. The supernatant was discarded and the pellets from each bottle were collected into 50 ml distilled deionized water and made up to 100 ml. Thirty three grams of ammonium sulfate (50% saturation) was added to this mixture, mixed well, and centrifuged at 5,000 X g in Sorvall RC2-B Centrifuge at 8 C for 10 min (washing step). Again, the pellets were collected into 50 ml distilled deionized water, made up to 100 ml, adjusted to one-half saturation with ammonium sulfate, mixed well, then stored at 4 C for further processing. The salted-out precipitate was designated as Fraction G and used for isolation of the major component (Fraction I protein).

Isolation of Fraction I Protein

A combination of ion-exchange and gel filtration chromatography was utilized to isolate the major component (Fraction I protein) from the salted-out protein, Fraction G.

DEAE cellulose (Whatman DE 32) was precycled with 0.5 N HCl and 0.5 N NaOH and equilibrated with 0.1 M Tris-HCl buffer, pH 8.0.

Followed by removing the fine materials and degassing under 30 in vacuum. The slurried cellulose was poured into a 2.5 X 45 cm Pharmacia column and packed under 60 cm hydrostatic pressure. The final dimension of the cellulosic bed was 2.5 X 30 cm. The column was washed with pH 8.0, 0.1 M Tris-HCl buffer until the pH and conductivity of eluate matched those of the buffer. A solution of Fraction G was placed on the sample support. The Fraction G sample was prepared by dissolving the pellet, collected by centrifuging 15 ml of the salted-out protein mixture, in 20 ml of 0.1 M Tris-HCl buffer, dialyzed to equilibrium against one-liter volumes (4X) of the same buffer for 24 hr at 4 C. A small precipitate was removed from the equilibrated solution at 1,200 X g for 10 min. A discontinuous buffer elution system was employed as follows: approximately 80 ml 0.1 M Tris-HCl, pH 8.0, followed by 280 ml pH 7.0 phosphate buffer (0.1M), and final 150 ml to 180 ml of phosphate buffer containing 0.3 M NaCl. The eluate was monitored at 254 nm with a ISCO Model UA-2 detector and the peak eluted near the break through of the NaCl-phosphate buffer was collected. The column was regenerated with 0.5 N NaOH and re-equilibrated with the starting buffer.

The fraction collected was concentrated by pervaporation and dialyzed against the Tris-HCl buffer containing 12.5 mM $MgCl_2$ for 24 hr at 4 C. Finally 10-15 ml of dialyzed protein solution was applied to Sephadex G-200 column with the aid of a three-way Pharmacia valve.

Sephadex G-200 was equilibrated in 0.1 M Tris-HCl buffer, pH 8.0, containing 12.5 mM $MgCl_2$ and 0.02% NaN_3 as a preservative. After 5 days the mixture was degassed for 20 min under 30 in vacuum and

poured into a Pharmacia column fitted with a flow adaptor and extension tube. The beads were allowed to settle overnight, then packed while flowing under 30 cm hydrostatic pressure. When the final column height was established, a top adaptor was fitted into the column. The column was washed in an up-flow mode with the same buffer, minus preservative, under hydrostatic head 30 cm. The final dimension of column support was 2.5 X 35 cm, possessing a void volume of approximately 65 ml.

The column elution was monitored at 254 nm with detector and appropriate fractions were collected. The collected fractions were salted-out by adding ammonium sulfate to half saturation. The precipitate was collected and stored at 4 C for subsequent studies.

Separation of Subunits of Fraction I protein

Purified Fraction I protein obtained from the G-200 column was mixed with 0.1 M Tris-HCl buffer, pH 8.6, containing 0.5 or 2% SDS and 0.1% ME. Followed by dialysis against the elution buffer, pH 8.6 Tris-HCl buffer containing 0.1 mM EDTA and 0.5% SDS, for 24 hr at room temperature. Approximately 10 to 20 ml of 1% protein solution were applied to a Sephadex G-100 column through a 3-way Pharmacia valve.

The preparation of Sephadex G-100 column was similar to that for the G-200 column described previously. Column elution maintained at 35 ml/hr was monitored at 254 nm and peak eluates were collected.

The SDS in the protein fractions was removed by ion-pair extraction described by Henderson et al. (1979). The SDS-protein samples were mixed with a freshly prepared solvent containing reagent-grade acetone, triethylamine, and acetic acid. The volume ratio of sample

to solvent was 1:20 and the proportion of acetone, triethylamine, acetic acid, and water in the final mixture was 85:5:5:4.5 in volume. The precipitate was separated by low-speed centrifugation and the solvent was removed by decantation. The pelleted protein was washed twice with fresh solvent containing 4.5% water, followed by two washings with acetone. Residual acetone was removed in a vacuum desiccator. The protein specimen was stored at 4 C for further use.

Chemical Analysis

Total Nitrogen

Nitrogen analyses were performed in replicate. A semimicro-Kjeldahl apparatus equipped with round bottom flasks and ball-and-socket ground glass joints was used. The digestion mixture contained 5.0 $CuSO_4 \cdot 5H_2O$ and 5.0 g SeO_2 in 200 ml concentrated sulfuric acid. Approximately 10 mg sample of dried protein were digested in 4 ml of digestion mixture over a gas flame for one hr. Flasks were cooled for a minimum of 30 min, then, 1 ml of 30% hydrogen peroxide was added and digestion resumed for an additional hour. Finally, flasks were removed from the digestion rack, cooled and rinsed with 10 ml of deionized water. The digests were neutralized with 25 ml of 40% of NaOH (w/v) and steam distilled into 15 ml of 4% boric acic containing 3 drops of indicator, consisting of 400 mg of bromocresol green and 40 mg of methyl red in 100 ml of 95% ethanol. Seventy five ml of distillate were collected and titrated with a standardized acid solution. A 95 to 99 per cent recovery was achieved from a tryptophan standard.

Non-Protein Nitrogen

The protein in a sample was precipitated by TCA in final concentration of 15%. The mixture was held at room temperature for 30 min, then centrifuged for 3 min at maximum speed on an International Clinical Centrifuge. The nitrogen content of an aliquot of the supernatant was measured as described above.

Hexose

The colorimetric method of Dubois et al. (1956) was used for hexose determinations. A carefully weighed sample was dissolved in water or weak ammonium solution. One ml of the solution was pipetted into a test tube and 1 ml of 5% phenol (redistilled reagent grade)-water mixture was added. Five ml of concentrated sulfuric acid were added directly against the liquid surface in order to obtain thorough mixing and maximum heat development. After 10 min at room temperature, the tubes were shaken and placed in a 25 C water bath for 20 min. Absorbance was read at 490 nm on a spectrophotometer.

Quantitation was achieved with a standard curve derived from a mixture of mannose and galactose ranging from 0 to 50 ug/ml (1:0.88). Blanks were prepared from the reaction mixture minus protein.

Sialic Acid

Sialic acid determinations were made according to Warren's thiobarbituric acid method (1959).

Protein samples, 10 ± 0.2 mg, were hydrolyzed in 0.1 N sulfuric acid for 1 hr at 80 C. Two hundred µl of hydrolyzed aliquot in duplicate was mixed with 0.1 ml of periodate solution, i.e., 0.2 M sodium

meta-periodate in 9 M phosphoric acid. The mixture was shaken and held at room temperature for 20 min. Following the addition of 0.1 ml of arsenite solution (10% sodium arsenite in a solution of 0.5 M sodium sulfate-0.1 N sulfuric acid), the solutions were agitated until the yellow-brown color disappeared. Then, 3 ml of thiobarbituric acid solution (0.6% in 0.5 M sodium sulfate) was added. The tubes were shaken, capped with glass beads and heated in a vigorously boiling water bath for 15 min. The tubes were removed and cooled for 5 min in cold water before adding 4.3 ml cyclohexane. After mixing through, the aliquots were transferred to 15 ml centrifuge tube and centrifuging for 15 min. Absorbance of the upper cyclohexane phase was determined at 549 nm. A standard curve covered the range 0 to 20 μg of N-acetyl-neuraminic acid.

Hexosamine

Total hexosamine content of Fraction I protein sample was determined according to the method described by Johansen et al. (1960).

Four to five mg of sample were weighed directly into a 5 ml ampoule. One ml of 4 N HCl was added and the mixture frozen in a dry ice-ethanol bath, evacuated, refrozen and sealed under vacuum. The samples were hydrolyzed for 6 hr at 100 C in a convection oven. Cooled hydrolyzates were transferred into distillation flasks. The ampoules were rinsed sequentially with 1 ml of 4 N NaOH and twice with 1 ml of distilled water.

Ehrlich's reagent was prepared by dissolving 2 g of p-dimethyl-aminobenzaldehyde in absolute ethanol, which contained 3.5% concentrated

HCl to a final volume of 250 ml. This solution can be stored at 4 C. The acetylacetone reagent was prepared by dissolving 1 ml of fresh distilled acetylacetone in 25 ml of 1 M Na_2CO_3 solution plus 20 ml of water, adjust to pH 9.8 and made the final volume to 50 ml. This solution should be used within 30 min after preparation.

Five and one-half ml of acetylacetone reagent was added to each sample, maintaining the pH of the mixture at 9.5-10. The flasks, with stoppers inserted, were heated in a vigorous boiling water bath for 20 min. After cooling in an ice-water bath, the flasks were connected to a micro-Kjeldahl distillation apparatus and heated over a mini-Bunsen flame. The steam-volatilized chromogen was collected in a 10 ml volumetric flask containing 8 ml of Ehrlich's reagent. Transmittances were read after 40 min at a wavelength of 548 nm with a Spectronic 21 spectrophotometer.

A mixture of glucosamine-galactosamine (1:1, w/w), ranging from 0 to 10 ug were used to establish the standard curve. A reagent blank consisted of 1 ml 4 N HCl, 1 ml 4 N NaOH, and 2 ml distilled deionized water. With a controlled standard, the average recovery was 94-97%.

Amino Acid

The amino acid analyses were performed on 24 hr protein hydrolyzates employing a Beckman Automatic Amino Acid Analyzer, Model 120C (Moore et al., 1958).

Four to five mg of samples were carefully weighed into 10 ml glass ampoules. Five ml of 6 N HCl were added and the mixture was

frozen in a dry ice-ethanol bath. The ampoules were evacuated, allowed to melt slowly under vacuum to get rid of gases, refrozen and sealed with a propane flame. The sealed ampoules were placed in an oil bath, which temperature equilibrated in a 110 C oven, and allowed to hydrolyze for 24 hr. The ampoules were removed and cooled, opened and 1 ml of a 2.5 μmole N-leucine solution was added to the hydrolyzate as an internal standard. The content of ampoule was quantitatively transferred to a 25 ml pear-shaped flask and evaporated to dryness in a rotary evaporator. The residue was redissolved in small amount of distilled deionized water and evaporated again until all HCl was removed. Finally, the dried hydrolyzate was made up to 5 ml with a 0.067 M citrate-HCl buffer, pH 2.2. Aliquots of 0.2 ml were applied to the Analyzer to analysis.

Oxidation and hydrolysis for cysteic acid and methionine sulfone as described by Lowis (1966) was used to determine the half-cystine and methionine contents of the sample.

