Soluble Protein Concentrate from Alfalfa by Low-Temperature Acid Precipitation

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A soluble protein fraction was obtained from chloroplast-free alfalfa juice (prepared by centrifugation of flash-heated whole juice) by precipitation at pH 3.5 and 2°. To retain the solubility, it was also necessary to maintain this temperature during washing at pH 3.5 and redissolving at pH 7. Acid precipitation at higher temperatures yielded protein with reduced solubility. The low-temperature fraction contained about 70% protein and could be concentrated to about a 6% solution. When freeze dried, the protein could be redissolved at room temperature, even after storage for several months at ambient temperatures.

Although green leafy plants contain most of the world's supply of protein, it is largely unavailable to monogastric animals, including man, because it is associated with large amounts of fiber. The Pro-Xan process, developed at this laboratory (Kohler et al., 1968; Spencer et al., 1970), was designed to recover a major portion of the proteins and carotenoid pigments free from the insoluble fibrous residue. In addition to this fibrous residue, still highly desirable as a ruminant feed, the major products of the process are a high-protein, high-xanthophyll concentrate (Pro-Xan) and an alfalfa solubles fraction. Although whole leaf protein concentrate (whole LPC) is nutritious, it is green in color and has a grassy flavor many people find objectionable. This has discouraged its use directly in many processed foods.

In order to prepare protein concentrates with a higher degree of acceptability as a food supplement, a modified Pro-Xan process has been developed. In this modification, called the Pro-Xan II process, the chloroplastic material responsible for the green color and much of the grassy flavor is centrifugally removed (de Fremery et al., 1973; Edwards et al., 1975). The remaining soluble protein is heat coagulated to yield a light-colored bland 90% protein concentrate. This product is highly nutritious and exhibits some desirable functional properties (Betschart et al., 1973), but the range of its application in the food industry is limited by its low solubility.

The experiments described in this paper report a method of acid precipitating the protein from the chloroplast-free juice under conditions which preserve its native solubility.

EXPERIMENTAL SECTION

The preparation of the chloroplast-free juice (referred to in this paper as "clear brown juice" or CBJ) containing the soluble white proteins has been previously described in detail (Edwards et al., 1975; Bickoff and Kohler, 1974). In brief, freshly expressed alfalfa juice is heated to 60°, held 20 sec, cooled, centrifuged to remove most of the chloroplastic fragments, clarified by filtration with diatomaceous earth, and held at 0°. In the present work, CBJ was either used within 24 hr or stored at −34° until needed. Frozen CBJ was thawed rapidly under cold running tap water. When necessary, it was centrifuged at 2° for 20 min at 37,000*g* to remove traces of insoluble material.

All of the experiments reported here were performed in the following manner. Thirty milliliters of CBJ was pipetted into a 50-ml plastic centrifuge tube containing a magnetic stirring bar and placed in a constant temperature water bath. The pH was adjusted to the desired level by the dropwise addition of 1 *N* HCl to the rapidly stirred solution. This pH was maintained during a 30-min equilibration period, the sample was centrifuged (1200*g* for 15 min), and the supernatant was decanted and saved. The precipitate was washed by mixing it with about 25 ml of distilled water which had previously been adjusted to the same pH and temperature as the precipitate. After centrifugation (1200*g* for 15 min), this supernatant was combined with the previous one, the total volume measured, and the sample analyzed for total nitrogen by standard AOAC methods (AOAC, 1970). Nonprotein nitrogen is that nitrogen which is soluble in 10% trichloroacetic acid (Cl_3CCOOH); protein nitrogen is calculated by difference. Ten to twenty milliliters of distilled water was added to the precipitate and, while maintaining the predetermined temperature, 0.1 *N* NaOH was slowly added with stirring to raise the pH to the desired value. The solution was stirred at the desired pH and temperature for 1 hr. The solution was centrifuged (37,000*g* for 20 min), the supernatant decanted, and its volume measured.

Total and nonprotein nitrogen contents of the supernatants were determined as before, and any residual pellet was also analyzed for nitrogen. From these data, the percent nitrogen that redissolved could be calculated.

RESULTS AND DISCUSSION

A light tan precipitate is obtained from the CBJ when the pH is adjusted below 5. As the pH is lowered to 3.5 at room temperature, the yield of protein nitrogen increases and the percent nitrogen in the precipitate decreases (Table I). Washing the precipitate at pH 8.5 increases the nitrogen content of the insoluble precipitate to 15–16%, regardless of the pH of precipitation, indicating that some acid-insoluble contaminant, low in nitrogen and soluble under alkaline conditions, is co-precipitating with the protein.

The solubility of an alfalfa protein preparation, precipitated at pH 4.5 at room temperature, was evaluated over the pH range of 6 to 9 (Figure 1). Although there was an increase in solubility with increasing pH, more than one-half of the protein remained insoluble at pH 9. This is in agreement with the work of Betschart (1974), who showed a comparable solubility profile, between pH 6 and 9, with an alfalfa protein precipitated at pH 3.5. She obtained nearly complete solubility under the conditions of her test at pH 10.

