Centrifugal Separation of White and Green Protein Fractions from Alfalfa Juice Following Controlled Heating

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The use of controlled heating to separate centrifugally the white and green protein fractions from freshly expressed alfalfa juice has been investigated. Heating in a batch-type operation, with a slug flow heat exchanger, or by direct steam injection has been conducted over a range of temperatures (30–64°), times (10 sec–20 min), and pH values (5.5–8.6). Results indicate a superiority of direct steam injection over the other methods of heating. At a pH of 8.3–8.6, heating for 10–20 sec at temperatures of 60–62° results in a precipitation of only 11–13% of the white proteins from the supernatant solutions, accompanied by the removal of essentially all of the green products. When the pH is lowered, the temperature must also be lowered to prevent excessive loss of the white protein products. The general technique appears to be adaptable to the large-scale treatment of alfalfa juice on a continuous basis.

The PRO-XAN process, as developed in the Western Regional Research Laboratory (Kohler *et al.*, 1968; Knuckles *et al.*, 1970, 1972; Lazar *et al.*, 1971; Miller *et al.*, 1972; Spencer *et al.*, 1970, 1971), is a process which separates a portion of the proteins and carotenoid pigments from the freshly expressed juice of alfalfa (*Medicago sativa*) as a dry product that is essentially devoid of fiber. The product, named PRO-XAN because of its high content of protein and xanthophyll, is presently being produced commercially by an alfalfa processor under the trade name X-PRO (Batley-Janss Enterprises, Brawley, Calif.) and has found wide acceptance in the feed industry as a nonruminant feed. PRO-XAN, however, is not considered suitable as a source of protein in human nutrition due to its strong grassy flavor and deep green color.

In attempting to modify this process to prepare protein concentrates suitable for human consumption, our approach has been to develop a method which will separate the soluble white proteins from the insoluble, or particle-bound, colored protein material. (These two fractions have often been described in the scientific literature as "cytoplasmic" and "chloroplastic.") Separation of these two classes of protein will solve the pigmentation problem already discussed. Since all of the chlorophyll is associated with the subcellular chloroplasts and chloroplastic fragments in freshly expressed alfalfa juice, a laboratory separation of the proteins can be made by the use of ultracentrifugation. Unfortunately, there is no commercially available equipment which can generate the high centrifugal forces necessary to perform this separation on a continuous economic basis. Consequently, we have sought other means to achieve this separation.

A search of the literature revealed that several different approaches had been taken to prepare chlorophyll-free protein concentrates from the expressed juice of several different plant species. Menke (1938) attempted a separation by differential precipitation with ammonium sulfate or dilute acids, and Yemm and Folkes (1953) achieved partial separation using calcium salts as a flocculating agent for the chloroplastic fragments. Chibnall (1939) recovered cytoplasmic proteins from leaves by diffusing them through the cell walls after altering permeability by treatment with ether–water. More recently, extraction of the expressed juice or crude protein concentrates with polar solvents to remove chlorophyll and other lipoidal pigments has been suggested as a means of producing bland, light-colored protein concentrates from green leafy plant tissue (Chayen *et al.*, 1961; Huang *et al.*, 1971; Wilson and Tilley, 1965). The most common method referred to, however, was differential heat treatment, with the chloroplastic fragments coagulating at lower temperatures than the white proteins (Byers, 1967; Cowlishaw *et al.*, 1956; Henry and Ford, 1965; Lexander *et al.*, 1970; Rouelle, 1773; Subba Rau *et al.*, 1969). Preliminary experiments indicated that this method offered the greatest promise of separating the green chloroplastic proteins from the soluble white proteins rapidly, efficiently, and economically. The present paper summarizes our laboratory-scale experiments on the effect of temperature, time, and pH on the separation of these two classes of proteins.

EXPERIMENTAL SECTION

Preparation of Juice. Juice was expressed from freshly chopped alfalfa by the method of Knuckles *et al.* (1970), except that ethoxyquin and ammonia were not added. The juice was passed through a 40-mesh screen to remove a small amount of residual fiber and then held in ice until the heat treatment could be instituted (less than 2 hr). When necessary, the pH was adjusted with 1 *N* HCl or 1 *N* NaOH immediately preceding the heat treatment. In some experiments, gaseous ammonia was added to the alfalfa prior to juice expression so that the final pH of the juice was 8.5.

Heat Treatment. In experiments with heating in a batch-type operation, aliquots of sample were placed in Erlenmeyer flasks to a depth of 5–7 mm (20–25 ml). The flasks were then placed in a water bath at the appropriate temperature, swirled until the flask contents were within 1° of the bath temperature, stoppered, held in the bath for selected times, and then placed in an ice–water bath for rapid cooling. After the aliquots were cooled, samples were removed for chemical analysis.

In other experiments, 1-l. batches of juice were heated in a slug flow heat exchanger as described by Stroup *et al.* (1969). Prior to passage through the heat exchanger and immediately following the heat treatment, samples were held in an ice–water bath.