A four to five mg sample of dried protein was weighed into 25 ml pear-shaped flask. Ten ml of the oxidant, performic acid, was added. After 15 hr at 4 C the oxidized mixture was evaporated, 5 ml of 6 N HCl was added, and the air was removed by vacuum. The flasks were placed in a 110 C oven for 20 hr and 2.5 μmole of N-leucine was added. Subsequent procedures were similar to those employed for the acid hydrolyzate. Half-cystine was evaluated as cysteic acid and methionine as methionine sulfone, both eluted with the pH 3.28 buffer.

The amino acid composition was expressed as either moles of residue per 100 moles of total residues, or as relative molar ratios based on phenylalanine.

Tryptophan

Procedure W in the pronase hydrolysis method of Spies (1967) was employed for the determination of tryptophan.

Three to five mg sample were carefully weighed into 2 ml glass screw-top vial, mixed with 0.1 ml pronase solution (10 mg of pronase per ml of 0.1 M phosphate buffer, pH 7.5) and agitated momentarily. Fresh pronase solution was prepared for each set of determination. The capped vials were incubated for 24 hr in a 40 C oven, cooled in ice bath and the addition of 0.9 ml of 0.1 M phosphate buffer. Vials were placed into 50 ml Erlenmeyer flasks, containing 30 mg of p-dimethylaminobenzaldehyde and 9.0 ml of 21.2 N sulfuric acid, tipped over and the contents quickly mixed by swirling. The flasks were covered with Parafilm and allowed to stand at room temperature for 6 hr in the dark. Finally, 0.1 ml of 0.045% (w/v) sodium nitrite was added and after 30 min, transmittance was read at 590 nm.

Duplicated samples of the pronase hydrolytic solution without protein sample were simultaneously analyzed and employed as a blank correction. A standard curve was developed from analysis of authentic tryptophan, 0-120 μg, as described above, but without the presence of pronase. The tryptophan content of the analyzed sample was combined with the amino acid data and recalculated to express composition as moles of residue per 100 moles of total residues.

Bio-Rad Protein Assay

The protein assay used in this study was described by Bradford (1976).

Dilute dye reagent was prepared by mixing one part Bio-Rad dye reagent concentrate (Bio-Rad Corp.) with four parts distilled water, filtered through Whatman No. 1 paper and stored at room temperature. To determine the protein concentration, simply 0.1 ml of sample solution, standard, or blank was transferred into test tubes, 5.0 ml dilute dye reagent were dispensed into each tube, followed by agitating carefully to avoid foaming. Transmittances were read at 595 nm on a Spectronic 21.

Concentration of protein was determined by referring to a standard curve constructed with bovine serum albumin. Blank consisted of 0.1 ml buffer solution and 5.0 ml dilute dye reagent.

Available Sulfhydryl Groups

The determination of protein sulfhydryl groups was by the method described by Habeeb (1972).

One ml of pH 7.7 phosphate buffer solution, containing 8.5 mg protein, was mixed with 3 ml of 0.1 M sodium phosphate buffer, pH 8.0, containing 0.04% EDTA. Absorbance was read at 412 nm 45 min after addition of 0.02 ml of dithionitrobenzoic acid (DTNB) solution (40 mg DTNB in 10 ml of phosphate buffer). The molar extinction coefficient of 13,600 reported by Ellman (1959) was used for quantitation. The calculation is as

$$\text{Number of -SH} = \frac{\text{Mol. Wt.} \times A \times D}{13{,}600 \times m}$$

where A is absorbance, D is total volume, m is the weight of sample.

Total Sulfhydryl Groups

About 3.0 mg of protein specimen was dissolved in 6 ml of 0.1M phosphate buffer, pH 8.0, containing 2% sodium dodecyl sulfate (SDS) and 0.04% EDTA. To 3 ml of the solution was added 0.1 ml of DTBN solution. Color was developed for 15 min and absorbance was recorded at 410 nm. Determinations were made in duplicate and a reagent blank was prepared concurrently with the sample.

Disulfide Groups

The principle of disulfide groups determination is based on the reduction of disulfide bonds to sulfhydryl groups by strong reducing agent. Then, total sulfhydryl groups are determined as described above. The method employed here was adopted from the procedure developed by Cavallini et al. (1966).

The determination conducted at least triplicate and absorbance was measured at 412 nm against an appropriate blank. A molar absorptivity of 12,000 M^{-1} cm^{-1} was used for calculating the number of sulfhydryl groups formed after reduction.

Total Lipid

Modification of the method of Mojonnier and Troy (1925) was used to determine total lipid concentration.

Fifty to sixty mg of protein specimen were weighed into conically-shaped centrifuge tubes and to which 1.5 ml of a 2% KCl solution was added. After agitation, 1.0 ml 95% ethanol was added in each tube. the tubes were sealed with stoppers, wrapped in Saran wrap and shaken 30 sec. The tubes were opened and 2.5 ml ethyl-ether (making certain

to rinse stoppers) were added. The tubes were resealed and shaken 30 sec. Followed by releasing the pressure and adding 2.5 ml petroleum ether in each tube, sealing the tubes and shaking 30 sec, prior to centrifuging for 1 min. Then, upper layer solution was removed with syringe and placed in a previously weighed evaporation dish. The extraction procedure was repeated twice. The pooled upperlayers were reduced by evaporation and dried in a 110 C vacuum oven for 30 min. The dishes were cooled in a desiccator and reweighed.

Ash

Porcelain crucibles were heated at 550 C in a muffle furnace, cooled in a desiccator and weighed to four decimal places. Fifty mg of specimen were weighed directly into crucibles, heated over a Bunsen burner in a hood until smoking ceased. The crucibles were placed in the muffle furnace at 550 C until a light gray ash results, or to constant weight (about 48 hr).

The crucibles were cooled in a desiccator and weighed. Ash in the sample was represented as percentage.

Enzymatic Activity

Ribulose-1,5-diphosphate carboxylase/oxygenase activity of Fraction I protein was assayed according to the direct spectrophotometric method described by Rice and Pon (1978).

The stock solutions were (1) 300 mM Tris-OAc buffer, pH 8.1, (2) 50 mM of MgOAc, and (3) 50 mM of $NaHCO_3$. The protein sample was concentrated from the fresh solution collected from the G-200 column and activated at 37 C for 40 min. The protein concentration of samples

was determined by the Bio-Rad protein assay.

The final volume of 3.5 ml in a quartz cuvette contained 525 μmol Tris-OAc, 35 μmol MgOAc, 35 μmol $NaHCO_3$, and 2 to 3 mg of ribulose-1,5-diphosphate. The matched reference cuvette contained all of the above reagent but ribulose-1,5-diphosphate in a volume identical to that of the sample cell. After equilibrating in a 25 C water bath, about 250 - 280 μg of activated protein specimen was added sequentially to reference and sample cuvettes to initiate the reaction. Absorbance were recorded every 30 sec at 280 nm.

Physical Analysis

PAGE in Discontinuous Buffer System

All electrophoretic experiments were conducted by using a 6 mm I.D., 2 mm walled, and 75 mm length glass tubes. The tubes were washed with detergent, immersed in chromic acid, rinsed with deionized water and treated with Photoflo (1:200) before using. Acrylamide and bis-acrylamide were recrystallized from acetone.

Disc gel electrophoresis was conducted as described by Melachouris (1969) with two modification: (1) the acrylamide:bisacrylamide ratio was kept at 19:1 to achieve 5% crosslinked gels, (2) no urea was incorporated into the gel formula. Electrophoresis was employed initially at 2 mA/tube and increased to 5 mA/tube when the tracking dye entered the running gels.

Gels were stained for 4 hr in a solution of Coomassie Brilliant Blue R (Weber and Osbone, 1969), or for 30-60 min in a solution of

Coomassie Brilliant Blue G-250 as described by Reisner et al. (1975). Both stained gels were destained by diffusion in a solution of 7% acetic acid and stored in the same solution.

Urea-PAGE

The basic procedure of Melachouris (1969) with the above modification was adopted. However, before adding the polymerizing reagent, solid urea was added to produce a final concentration of 5 M in both the running and spacer gels. Samples were equilibrated with gel buffer containing 5 M urea for 24 hr at room temperature prior to applying to the gel.

PAGE in High pH, Continuous System

The gel buffer was a solution of 0.05 M phosphate buffer, pH 11, containing 9% of Cyanogum 41 and 0.6% N,N,N',N'-tetramethylethylenediamine (TEMED). A similar buffer was employed in the electrode reservoirs and was prepared by dissolving 14.1 g Na_2HPO_4 in 2 l of distilled-deionized water with sufficient NaOH to yield a final pH of 11.

Gels were prepared by mixing 20 ml of gel-containing buffer with 0.01 ml of 1% freshly prepared ammonium persulfate. The mixture was added to the tube to yield a 5.5 cm gel column. A water layer was placed on the top of the gel solution by means of a syringe as outlined by Davis (1964). Polymerization of gels were permitted to proceed 1 hr prior to electrophoresis. The experiment was conducted under 4 mA/tube of constant current for 4 hr at which time the dye front had migrated approximately 5.0 cm. Gels were removed and stained with Coomassie

Brilliant Blue G-250 as described previously.

Protein samples were prepared to about 1% concentration and dialyzed against the gel buffer solution for 24 hr at 4 C before applying to the gels.

SDS-PAGE

Gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was prepared according to the method of Weber and Osbone (1969).

One to four mg of protein sample was weighed into 5 ml vials and 1 ml 0.01 M phosphate buffer, pH 7.2, containing 1% SDS and 0.1 mercaptoethanol (ME) was added. These mixture were allowed to equilibrate for 24 hr at room temperature before 10% sucrose and 2 drops of tracking dye was added and incubated for 5 min at 100 C. Forty microliter portions (300-400 μg) were applied to the top of the gels.

Molecular weights of the subunits were estimated from a plot of relative mobility (R_f) vs the log of the molecular weights of standard proteins. The standard proteins used in this study were phosphorylase B (94,000), bovine serum albumin (68,000), oval albumin (43,000), pepsin (35,000), carbonic anhydrase (29,000), trypsin (23,300), and lysozyme (14,300). The relative mobility was calculated as follows:

$$\text{Relative Mobility; } R_f = \frac{\text{Distance of protein migration}}{\text{Distance of the dye migration}} \times \frac{\text{Length of gel before staining}}{\text{Length of gel after staining or destaining}}$$

Sedimentation Coefficient

Sedimentation velocity experiments were conducted in a Beckman-Spinco Model E ultracentrifuge using a rotor speed of 39,460 rpm. All

experiments were performed in a double sector, synthetic boundary cell at 20 C.

The sedimentation coefficient of the Fraction I protein from alfalfa leaves was determined on non-lyophilized, fresh samples since significant physical interaction occurred during storage which resulted in altered sedimentation patterns. The freshly isolated protein was concentrated by evaporation, dialyzed 2 days against the same buffer (Tris-HCl, pH 8.0, containing 12.5 mM $MgCl_2$, μ = 0.1). Just before centrifugation, the concentration of protein in solution was measured by the Bio-Rad method.

The apparent sedimentation coefficient (S_{app}) was calculated from following formula:

$$s = S_{app} = \frac{2.303}{60 \cdot w^2} \cdot (d \log x / dt)$$

where w is anglar velocity, in radians/sec. This is $2\pi N/60$, where N is in rpm, revolutions per min. The distance of the boundary from the rotation axis is x in cm, and t is the time in sec. The sedimenting rate, $d \log x / dt$, was the slope of the plot of logarithm of the distance of boundary moved vs the time of sedimentation and was computed by linear regression.