The effect of temperature on solubility was examined next (Figure 2). As the temperature is decreased, the solubility of the acid-precipitated protein is increased. The lowered solubility of the products prepared at the higher temperatures does not appear to be affected by time of holding at pH 3.5 from 5 min to 2 hr. Alfalfa protein can be acid precipitated and washed, and will remain over 90% soluble (pH 7) when a temperature of 2° or less is main-

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Table I. Composition of Freeze-Dried Alfalfa Protein Fractions

Fraction	pH of precipitation	Yield,[a] %	Composition,[b] %		
			Nitrogen	Protein[c]	Ash
Clear brown juice			4	25	18
Room temperature	4.5	63	13.6	85.0	<1
acid precipitate,	4.0	77	12.7	79.4	<1
washed at pH of precipitation	3.5	87	11.8	73.8	<1

[a] Yield expressed as percent of total Cl_3CCOOH precipitable protein in CBJ. [b] Dry basis. [c] Percent nitrogen × 6.25.

Table II. Composition of Freeze-Dried Alfalfa Protein Fractions

Fraction	Yield,[a] %	Composition,[b] %		
		Nitrogen	Protein[c]	Ash
Acid precipitated at pH 3.5, washed and redissolved at pH 7 (2°)	83	11.2	69.9	5.5
After diafiltration (1:10)[d]		15.0	93.7	1.7
Ultrafiltered CBJ (19-fold conc.)[d]	90	9.8	61.2	8.4
Retentate from diafiltration (1:10)[d]	90	14.9	93.4	1.6
Heat precipitated and washed protein[e]	91	14.2	88.7	< 0.5

[a] Yield expressed as percent of total Cl_3CCOOH-precipitable protein in CBJ. [b] Dry basis. [c] Percent nitrogen × 6.25. [d] From Knuckles et al. (1975). [e] From Edwards et al. (1975).

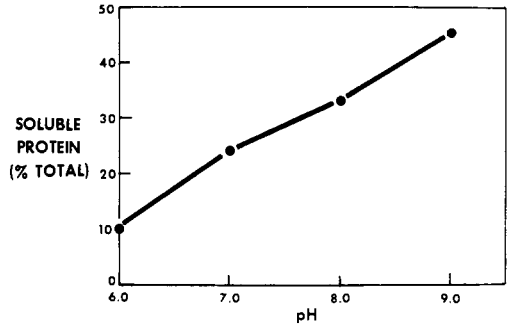

Figure 1. Solubility of alfalfa protein precipitated at pH 4.5 and room temperature.

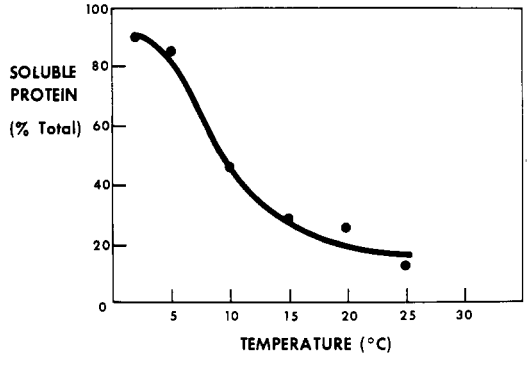

Figure 2. Solubility of alfalfa protein precipitated at pH 3.5 and redissolved at pH 7.0. Temperature maintained at indicated level during precipitation, washing, and redissolving.

tained throughout the experiment. If maximum solubility is desired, it is essential that the temperature remain low until the precipitate is completely redissolved.

The solubility of the protein precipitated at pH 3.5 and

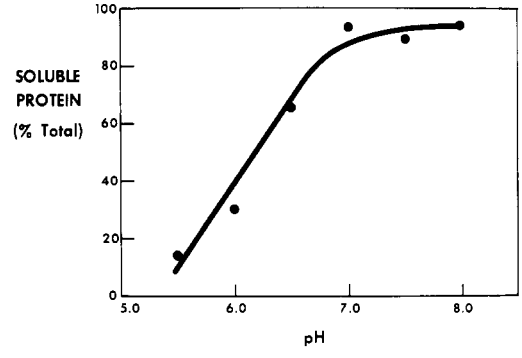

Figure 3. Solubility of alfalfa protein precipitated at pH 3.5 and redissolved at various pH levels. Temperature maintained at 2° during precipitation, washing, and redissolving.

2° was determined at several pH values (Figure 3). The solubility increased as the pH was increased from 5.5 to 7.0, at which point more than 90% of the precipitate had dissolved. There was no further increase in solubility at higher pH values. The redissolved protein will remain in solution for several days at ice temperature before the appearance of traces of sediment. When warmed to room temperature, a sediment begins to form after several hours. Freeze-dried protein will remain soluble for several months, and solutions containing up to 5% protein can be prepared at room temperature. The dried product contains about 70% protein and 5% ash (Table II).