In some experiments, heating was accomplished very rapidly by means of direct steam injection into a flowing stream of fresh juice. The steam injector was built from an 18-in. length of ½-in. galvanized pipe through which the juice flowed. The pipe was constricted moderately at the exit port by a series of bell reducers to a final ⅛-in. nipple 1½ in. long. Steam, at 120 psig, was admitted to the pipe 5½ in. from the exit port through a 0.136-in. diameter hole. The hole was drilled tangentially to the cross

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section of the pipe; it was angled slightly forward in the direction of fluid flow. When operated at a flow rate of 1 gpm, the residence time of heated juice was about ½ sec. Temperature, monitored by a thermocouple or thermometer inserted into the fluid stream at the exit port, was controlled with a needle valve which regulated the flow of steam into the injector. The heated juice was collected in a preheated Dewar flask, held for a fixed period of time, and then cooled rapidly by pouring into a precooled aluminum kettle immersed in an ice-water bath. The time required to collect and cool the sample ranged from 8 to 12 sec; this time is *not* included in the holding times listed in Tables III and IV.

Centrifugal Separation. When the total soluble (white) proteins were to be measured, a sample of juice that had not been heat-treated in any way was centrifuged in an ultracentrifuge (~100,000 × *g*) for 2 hr at 1°. The clear supernatant was decanted carefully so as not to disturb the uppermost layer of the pellet and then filtered to remove the waxy particles that floated to the top during centrifugation.

In experiments A, B, and C, the samples were centrifuged in a refrigerated laboratory centrifuge using conditions that were rapid and that would be related to the centrifugal forces and residence times that could be expected in commercial centrifuges. The rotor was brought to 10,000 × *g* (~1 min) and then allowed to coast to a stop under the action of the electrical brake (~4 min). Appearance of the supernatant was observed visually.

In experiments D and E, the samples were centrifuged at 1–5° in a Sharples laboratory super centrifuge (The Sharples Corp.) at 50,000 rpm. Appearance of the supernatant was observed visually.

In experiments with samples heated by steam injection, centrifugal separation was evaluated using a Gyro-Tester (DeLaval Separator Co.). This centrifuge is designed in such a way that the centrifugal performance of a small portion of the suspension in question can be used to predict roughly the performance of any of a number of large-capacity continuous centrifuges. According to the manufacturer's suggestions, the clarity of the supernatant solution is observed after centrifugation for periods of time up to 80 sec. In our case, the volume of suspended solids remaining in the supernatant after centrifugation for 40 sec was used as a guide to the sedimentability of insoluble chloroplastic material in the possible application of our work to large-scale protein separations. In addition, the heat-treated samples were centrifuged at 37,000 × *g* for 15 min at 1°. The supernatant solutions, which were consistently free of suspended particles, were then sampled for determination of protein concentration. This severe centrifugal technique was used not to evaluate the ease of separation following steam injection but rather to measure the loss of soluble white proteins following the heat treatment.

Analytical Methods. Total nitrogen was determined in all samples by the standard Kjeldahl method. Nonprotein nitrogen was assumed to be that nitrogen which was soluble in 10% trichloroacetic acid, and protein-nitrogen was determined by difference. Protein concentration was assumed to be protein-nitrogen × 6.25. Calculations included a correction for the dilution caused by uptake of steam during steam injection heating.

RESULTS AND DISCUSSION

Unheated Juice. When samples of freshly expressed alfalfa juice are chilled and centrifuged for 15 min at 1° at several different centrifugal forces, the amount of protein that is sedimented increases rapidly up to about 20,000 × *g* (Figure 1). Beyond that point, the amount of protein that can be sedimented increases only slightly (up to 40,000 × *g*). In all cases, the supernatant solution is turbid and dark green. At forces below 10,000 × *g* the pellet is soft; uppermost layers of it tend to drain off with the supernatant solution. When samples of fresh juice are ultracentrifuged (~100,000 × *g* for 2 hr), 35–40% of the total proteins are sedimented, and the supernatant solution is a clear yellow or brown and has no trace of green pigmentation. In the absence of commercial equipment that could generate the large centrifugal forces necessary to sediment the chloroplastic material on a continuous basis, we initiated experiments on the heat treatment of juice that would allow the rapid and economical centrifugal sedimentation of essentially all of the chloroplastic material.

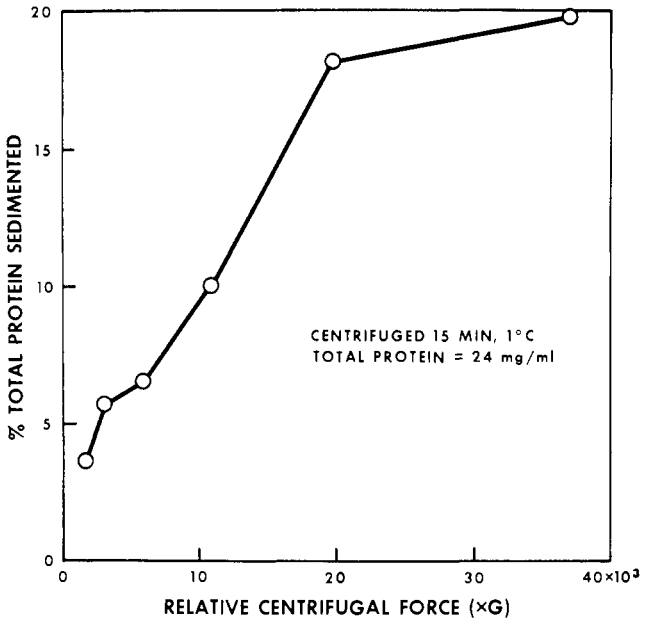

Figure 1. Sedimentability of proteins from freshly expressed alfalfa juice.