The value of the observed sedimentation coefficient (S_{obs}) was obtained as the intercept of the plot of S_{app} values against the protein concentrations. $S_{20,w}$ is the value which the protein would have in a solvent with the density and viscosity of water at 20 C and is usually reported as sedimentation coefficient. The following equation

was used to correct the S_{obs} to this standard condition.

$$S_{20,w} = S_{obs}\ (n_{t.w}/n_{20,w})(n_{sol}/n_w)\left(\frac{1 - \bar{v}\rho_{20,w}}{1 - \bar{v}\rho_{t,sol}}\right)$$

where $n_{t,w}/n_{20,w}$ is the ratio of the viscosity of the water at experimental temperature to that at 20 C, n_{sol}/n_w is the relative viscosity of the solvent to that of water at any temperature. The term, $\rho_{20,w}$, is the density of water at 20 C while $\rho_{t,sol}$, is that of the solvent at experimental temperature. The partial specific volume, $\bar{v}$, of the protein was assumed a constant value (0.73) in all solvent system performed.

Diffusion Coefficient

The diffusion property was determined in a double sector, synthetic boundary cell at a rotor speed of 4908 rpm in Beckman-Spinco Model E Ultracentrifuge at 20 C.

The preparation of the protein samples was the same as that in sedimentation experiments. The maximum height and area of the schlieren pattern are measured. The apparent diffusion coefficient (D_{app}) was obtained from the slope of the plot of $(1/4\pi)(A/H)^2$ against the time, t, timing $(1 - w^2st)$. The slope may be computed by either linear regression or the following relationship:

$$D_{app} = \frac{(1/4\pi)(A_1/H_1)^2 - (1/4\pi)(A_2/H_2)^2}{t_1 - t_2} \times (1 - w^2st)$$

where A is the area enclosed by the schlieren pattern above its base line in cm^2, H is the maximum height of the peak in cm, t is sec, is

the time measured from the start of centrifugation and $(1 - w^2st)$ is very close to 1.

Upon the plotting the D_{app} values vs different concentrations of protein solution, the D_{obs} was obtained by extrapolating to zero protein concentration. The observed diffusion coefficient was corrected for the effects of solvent as follows:

$$D_{20,w} = D_{obs} \ (293/(273 + t))(n_{t,w})(n_{20,w})(n_{sol}/n_w)$$

The terms in above equation were described previously.

Preparation of Rabbit Anti-sera

A 1% Fraction I protein solution in saline was diluted 1:1 with Freund's complete adjuvent and was emulsified by drawing into and squirting out of a syringe several times. Injections of 1.0 ml of this emulsion were given subcutaneously near groin nodes of a rabbit. Subsequent injections, 1, 2, 3, weeks after the first injection, consisted of a lower amount of antigen, i.e. 0.5% in saline.

One week after every injection, 30 to 40 ml of blood was collected from the marginal ear vein. The blood was centrifuged for 30 min at maximum speed in the Clinical centrifuge to remove red cells. The supernatant serum was decanted, stabilized with 0.02% NaN_3 and stored at 3-5 C until used (<4 weeks).

Immuno-Double Diffusion

Ouchterlony's double diffusion method (1968) was used in this study. A 2% solution of agar in phosphate-buffered saline, containing 0.02% NaN_3, was prepared and stored at 4 C.

Vacuum grease was placed on the botton of petri dishes (6.5x 1.4 cm) and spreaded evenly and invisibly with Kimwipes. Ten to twelve ml of melted agar solution was poured into each dish and allowed to solidify. After solidifcation, a five-hole pattern was cut with a Feinberg agar gel cutter.

Rabbit anti-Fraction I protein serum was placed in the central well and protein samples, dissolved in phosphate-buffered saline solution, were added to the outer wells. Diffusion was permitted to occur for 2-3 days to form precipitin lines. The results were sketched.

RESULTS AND DISCUSSION

Isolation of Fraction I Protein

Fraction G protein was equilibrated with 0.1 M Tris-HCl buffer, pH 8.0, and applied to DEAE-cellulose column. Elution was achieved as previously described. A typical elution pattern is shown in Figure 2A. The elution volume indicated by the horizontal bracket was collected, characterized with PAGE, and retained for further purification by gel filtration on Sephadex G-200. Gel A in Figure 3 shows the electropherogram of fraction G which indicates that the protein was heterogeneous. The electropherogram of the DEAE-resolved fraction (Figure 3, gel B) indicated that the major component was recovered in relatively high purity. Gel filtration was conducted on the fraction collected from the DEAE column following dialysis and concentration as previously described. Figure 2B represented the gel filtration chromatogram and indicated that most of the materials absorbing at 254 nm appeared in a single zone near the void volume of the column. The smaller, trailing peak was examined and did not contain protein based on (1) the lack of a precipitate upon the addition of TCA to 15% (w/v), (2) the absence of a protein zone in disc PAGE, and (3) no precipitate upon heat treatment at 100 C. Generally, the middle portion of the large peak was collected for subsequent analysis.

The sedimentation velocity patterns (Figure 4) revealed a single boundary of approximately 18.7S and a very small area of slower

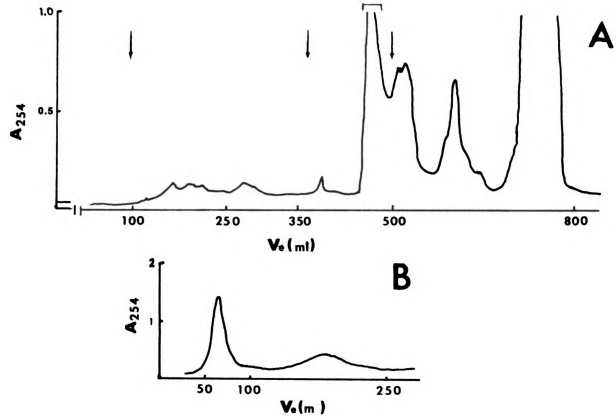


Figure 2. Chromatograms obtained during isolation of Fraction I protein from alfalfa leaves: (A) DEAE cellulose column. Arrows indicate buffer change during elution of fraction G. Bracket indicates fraction collected for further analysis. (B) Sephadex G-200 column chromatogram of protein fraction obtained from DEAE cellulose column.

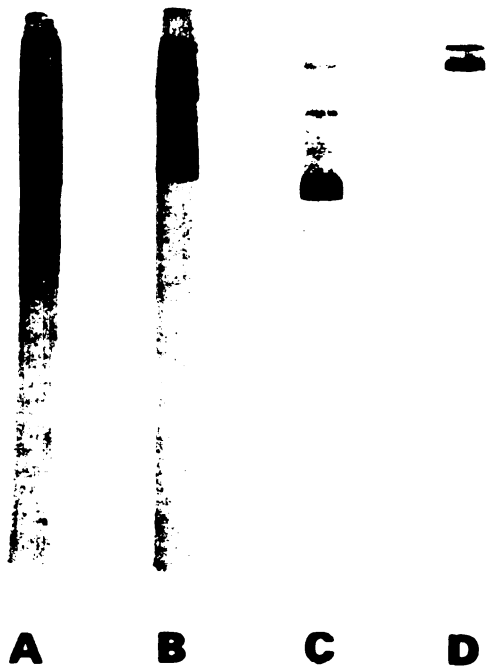


Figure 3. Disc PAGE patterns of fraction G (A), DEAE resolved fraction (B), final preparation obtained from the G-200 column-Fraction 1 protein (C and D). Total gel concentrations (T%) for patterns A, B and C were spacer gel (5%), running gels (7%); for pattern D spacer gel (5), running gel (10%).

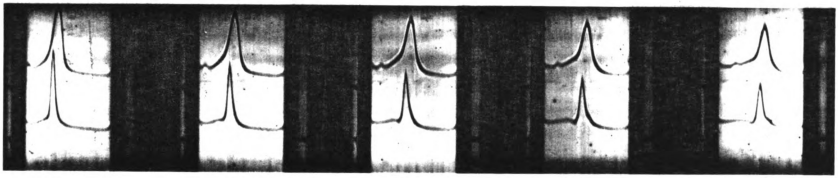


Figure 4. Sedimentation velocity patterns of alfalfa leaf Fraction 1 protein.
Velocity: 39460 rpm; Temperature 20°C
Protein concentrations: top pattern = 13.5 mg/ml; bottom pattern = 6.75 mg/ml
Buffer = 0.1M Tris - HCl, pH 8.0, containing 12.5 mM $MgCl_2$ (μ=0.1)

sedimenting species. Figure 3 shows the electropherogram of the purified Fraction I protein, gels C and D, and indicates that most of the material migrated as one zone, but a small "eyebrow" appeared in back of major zone. In 10% T (total gel concentration) PAGE (Figure 3, gel D), the minor zone is just below the spacer-running gel interface. Additional gel filtration or ammonium sulfate fractionation did not remove this minor component which increased in intensity after a period of storage at 4 C. Sedimentation velocity and gel electrophoresis analysis on this protein support the conclusion that the protein is homogeneous and suitable for further study.

Experimental observations as well as a review of the literature indicate that physical analysis of the alfalfa leaf Fraction I protein must be conducted immediately after isolation. Following a period of storage (3 - 4 weeks) the protein showed two boundaries of equal area in sedimentation velocity experiment (Hood, 1973). The solution was also slightly cloudy indicating the presence of a fine precipitate. These phenomena have been noted elsewhere (Stahmann, 1963). In preparation for the evaluation of physical parameters, the fine precipitate was removed from a small volume of protein solution by filtration through a Millipore filter (0.5 μm pore) fitted with a glass syringe. Except where indicated, physical and chemical analyses were performed on freshly prepared, non-lyophilized protein solutions. Protein concentrations were measured by the Bio-Rad assay (Bradford, 1976).

Enzymatic Nature

To utilize the direct spectrophotometric assay, cuvettes must be matched and pipetted volume must be precise. Additionally, the enzyme

in Fraction I protein should be activated. Magnesium served as an activator of RuDP carboxylase (E.C. 4.1.1.39.) (Johal and Bourque, 1979; Rice and Pon, 1978) and was included in the assay solution (12.5 mM $MgCl_2$) which was then activated for at least 40 min in a 37 C water bath. The reaction preceeded for 14 - 15 min after addition of enzyme.

The fresh sample eluted from the column gave a positive reaction for RuDP carboxylase (Figure 5, open-circle). The plot connecting the close-circles illustrates the result of lyophilizing the sample. Presumably the protein was denatured and completely lost its specific activity. Hood (1973), employing the assay procedure of Paulsen and Lane (1966), also got a positive result for the major component of alfalfa protein. Tomimatsu (1978) obtained a similar result for the final extract obtained from a G-200 column.

The specific activity of this enzyme in the sample under investigation was 1.24 units/mg protein which was higher than the 0.64 units/mg protein reported by Pon (1967) and the 0.4 units/mg protein reported by Tomimatsu (1978) and seems to be harmoniuos with the state of its purity (Rice and Pon, 1978). It exceeds the scope of this study to investigate the kinetic properties of this enzyme in detail. However, the enzymatic nature and physical properties discussed below indicate that the final product is identical to Fraction I protein described by Kawashima and Wildman (1970).