The technique of low-temperature acid precipitation makes it possible to obtain protein solutions at concentrations five- to tenfold higher than CBJ. At this degree of concentration, approximately 6%, the viscosity is quite high. Knuckles et al. (1975) reported similar high viscosity at this concentration in solutions prepared by ultrafiltration.

The composition of soluble protein prepared by low-temperature acid precipitation, ultrafiltration, or diafiltration is shown in Table II. The acid-insoluble impurity present in acid-precipitated protein apparently has a molecular

weight less than 30,000 (the cut-off point in the ultrafiltration experiments of Knuckles et al., 1975) since it is absent in the diafiltered retentate. This same impurity is also noncoagulable from neutral or alkaline solutions since it is absent in the heat coagulated protein of Edwards et al. (1975) (see Table II).

Reducing agents, such as sodium bisulfite and 2-mercaptoethanol, are frequently used when attempting to isolate soluble active enzymes and undenatured protein fractions (Anderson and Rowan, 1967; Wolf, 1972). In attempting to improve the solubility of the protein precipitated at room temperature, several reducing agents were added to the CBJ (before precipitation) at the following levels: sodium bisulfite, 0.156 *M*; sodium dithionite, 0.078 *M*; and mercaptoethanol, 0.40 *M*. The samples were precipitated and washed at pH 3.5 at room temperature, and the protein reslurried in pH 8.5 borate buffer (0.1 *M*) for 1 hr. There was no increase in solubility when using the reducing agents compared to the untreated control. In related work, Betschart (1974) reported that mercaptoethanol was ineffective in increasing the solubility of freeze-dried, acid-precipitated alfalfa protein.

By acid precipitating the alfalfa protein at 2°, the native solubility is preserved. This protein concentrate, being free from the dark green color and most of the grassy flavor of the typical LPC, and still retaining its native solubility, should have many uses in the food industry. The protein can be further purified by membrane filtration if necessary.

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Received for review February 24, 1975. Accepted July 7, 1975. Presented in part at the 1973 Pacific Conference on Chemistry and Spectroscopy, San Diego, Calif., Nov 1–3.

Changes in Concentrations and Interrelationships of Phytate, Phosphorus, Magnesium, Calcium, and Zinc in Wheat during Maturation

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Changes in concentrations and interrelationships of phytate, P, Mg, Ca, and Zn were studied in the heads of 11 varieties (Roshan, Jolgeh, Koohrang, Derakhshan, Ommid, Navid, Deihim, Siano, Penjamo, Tobari, and Akowa) of wheat (*Triticum aestivum* L.) during maturation. The maturation period was divided into prechlorophyll, chlorophyll, and postchlorophyll destruction stages. Concentrations of total P, Mg, and Zn showed an upward trend and that of Ca fluctuated during maturation. Phytate P concentration doubled mainly at the expense of nonphytate P during the chlorophyll destruction stage. Concentration of total P was related to nonphytate and phytate P during prechlorophyll and chlorophyll destruction stages, respectively. Phytate P, Mg, and Ca concentrations were interrelated during the postchlorophyll destruction stage. By the end of this period, phytate P constituted 75% of the total P of the wheat heads. The rate of phytin synthesis in wheat during maturation, together with the need of chlorophyll data in such studies, are discussed.

As early as 1934 it was suggested that cereal P was physiologically unavailable, because a major portion of it was present as inositol hexaphosphate or phytate (Bruce and Callow, 1934). Since then similar results have been reported by other investigators (Gillis et al., 1957; Nelson, 1967; Hintz et al., 1973). It was found that in wheat, phytate P varied from 49 to 80% of the total P of the grain (Knowles and Watkins, 1932; Booth et al., 1941; Asada et al., 1968; O'Dell et al., 1972; Abernethy et al., 1973). The mixed Mg, Ca, and K salts of phytate are termed phytin (Averill and King, 1926). Phytin is the principal storage form of P in most seeds (Earley and DeTurk, 1944).

A number of investigations have demonstrated that phytate might be responsible for decreased physiological availability of dietary Ca (Harrison and Mellanby, 1939; McCance and Widdowson, 1942; Krebs and Mellanby, 1943; Hoff-Jorgensen et al., 1946; Cullumbine et al., 1950; Nelson et al., 1968; Berlyne et al., 1973; Reinhold et al., 1973), Zn (O'Dell and Savage, 1960; Prasad et al., 1963; O'Dell, 1969; Reinhold, 1971; Halsted et al., 1972; Reinhold et al., 1973), and Mg (McCance and Widdowson, 1942; Roberts and Yudkin, 1960; Likuski and Forbes, 1965).

This study was carried out in an attempt to provide data on changes in concentrations and interrelationships of phytate P, nonphytate P, total P, Mg, and Zn in wheat during maturation.

MATERIALS AND METHODS

Seven Iranian (Roshan, Jolgeh, Koohrang, Derakhshan, Ommid, Navid, and Deihim) and four foreign (Siano, Penjamo, Tobari, and Akowa) varieties of wheat (*Triticum aes-*

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