UV Spectrum

The UV absorbance spectrum for Fraction I protein of alfalfa leaves shown in Figure 6 is typical for a protein, having an A_{max} at

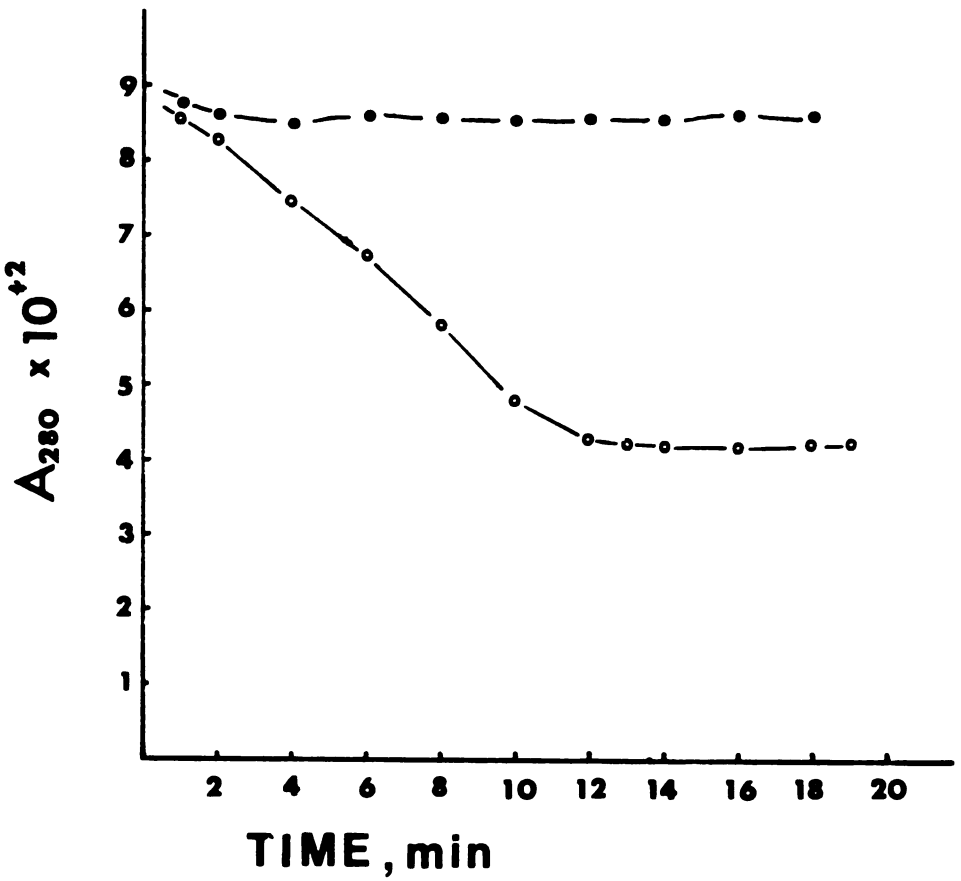


Figure 5. The direct spectrophotometric assay at 280 nm for RuDP (13 nmole/ml) over a period of time. The lower line (o-o) shows the decrease in absorbance of RuDP with time in presence of 75 μg/ml fresh activated enzyme (Fraction I protein). The reaction ended 14 min after enzyme addition. The upper line (o-o) represents the reaction of RuDP with lyophilized enzyme (Fraction I protein).

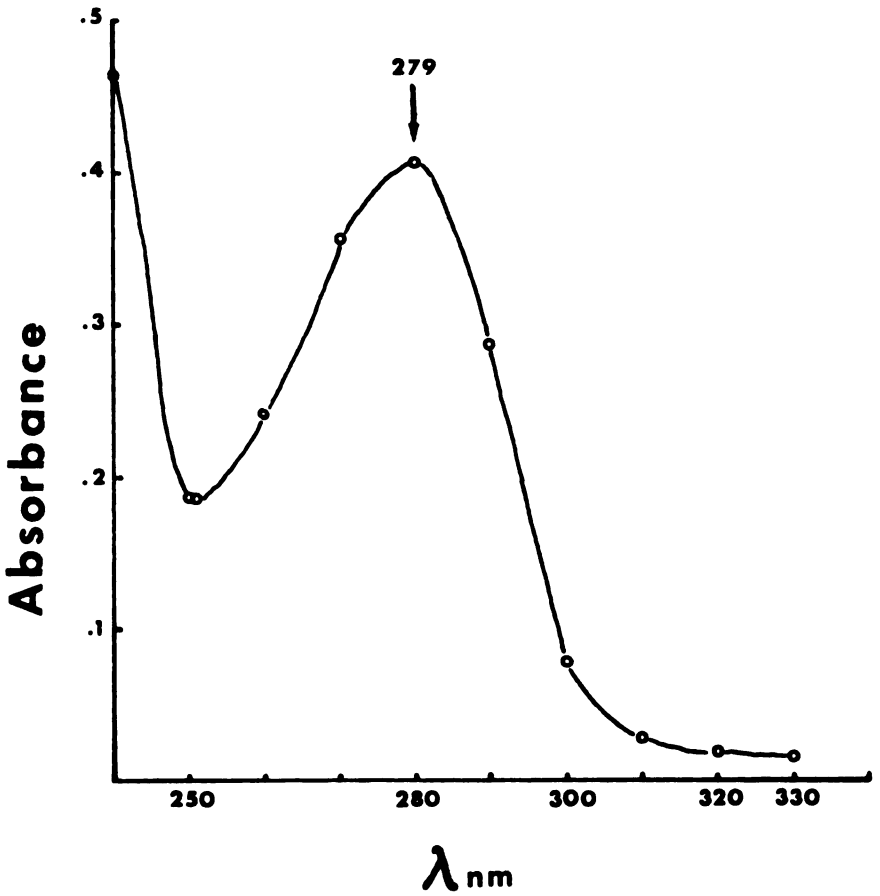


Figure 6. UV absorption spectrum of purified Fraction I protein. Protein concentration was 0.35 mg/ml in 0.1M Tris-HCl buffer, pH 8.0, containing 12.5 mM $MgCl_2$. The maximum in absorbance was at 279 nm.

279 nm. The ratio of A_{280} to A_{260} was 1.70 to 1.75 for several samples collected from the G-200 column (0.30 - 0.41 mg protein/ml), employing the elution buffer as a reference. These values indicate an absence of nucleic acids in the preparation which is in agreement with previously reported studies (Pon, 1978; Kawashima and Wildman, 1970; Sarkar et al., 1975; Jones and Lyttleton, 1972; Hood, 1973; Chollet et al., 1975) and support the decision that no further purification was necessary. Table A2 represents the results of analyses of alfalfa Fraction I protein from different cutting in this study.

Chemical Analysis

Table 1 summarizes the results of chemical analyses of alfalfa Fraction I protein. Isolated alfalfa Fraction I protein obtained from Sephadex G-200 column contained 16.4% protein nitrogen, 1.85% hexose and no hexosamine. There was no sialic acid in the sample as determined by Warren's standard procedure. Additionally, isolated material was hydrolyzed with 0.1 N H_2SO_4 at 80 C for 1 hr to free the bound sialic acid, if present. Results indicated an absence of the bound acid. Therefore, it is concluded that alfalfa Fraction I protein does not contain sialic acid. Ridley et al. (1967) reported that Fraction I protein from spinach beet leaves contained 16.75% nitrogen as calculated from the amino acid analysis and the conversion factor of 5.97. Employing this factor, the final preparation in this study contained 97.9% protein, a value slightly lower than the 99.1% found by Hood (1973).

The protein isolate in this study contained approximately 1.85% hexose but lacked other carbohydrates normally occurring in

glycoprotein. Akazawa et al. (1965) reported that Fraction I protein isolated from rice leaves was a glycoprotein. Ridley et al. (1967) also found the existence of the carbohydrates, glucose, xylose, and galactosamine in spinach beet leaf Fraction I protein. But Paulsen and Lane (1966) and Pon (1967) reported its absence. Hood (1973) obtained similar results to those in this study and suggested that the hexose was fortuitously bound with protein during isolation. Trown (1965) noted that Fraction I protein tends to bind other materials during the isolation process. Also, plant materials are an excellent source of phenols which could contribute to a positive hexose measurement with the method used in this study (Dische, 1955). Thus, Fraction I protein isolated from alfalfa leaves can not be designated as a typical glycoprotein based upon the small amount of hexose detected and the absence of other carbohydrates commonly found in glycoprotein. The content of hexose in the alfalfa Fraction I protein is considered to be an isolation contaminant in the present experiment.

No lipid occurred in the alfalfa Fraction I protein as isolated. Trace lipids were found in the enzyme isolated from spinach beet leaves but were likely only a contaminant (Ridley et al., 1967). The traces of ash found in this study may have been a result of the copper and iron which have been found in this enzyme (Johal and Bourque, 1979; Chollet et al., 1975). However, no analyses were performed in the present study to evaluate this hyopthesis.

The data in Table 1 represent the results of analyses for available sulfhydryl, total (unexposed) sulfhydryl, and reduction-induced sulfhydryl groups. There was approximately 0.8 available sulfhydryl

Table 1. Chemical composition of Fraction I protein from alfalfa leaves

Component	Content (%)
Nitrogen	16.4
Protein (5.97[a] x %N)	97.9
Carbohydrate	
Hexose	1.85
Hexosamine	none
Sialic acid	none
Tryptophan	2.59
Ash	trace
Lipid	none
Available -SH[b]	0.8
Total -SH[b]	37
Total -SH after reduction of S-S[b]	99

[a]Ridley et al. (1967)

[b]Expressed as number of -SH groups per mole (573,000 daltons)

group per 573,000 daltons of protein in the native conformation. The protein, after treatment with 0.2% SDS, produced a total of 37 detectable sulfhydryl groups, whereas the protein treated with a strong reducing agent, $NaBH_3$, produced a total of approximately 99 sulfhydryl groups per protein molecule. This suggested that there are 31 disulfide bridges distributed throughout the interior of the molecule or its subunits.

The 37 free sulfhydryl groups found in this study is in agreement with 30 - 40 free sulfhydryl groups detected by other techniques (Sugiyama et al., 1968). It is lower than the 46 for alfalfa Fraction I protein reported by Hood (1973) who used the same method of analysis. Hood (1973) used Flavin's (1962) extinction coefficient of 12,000 to quantitate his absorption data. If his data are interpreted using Ellman's molar absorptivity (13,600), as used in this study, alfalfa Fraction I protein would have 40 total sulfhydryl groups. If his data are modified in accordance with the molecular weight found in this study, 573,000, the unexposed -SH groups would be 34 per molecule. Whether it be 34 or 40 sulfhydryl groups, either value is in better agreement with other reported values (30-40) than is the higher value of 46.

Kawashima and Wildman (1970) stated that the enzyme is lacking disulfide bonds. This conclusion was supported by the result of Sugiyama and Akazawa (1967) who used a PCMB titration and found 96 free sulfhydryl groups which, if accurate, would negate the possibility for disulfide bonds in carboxydismutase. It is interesting that both the adjusted data of Hood (1973) and this study indicate the existence of

disulfide bonds in alfalfa Fraction I protein: 26 and 31 disulfide bonds, respectfully. The literature relating to the presence of disulfide bonds is further complicated by the finding that the molecule actually consists of non-identical subunits (Moon and Thompson, 1969; Rutner and Lane, 1967). The techniques applied here do not distinguish between intra- and inter-molecule disulfide bonds. However, results presented and discussed with regards to effects of dissociating agents indicate that the disulfide bonds can be considered to be of the intra-chain type, because mercaptoethanol treatment of the protein did not release significant amounts of electrophoretically distinct protein zones when compared to PAGE in urea-containing gels or when exposed to high value of pH (i.e. >10).

Amino Acid Composition

The amino acid composition of alfalfa Fraction I protein is reported in Table 2 and is expressed in two ways: (1) as moles of residue per 100 moles of total residues, and (2) as relative molar ratio of each amino acid compared to phenylalanine.

Based upon the data of moles/100 moles amino acid, the ratio of the acidic residues, Asp and Glu, to the basic residues, Lys, Arg, and His, is approximately 1.1. The ratio of the hydrophilic residues, Ser, Thr, Tyr, Asp. Glu, Lys, ½Cys, and His, to the hydrophobic residues, Leu, ILeu, Val, Pro, Phe, and Met, is 1.2. This illustrates that Fraction I protein from alfalfa leaves possesses an acidic and hydrophilic nature. Hood (1973) drew the same conclusion although he did not measure the ½cystine and methionine in performic acid oxidized

Table 2. Amino acid composition of Fraction I protein from selected plant species

	Alfalfa[a] (Moles/100) moles	Molar ratio relative to phenylalanine				
		Alfalfa[a]	Alfalfa[b]	Spinach beet[c]	Spinach[d]	Spinach[e]
Phenylalanine	4.37	1.00	1.00	1.00	1.00	1.00
Aspartic acid	7.04	1.61	1.60	1.76	2.01	2.37
Serine	3.00	0.69	0.61	1.08	0.68	0.87
Threonine	4.83	1.11	1.07	1.41	1.37	1.54
Glutamic acid	6.78	1.55	1.92	2.08	2.18	2.55
Proline	4.10	0.94	1.09	1.17	1.24	1.29
Glycine	7.66	1.75	1.70	1.95	2.05	2.22
Alanine	7.32	1.68	1.64	1.80	1.89	2.06
Half-cystine	1.39	0.32	0.24	0.40	0.46	-
Valine	6.31	1.44	1.31	1.80	-	1.39
Methionine	1.34	0.31	0.08	0.40	0.45	0.50
Isoleucine	3.99	0.91	0.88	1.00	0.84	0.65
Leucine	7.62	1.74	1.58	1.89	2.00	2.12
Tyrosine	3.31	0.76	0.86	0.89	1.07	1.10
Lysine	4.73	1.08	1.05	1.19	1.15	1.18
Histidine	2.62	0.60	0.82	0.89	0.67	0.67
Arginine	5.40	1.24	1.35	1.00	1.34	1.42
Tryptophan	2.12	0.49	0.34	0.30	0.64	-

[a]This study [b]Hood (1973). [c]Ridley et al. (1967).

[d]Rutner and Lane (1966). [e]Kawishima (1969).

specimens. He concluded that this compositional characteristic confirmed the theory of Trown (1965) who suggested that carboxydismutase had a hydrophilic character based upon its large charge/mass ratio and its tendency to bind ionic substances during isolation.

The amino acid composition of RuDP carboxylase is conventionally reported in ratios related to phenylalanine (Kawashima and Wildman, 1970). Therefore, the amino acid composition of alfalfa RuDP carboxylase (Fraction I protein) was calculated for comparison to Hood's data (1973) and for comparison with Fraction I protein of other species. These comparisons show that the molar ratios of alfalfa Fraction I protein are significant different from those of other plant species. This table indicates that the primary structure of Fraction I protein from different plant species is not identical. The values of molar ratios in Table 2 also indicate a slight difference between Hood's and this study except ½Cys and Met which were quite different. The ratio values of ½Cys and Met obtained by using separate analysis in this study (not applied by Hood) were close to the values yielded by Ridley et al. (1967) and Rutner and Lane (1967) who also determined these residues by a separate method.

Based on the amino acid chemical scoring, the sulfur-amino acids are the limiting residues in alfalfa Fraction I protein. The results of these calculations are shown in Table 3. The distribution of essential amino acids in alfalfa leaf Fraction I protein is similar to that of casein. For example, leucine is the essential amino acid in highest content in both proteins, i.e. 8.89 g/16 g N in alfalfa Fraction I protein and 10.0 g/16 g N in casein. Also, sulfur amino

Table 3. Amino acid chemical scoring for alfalfa Fraction I protein

Amino Acid	FAO/WHO[a] (g/16 g N)	Whole egg (g/16 N)	Fraction I protein (g/16 g N)	Scoring, % A[d]	Scoring, % B[e]
Lysine	5.5	6.4	6.25	100	98
Tryptophan	1.0	1.6	4.07	100	100
Threonine	4.0	5.1	5.04	100	99
½ Cystine & Methionine	3.5	5.5	3.27[b]	93	59
Valine	5.0	7.5	6.45	100	88
Isoleucine	4.0	1.6	4.65	100	100
Leucine	7.0	8.8	8.89	100	100
Tyrosine & Phenylalanine	6.0	5.8	12.20[c]	100	100

[a]FAO/WHO reference (1973).

[b]½ Cystine 1.46, Methionine 1.81.

[c]Tyrosine 5.57, Phenylalanine 6.63.

[d]Compared to FAO/WHO reference.

[e]Compared to whole egg reference.

acids are the limiting residues in both protein. The tryptophan content of the proteins is significant different.

From the ratio of the moles ½Cys to moles protein, using a molecular weight of 573,000, it is found that alfalfa leaf Fraction I protein contains 82 SH groups per mole. This value is very close to the value of 84 obtained from Kawashima and Wildman's calculation (1970). Although this value of 82 is different from the experimental value of 99 obtained by direct analysis of cysteic acid residue in this study, it still supports the conclusion of the existence of disulfide bonds in alfalfa Fraction I protein since only 37 free sulfhydryl groups were found before reduction.

Sedimentation Coefficient

Table 4 lists the apparent sedimentation coefficients (S_{app}) of the sample protein in pH 8.0 Tris-HCl buffer (μ = 0.1) at various protein concentrations.

The apparent sedimentation coefficient of the major boundary displayed a normal concentration dependence, increasing with decreasing concentration of protein which is in agreement with other reports (McArthur et al., 1964; Kawashima and Wildman, 1970; Ridley et al., 1967; Pon, 1967; Hood, 1973). Trown (1965) reported that the sedimentation coefficient of carboxydismutase (Fraction I protein) was proportional to the concentration of enzyme, decreasing with decreasing protein concentration. Pon (1967) reported that the value for Fraction I protein from spinach leaves increased with decreasing concentration of protein, but below 0.34 mg/ml, it decreased with decreasing

Table 4. Apparent sedimentation coefficients for Fraction I protein. Buffer: 0.1 M Tris-HCl, pH 8.0, containing 12.5 mM $MgCl_2$ (μ = 0.1)

Protein Concentration (mg/ml)	Sedimentation Coefficient
13.5	15.4
6.75	16.1
3.37	16.7

concentration. These observations may indicate the dissociation of the protein.

Figure 7 shows that S_{obs} of Fraction I protein is approximately 17.1 when extrapolated to zero protein concentration. A $S_{20,w}$ of 18.7 was computed when corrected for buffer effects. This value is within the range of the value 18.2 to 18.7 reported for alfalfa (McArthur et al., 1964; Sarkar et al., 1975) and other species of plant (Kawashima and Wildman, 1970). Hood (1973) reported a $S_{20,w}$ of 25.3 for alfalfa Fraction I protein. His relatively high sedimentation coefficient value apparently arose from appreciable concentration dependency.

Diffusion Coefficient

The apparent diffusion coefficients of alfalfa leaf Fraction I protein were determined at various protein concentrations and the results listed in Table 5. The data plotted in Figure 8 show that the diffusion coefficient of the protein sample was slightly concentration dependent. This is contrary to normal behavior for undissociated species. Hood (1973) obtained the same phenomenon in alfalfa Fraction I protein and suggested that protein dissociation probably occurred at low concentration. The observed diffusion coefficient (D_{obs}) of 2.75 Ficks Units was obtained by extrapolating the plot to zero concentration of protein. A value of 2.97 for $D_{20,w}$ was calculated after correction for buffer effects. This value is close to the 2.98 found by Hood (1973), 2.93 found by Trown (1965), and within the range of 2.75 to 3.01 F.U. reported by previous investigators (Lyttleton, 1956; Pon, 1967; Kawashima and Wildman, 1970).

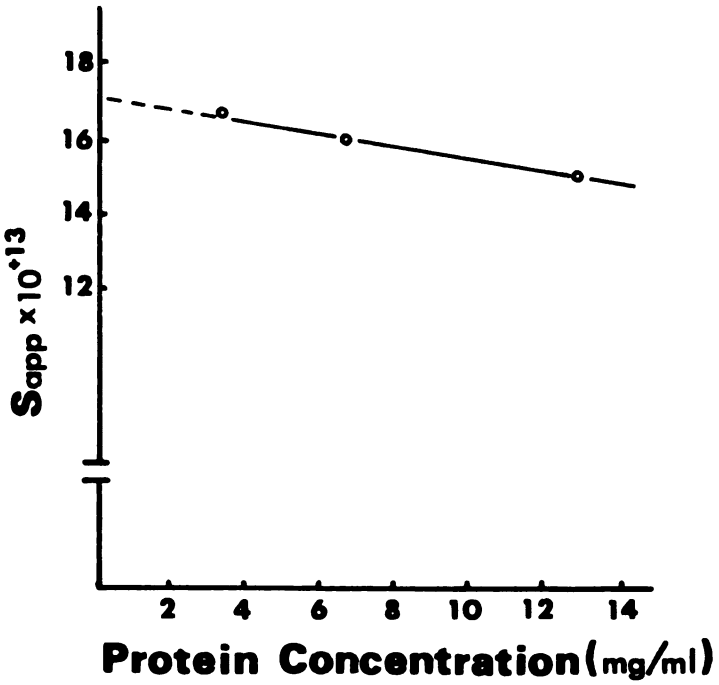


Figure 7. Sedimentation coefficient of alfalfa Fraction I protein at several concentrations. Buffer: 0.1M Tris-HCl, pH 8.0, containing 12.5 mM $MgCl_2$ (μ = 0.1).

Table 5. Apparent diffusion coefficients for Fraction I protein in 0.1 M Tris-HCl, pH 8.0, containing 12.5 mM $MgCl_2$ (μ = 0.1)

Protein Concentration (mg/ml)	Diffusion Coefficient
13.5	2.46
6.75	2.55
3.37	2.70

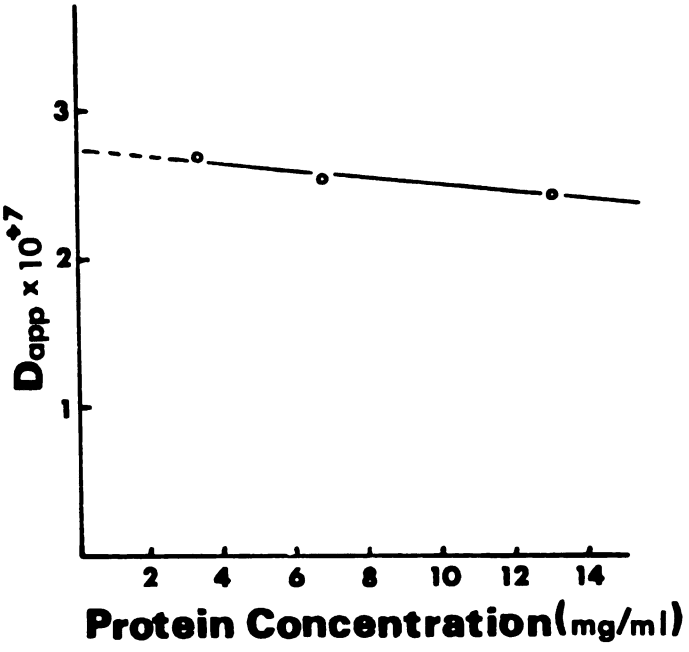


Figure 8. Diffusion behavior of alfalfa Fraction I protein at several concentrations. Buffer: 0.1 M Tris-HCl, pH 8.0, containing 12.5 mM $MgCl_2$ (μ = 0.1).

Estimation of Molecular Weight

The Svedberg equation can be written, with diffusion coefficient (D) as:

$$\text{Molecular Weight} = RTs/D(1 - \bar{v}\varrho)$$

in which R is the gas constant, 8.315×10^7, T is absolute temperature, $\bar{v}$ is the partial specific volume of the macromolecule, and ϱ is the density of protein solution. The molecular weight of the macromolecule can be calculated from $S_{20,w}$ and $D_{20,w}$. The density of the protein solution after dialysis was assumed to be close to that of the dialyzing buffer. A value of 1.004 for the density of 0.1 M Tris-HCl, pH 8.0, containing 12.5 mM $MgCl_2$ (μ = 0.1) after dialyzing against the protein solution was measured by pycnometer. The value of 0.73 for $\bar{v}$ as reported by Trown (1965) was used in the calculation.

The molecular weight of alfalfa leaf Fraction I protein in this study was estimated with the above formula as approximately 573,000. It is slightly greater than the 548,000 daltons reported for alfalfa RuDP carboxylase (Fraction I protein) by Tomimatsu (1978) using the same means of estimation. The value falls in the range of 480,000 to 590,000 reported for the enzyme from other plant species (Trown, 1965; Paulsen and Lane, 1966; Ridley et al., 1967; Pon, 1967; Kawashima and Wildman, 1970, 1971b). These workers obtained the molecular weight by employing the sedimentation-equilibrium method which is considered more reliable than values derived from a sedimentation-diffusion coefficient estimation. Hood (1973) obtained a higher molecular weight (786,800) due to the higher sedimentation coefficient obtained

in his experiment. Attempts to study the molecular weight of alfalfa Fraction I protein by equilibrium analysis in this study were unsuccessful. It is probable that the Fraction I protein isolated from alfalfa leaves contained several free sulfhydryl groups which may cause sulfide interchange leading to aggregation of protein molecules during equilibrium experiments. Other researchers have incorporated reducing agents such as cysteine or mercaptoethanol in the buffer used for centrifugal analysis to avoid the aggregation of protein caused by disulfide interchange. These reducing reagents were not included in the present studies.

Paulsen and Lane (1966) obtained a molecular weight of 557,000 for Fraction I protein from spinach leaves by sedimentation equilibrium experiment. The $M_{S/D}$ calculated from the use of Paulsen and Lane's $S_{20,w}$ (21.0) and from the $\bar{v}$ and $D_{20,w}$ used in this study yielded a molecular weight of 644,000. This value is 15% higher than that obtained by sedimentation equilibrium. If we assume this 15% difference is transferable to the present results, a molecular weight 487,000 is obtained. In this case, the value 487,000 is close to 475,000 obtained by Pon's sedimentation-equilibrium experiments and the value of 497,000 calculated from light scattering measurements by Tomimatsu (1978). Though the value of 487,000 is lower than the 511,000 - 515,000 values reported by Kawashima and Wildman (1970), it is still within the range of reported molecular weights for Fraction I protein from various species of plant (480,000 - 590,000). Table 6 shows the ultracentrifugation data for Fraction I protein from different plants.

Table 6. Some physical parameters of Fraction I protein from various plant leaves

Plant	$S_{20,w}$	$D_{20,w}$	$M_{S/D}$ [a]	M_{eq} [b]	Reference
Alfalfa	18.7	2.97	573,000		This study
Alfalfa	25.3	2.98	786,800		Hood (1973)
Spinach	18.6	2.93	559,000	515,000	Trown (1965)
Spinach	18.7	3.01	559,000	475,000	Pon (1967)
Spinach beet	18.3			557,000	Ridley et al. (1967)
Clover	16.2	2.75	600,000		Lyttleton (1956)
Chinese cabbage	17.0			511,000	Kieras and Haselkorn (1968)
Avena sativa L.	18.3		570,000		Steer et al. (1968)
Tobacco	18.3			525,000	Kawashima and Wildman (1971b)

[a] Molecular weight calculated from $S_{20,w}$ and $D_{20,w}$.

[b] Molecular weight obtained by sedimentation-equilibrium.

Effects of Dissociating Agents

The effect of five dissociating condition: (1) mercaptoethanol (ME), (2) urea, (3) urea plus ME, (4) pH 11 buffer, and (5) SDS on the protein sample was studied by PAGE. The results are shown in Figure 9. Gel A and B represent the undissociated protein sample in 7% and 10% total gel concentration (T) run according to Melachouris's method (1969). Mercaptoethanol appears to have very little effect on Fraction I protein. Gel C represents the PAGE pattern after the purified protein was exposured to 10 mM ME for 30 min. There is no significant difference between gel B and C. Gel D shows the pattern of protein isolate equilibrated with 5 M urea plus 10 mM ME and applied to 5 M urea gel system. The results of adding 5 M urea to the sample (without ME) and a 5 M urea gel system (not shown) is similar to that seen in gel D. Diffuse zones appear in both gel patterns which were absent in gel B and C. This may indicate that urea causes unfolding of the protein structure. The presence of ME in the urea gel system did not reveal a sharpening of zones which was observed by Hood (1973). Sugiyama and Akazawa (1967) were also unsuccessful in obtaining a clear dissociation of protein into subunits with urea treatment.

The gel E pattern suggests that pH 11 buffer is an effective dissociating system for alfalfa Fraction I protein. More than 5 separate zones were apparent in this high alkaline gel system. Furthermore, high pH treatment resolved the protein into more components or subunits than 5 M urea-ME treatment. Hood (1973) observed approximately 7 bands in the same system and in the system containing ME. He suggested that this phenomenon may be cuased by disulfide bond-splitting at the high

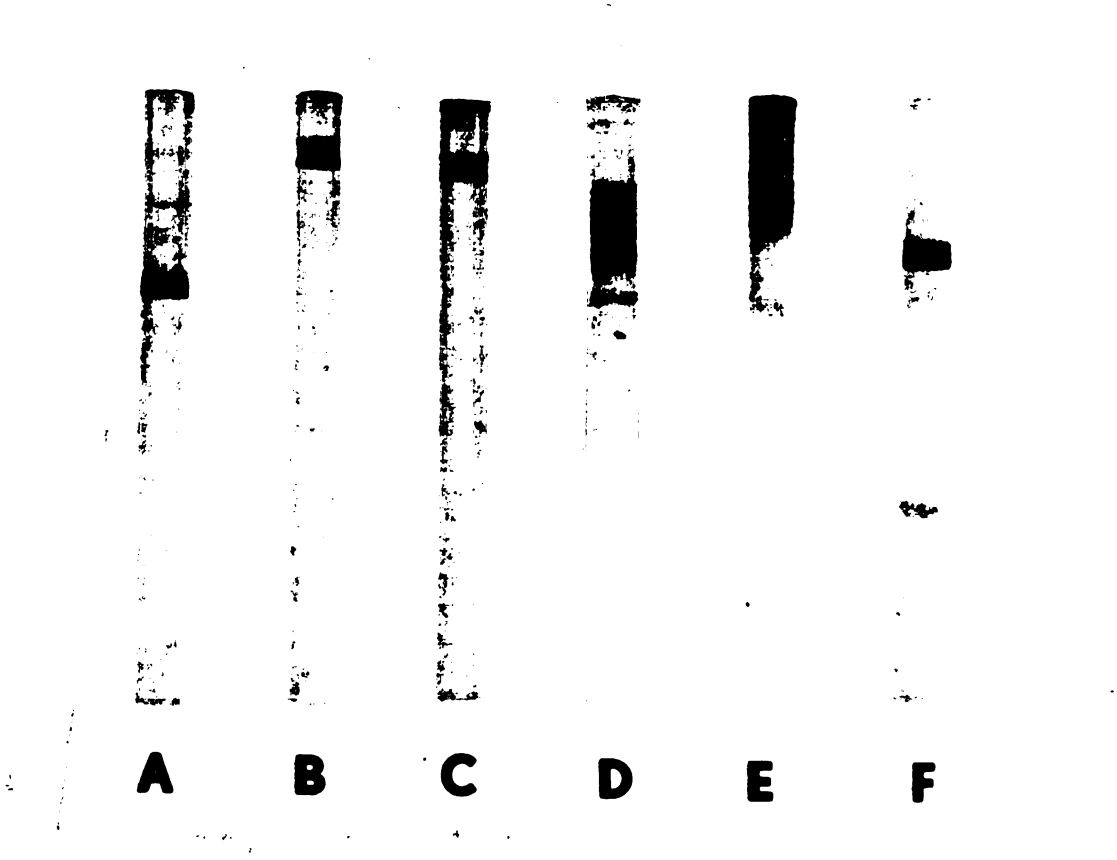


Figure 9. Electrophoretic patterns of alfalfa leaf Fraction I protein with and without various dissociating agents. (A) no dissociating agents (7% T), (B) no dissociating agents (10% T), (C) sample equilibrated against 10 mM ME (10% T), (D) sample equilibrated against 10 mM ME and 5 M urea, gel containing 5 M urea (7.5% T), (E) sample equilibrated against pH 11 phosphate buffer, gel running in same buffer (10% T), (F) in 0.1% SDS according to Weber and Osbone (1969).

alkaline pH condition. Sarkar et al (1975) reported that alfalfa Fraction I protein was partially dissociated at pH 11.3 and completely dissociated at pH 11.7.

Gel F represents the result of SDS-PAGE of the protein isolate run according to the method of Weber and Osbone (1969). Two major protein bands were observed. A comparison of the relative mobilities for the two principal bands with those in the standard curve in Figure 10 reveal molecular weights of approximately 52,000 for large subunit (upper band) and 12,500 for the small subunit (lower band). RuDP carboxylase isolated from several different sources consists of two distinct subunits, a large one of approximately 50,000-60,000 daltons and a small one of approximately 12,000-16,000 daltons (Rutner and Lane, 1967; Akazawa et al., 1972; Rutner, 1970; Sugiyama et al., 1971; Moon and Thompson, 1969; Ellis, 1973; Kung, 1976). Hood (1973) obtained three zones in SDS-PAGE with molecular weights of 56,000, 49,000, and 24,000. The size of the large subunit obtained in this study was close to 56,000, whereas the small one, 12,500, is quite different from 24,000. However, the molecular weights of large and small subunits of alfalfa Fraction I protein found in this study are in reasonable agreement with those previously reported.

Rutner and Lane (1967) and Sugiyama et al. (1971) found two subunits in the enzyme from spinach leaves and Chlorella ellipsodia. Most investigators believe that Fraction I protein has two subunits differing in molecular weight (Kawashima and Wildman, 1970; Kung, 1976; Chen and Sand, 1979; Baker et al., 1977a,b). In 1967, Sugiyama and Akawawa found that carboxydismutase from wheat was dissociated into several

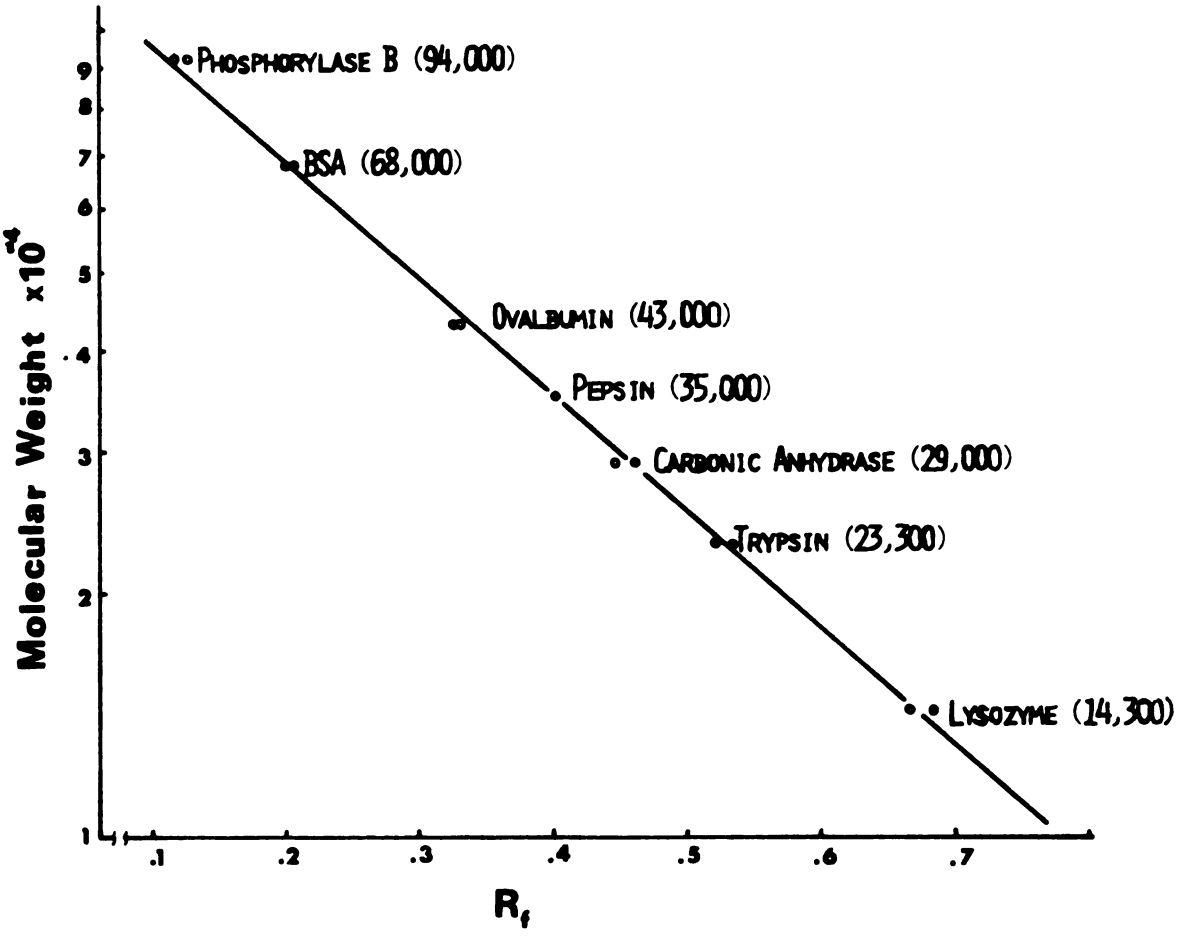


Figure 10. Standard curve for the estimation of molecular weights in SDS-PAGE (according to the method of Weber and Osborne, 1969).

subunits whose number depended on the concentration of SDS used. Sugiyama et al. (1968) observed that SDS concentration affected the dissociation of protein from spinach leaves. Sarkar et al. (1975) also observed a third protein zone just below the large subunit corresponding to the subunit of 49,000 daltons observed by Hood (1973). But they concluded that the appearance of a third band in SDS-PAGE was due to incomplete dissociation of the intact protein by SDS. In this study, there was a shadow (zone) just below the large subunit (Figure 9, gel F). Its molecular weight was estimated at 46,000 which is similar to the 49,000 species observed by Hood (1973). Alfalfa Fraction I protein possessed at least two non-identical subunits which are similar to the subunits of the enzyme from other sources. The possibility of the existence of a third subunit will be discussed later.

Subunits

From the above discussion, it can be concluded that alfalfa Fraction I protein contains at least two subunits whose sizes are similar to the subunits of Fraction I protein from other species. A G-100 Sephadex column was utilized to separate these components. Figure 11 shows a typical elution pattern for the separation. Fractions collected were designated as Fraction A and B. The electropherograms of these fractions in SDS-PAGE are shown in Figure 12 (gels 4,5, and 6).

Fraction I protein samples were treated with 0.5% and 2% SDS at pH 8.6. Gels 2 and 3 in Figure 12 show the SDS-PAGE patterns of treated sample before application in the G-100 column. In comparison with gels 2 and 3 patterns, gel 1 (same as gel F in Figure 9) shows

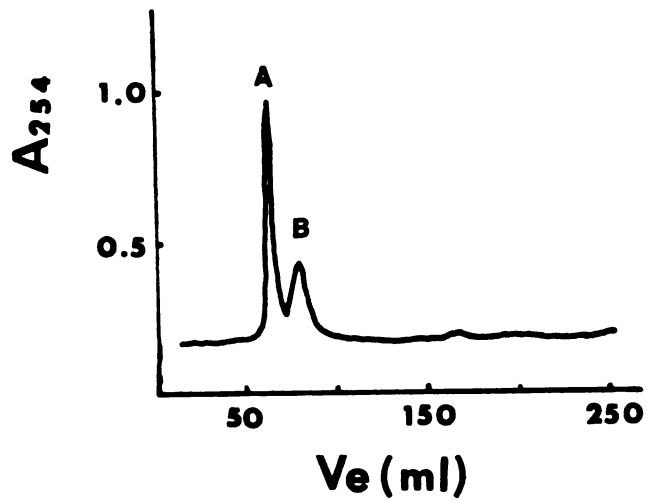


Figure 11. Separation of subunits from alfalfa Fraction I protein by gel filtration on Sephadex G-100. Sample was treated with 2% SDS-buffer. Peaks A and B were collected for further analysis.

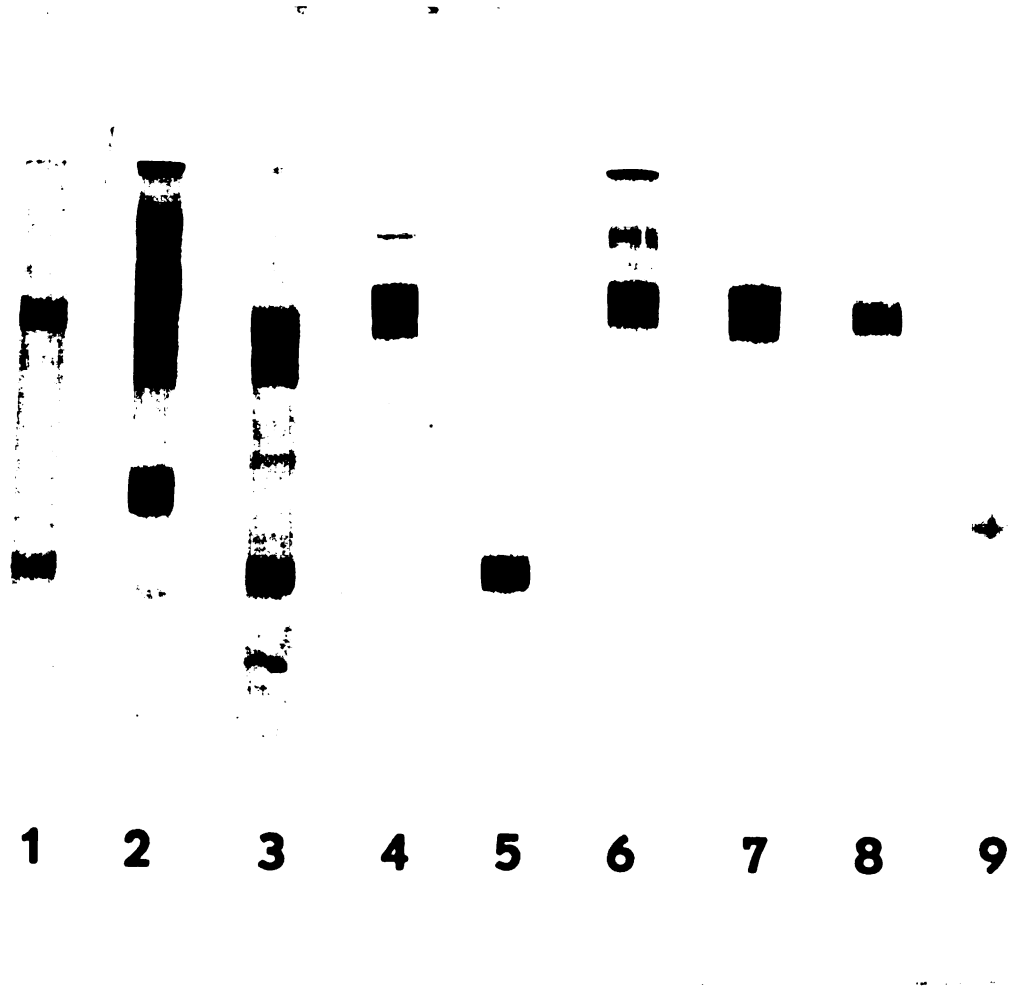


Figure 12. SDS-PAGE patterns of Fraction I protein and its subunits. The experimental conditions are described in the text. Gel 1, 2, 3: Fraction I protein. Gel 4, 6, 7, 8: Subunits collected from peak A of G- 100 column. In gel 8, a faint protein band which failed to appear in the photograph is at the position of molecular weight 12,300 (Fraction B). Gel 5, 9: Subunits collected from peak B of G- 100 column.

fewer bands. Also, there are many bands, i.e. the uppermost band, which appear in gel 2 but are absent in gel 3. Relating the relative molibilities of these bands to the standard curve (Figure 10), the estimated molecular weights (from top to bottom) are 85,000, 68,500, 54,000, 52,000, 47,500, 37,500, and 12,000 in gel 2 and 52,500, 49,000, 36,500, 34,000 and 12,500 in gel 3. Since the molecular weights of the two major subunits are 52,000 (large), and 12,500 (small), the protein sample was not completely dissociated. For example, the 85,000 zone could contain one large and two small subunits, and 68,500 could contain one large and one small subunits. Sugiyama and Akazawa (1967) employed different concentrations of SDS (5×10^{-4} to 5×10^{-2} M) to dissociate Fraction I protein of wheat leaves. They observed more than two components in SDS-PAGE patterns.

In Figure 12, gels 4, 5, and 6 represent SDS-PAGE patterns of a fresh preparation of fraction A and B. The gels indicate that the G-100 column separated only the smallest subunit (12,500) from the mixture.

Further treatment with 5 M urea was employed on both fractions. Fraction B is not dissociated by this treatment (gel 9) and the estimated molecular weight was 12,600. Due to the absence of most of the protein zones which appeared in gel 4 and 6, gel 7 and 8 indicate that fraction A, after 5 M urea treatment, is further dissociated. Therefore, it is concluded that the concentration of SDS affects the dissociation of alfalfa Fraction I protein into its subunits. The molecular weights of the protein zones in gel 4 and 6 are 53,500 and 50,000, respectfully, for the upper major band and 45,000, 46,500 for the lower

major band. The molecular weights of the bands in gel 7 and 8 correspond to the above range. The protein component at 45,000 to 46,500 is similar to that observed in gels 1 - 4 and 6 - 8. Increasing the concentration of urea did not alter the pattern.

Sugiyama and Akazawa (1967) reported that high SDS concentration resulted in protein degradation. The third band, molecular weight of 46,000, was always observed just below the large subunit (52,000 daltons) in the above SDS-PAGE system, suggesting that this protein component is not a degradation product of the large subunit resulting from a high SDS concentration. This observation also suggests that the component is not an undissociated small subunit at lower SDS concentration. After re-application of fraction A to the G-100 column, the SDS-PAGE pattern of the new peak A still revealed a protein band with a molecular weight of 45,500. The new fraction A was equilibrated with pH 7.2 buffer containing 1% SDS for 24 hr and its SDS-PAGE pattern was found to be similar to gel 8 in Figure 12. A band with a molecular weight 46,000 was observed and not intensified. These observations indicate that no significant dissociation or association resulted when protein was assayed between pH 7.2 and 8.6.

The evidence presented in this study suggests that Fraction I protein from alfalfa leaves consists of three subunits with molecular weights of 52,000, 46,000, and 12,500. The commonly accepted model for the enzyme assumes two subunits with molecular weights of 52,000 and 12,500 occur in alfalfa RuDP carboxylase. However, an additional subunit with a molecular weight of 46,000 daltons has been found in SDS-PAGE by Hood (1973) and in this study. This suggests that further

work is necessary regarding the number and nature of subunits comprising the enzyme. It is possible that artifacts are produced in Fraction I protein by means of protease activity. The addition of a reagent to inhibit protease activity during isolation and purification might eliminate this possibility.

Immunological Properties

Figure 13 shows the immuno-double diffusion patterns of selected alfalfa protein fractions. In pattern A, the identity of Fraction I protein isolated from different preparations is shown (sample 3). Two precipitin lines indicate that Fraction I protein possesses two determinant groups.

The DEAE-derived fraction (sample 2) also reacted with Fraction I protein-antisera and its reaction is different from that of Fraction I protein. Its determinant groups show partial identity to that of Fraction I protein. This result suggests that DEAE-derived fraction is composed of Fraction I protein and other materials as well. The result of heterogeneity in the DEAE-derived fraction in disc PAGE pattern (Figure 3, gel B) confirmed this conclusion. No precipitate formed between the anti-sera and fraction G (sample 1), whereas a precipitate did form inside the No. 1 well. Fraction G molecule is too large to penetrate the 2% agar gel since fraction G solution (yellow color) contained other substances, i.e. pigments or polyphenols which may combine with protein molecules to produce a very large complex.

Pattern B illustrates that determinants of the large subunits (sample 5) are identical to those of the small subunit (sample 4). The

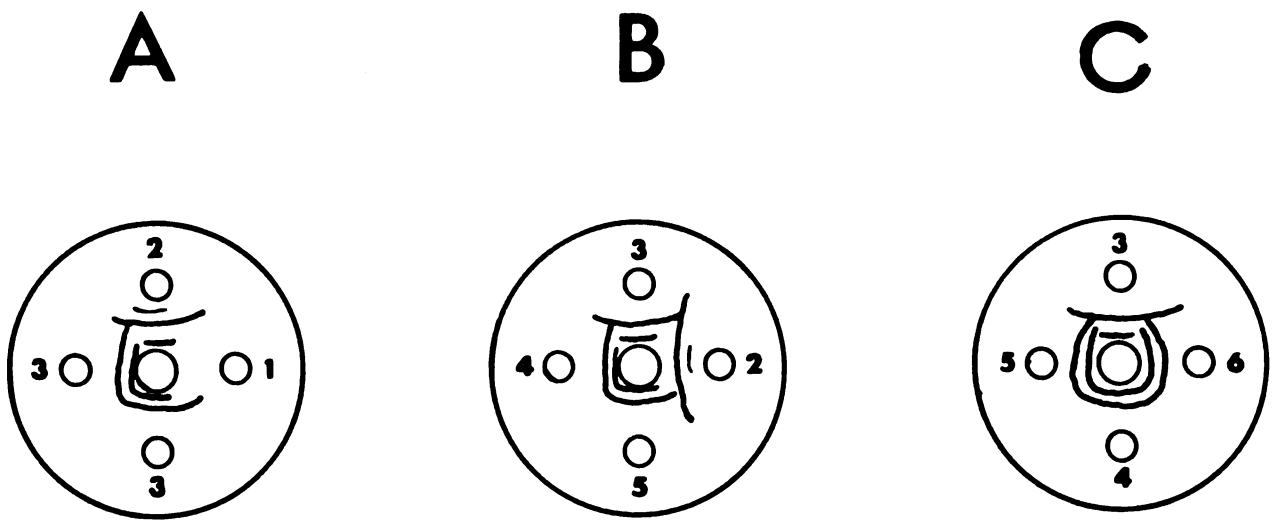


Figure 13. Diagramatic representation of immuno-doublt diffusion patterns of alfalfa protein fractions. Center well contained whole anti-sera derived from a rabbit previously sensitized to alfalfa Fraction I protein. Antigen wells contained (1) Fraction G, (2) DEAE-derived fraction, (3) Fraction I, (4) Subunit B from G-100 column, (5) Subunit A from G-100 column, and (6) SDS-treated Fraction I protein.

determinant groups of both subunits show partial identity to those of Fraction I protein sample (No. 4 and 3 in pattern B; No. 3, 5, and 6 in pattern C). It is interesting that the precipitin patterns of both subunits are identical and diffusion rates are close, though the sizes of the subunits are quite different. Possibly, advanced double diffusion experiments involving the subunits and their antisera would explain this phenomenon.

SUMMARY

Fraction I protein was purified from fraction G (ammonium sulfate fractionation) of alfalfa leaves by a combination of DEAE-cellulose and Sephadex G-200 chromatography. The final protein product was homogeneous as shown by a single band in PAGE and a single boundary schlieren pattern in sedimentation velocity experiment. The protein possessed a $S_{20,w}$ of 18.7 in 0.1 M Tris-HCl buffer, pH 8.0, containing 12.5 mM $MgCl_2$ (μ = 0.1). An approximate molecular weight of 573,000 daltons was estimated, using values of 18.7 for $S_{20,w}$ and 2.97 for $D_{20,w}$. The final preparation had the enzyme activity of ribulose-1,5-diphosphate carboxylase which is involved in primary CO_2 fixation in photosynthesis. The specific activity of fresh alfalfa Fraction I protein was 1.24 units/mg protein while lyophilization resulted in a complete loss of activity.

Electrophoresis in the presence of 5 M urea revealed a diffuse zone, whereas at least 5 separate zones appeared in electrophoresis at pH 11. This indicates the molecule was unfolded and resolved into components or subunits. Mercaptoethanol did not affect the characteristics of the protein in PAGE, indicating that disulfide bonds do not occur between subunits. Gel electrophoresis of the protein in the presence of SDS revealed resolved subunits. The SDS-PAGE pattern of the protein showed three protein zones with molecular weights estimated

at 52,000, 46,000, and 12,500.

Fraction I protein from alfalfa leaves had an A_{max} at 279 nm with a A_{280}/A_{260} ratio of 1.75, indicated that the protein was devoid of nucleic acids. Chemical analysis of the protein revealed 16.4% protein nitrogen, 1.85% hexose and the absence of lipid. Other carbohydrate moieties commonly associated with glycoprotein were absent. The protein contained 37 free sulfhydryl groups and a total of 99 reduced sulfhydryl groups per mole with molecular weight of 573,000 daltons. This result indicated that 31 disulfide bonds are present in the protein. Amino acid analysis revealed that Fraction I protein from alfalfa leaves had a slightly acidic and hydrophilic nature, containing Asp, Gly, Ala, Glu, and Leu in highest concentration. Sulfur-amino acids are the limiting amino acid as determined by chemical scoring. The relative distribution of all amino acid residues compared favorably with previously reported data.

APPENDIX

Table A1. Some important chemicals used in this study and their source

Chemical	Source
Acrylamide	Ames
N,N'-methylene bisacrylamide	
Sodium dodecylsulfate (SDS)	Bio-Rad
SDS-PAGE molecular weight standard	
Protein assay dye	
Pronase	
N-acetyl neuraminic acid	Calbiochem
N,N,N',N'-tetramethylethylenediamine	Eastman
p-Dimethylaminobenzaldehyde	
2- Thiobarbituric acid	
Sodium bicarbonate	Mallinckrodt
Pepsin	Nutritional Biochemical
Trypsin	
Tris (hydroxymethyl) aminomethane (Sigma 7-9)	Sigma
Coomassie brilliant blue G-250	
Coomassie brilliant blue R	
Ribulose-1, 5-diphosphate	
DL-tryptophan	
Dithionitrobenzoic acid	

Table A2. Characteristics of Fraction I protein from alfalfa leaves

Component	1[a]	2[b]	3[c]
Nitrogen (%)	16.2	16.4	16.4
Protein (5.97[d] x %N)	96.7	97.9	97.9
Carbohydrate (%)			
Hexose	1.85	1.89	1.82
Hexosamine	none	none	none
Sialic acid	none	none	none
Tryptophan (%)	-	2.59	2.59
Ash (%)	-	trace	trace
Lipid (%)	-	none	none
Available -SH[e]	-	0.8	0.9
Total -SH[e]	-	37.1	37.3
Total -SH after reduction of S-S[e]	-	99.1	100.0
Enzymatic activity (RuDP carboxylase, unit/mg)	-	1.24	-
$S_{20,w}$ (Sevdberg)	-	18.7	-
$D_{20,w}$ (F.U.)	-	2.97	-
Mol. wt. (dalton)	-	573,000	-

[a] 1: First cut on May 30, 1978.

[b] 2: Second cut on July 27, 1978.

[c] 3: Third cut on Feb. 2, 1979.

[d] Ridley et al., 1967.

[e] Expressed as number of -SH groups per mole (573,000 daltons).

Table A3. Protein recovery at sequential steps in the isolation of Fraction I protein from alfalfa leaves (first cutting)

Fraction	Protein[b]		% extracted protein
	%	g	
Fresh tissue[a]	23	12.05	-
Filterex extract	31.9	8.5	100
Clairified extract	17.5	5.62	66
Dialyzed extract	30	4.49	52.8
Fraction G	84.4	1.94	22.8
Fraction I	96.7	1.45	17.1

[a]Starting material consisted of 295 g of fresh leafy tissue.

[b]%Nx5.97 on dry basis

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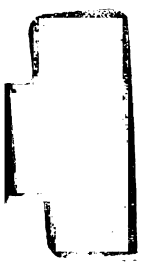

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