Batch Heating. Previously published work on controlled heating (see introductory section) had indicated that temperatures of 50–55° at a pH of about 6 for several minutes coagulate all of the green protein fractions. Cowlishaw *et al.* (1956) used a temperature of 65° and experienced extensive, though quantitatively unspecified, coagulation of soluble protein. In our experiments on the batch heating of alfalfa juice, we incubated samples at 30°, 40°, or 50° for several time periods and measured the amount of protein that remained in solution following centrifugation at 10,000 × *g* for 1 min (Table I). With the exception of the 20-min sample in experiment C, all supernatant solutions were green with variable amounts of turbidity.

None of the heat treatments in Table I can be considered satisfactory for the separation of green material from the soluble white proteins. Only one sample yielded a clear yellowish supernatant solution (experiment C, 20-min incubation). The low recovery of soluble protein in this sample is attributed not only to the increased coagulation of these proteins but also to the increased proteolysis that occurs during incubation. Evidence for increased proteolysis comes from the fact that nonprotein nitrogen increased from 27% of total nitrogen (unheated juice) to 44% following incubation. These results are not surprising when one considers that 50° and pH 6 are close to the optimum temperature and pH for proteolysis (de Fremery *et al.*, 1972). Based on a value for soluble protein of 60–65% of total protein (as determined in the ultracentrifuge on unheated samples), loss of soluble protein during this heat treatment amounted to about 50% of the initial soluble protein.

Table I. Effect of Batch Heating Conditions on Supernatant Protein Concentration

Heating conditions,[a] °C, min	Supernatant protein concentration,[b] % total juice protein
Experiment A	
0, 60	84
30, 17	70
40, 7	54
50, 2	43
Experiment B	
50, 0	49
50, 1	39
50, 0	57
50, 1	43
Experiment C	
0, 60	88
50, 5	48
50, 20	32

[a] pH was 5.5 in experiment A and first two samples of experiment B; pH was 6.0–6.1 in last two samples of experiment B and in experiment C. [b] Samples centrifuged at 10,000 × *g* for 1 min at 1°.

Table II. Effect of Heating Conditions in Slug Flow Heat Exchanger on Supernatant Protein Concentration

Heating conditions,[a] °C, min	Supernatant protein concentration,[b] % total juice protein
Experiment D	
0, 60	57
45, 4	44
50, 4	35
Experiment E	
50, 4	39
50, 4	44
52, 3	43

[a] pH was 5.5 in experiment D and in first sample of experiment E; pH was 6.3 in remaining samples. [b] Samples centrifuged at 50,000 rpm in a Sharples Laboratory Super Centrifuge at 1–5°.

Slug Flow Heat Exchanger. A slug flow heat exchanger has an important advantage over batch-type heating in that heating and cooling are very rapid and, consequently, the exposure of the sample to the critical temperature can be controlled quite accurately. When freshly expressed alfalfa juice is held for 3–4 min at temperatures of 45–52°, the proportion of total proteins that remains in the supernatant in the Sharples Super Centrifuge ranges from 35 to 44% (Table II). The low concentration of protein in the supernatant from the unheated sample can be attributed to the greater centrifugal separation efficiency of the Sharples centrifuge compared to the gentle technique used in experiments A, B, and C. Although the amount of protein that remains soluble in the supernatant is relatively low, the use of this type of heat exchanger in large-scale processing is possible since all of the heat-treated samples yield a clear yellowish supernatant solution following centrifugation. However, the unavailability of large-scale slug flow equipment, coupled with the somewhat lower yields of soluble protein in this method, prompted us to study direct steam injection as an alternate method of rapidly heating juice to the experimental temperature.

Direct Steam Injection. The results of an experiment in which samples of ammoniated alfalfa juice were heated to 56–64° for periods of time ranging from 0 to 60 sec (in addition to the collection and cooling time of 8–12 sec) are presented in Table III. Those samples which have a suspended solids content exceeding 0.8% (vol) (following a 40-sec test in the Gyro-Tester) are generally greenish in color. If the suspended solids content is 0.8% (vol) or less, not only is the turbidity less but the greenish color is either absent or minimal. With respect to possible application to large-scale processing of juice, this level of suspended solids should be easily removable by filtration with suitable filter aids.

Table III. Effect of Steam Injection Heating Conditions on Supernatant Protein Concentration

	Supernatant protein concentration, % total juice protein,[a] sec				
Temp, °C	0	10	20	40	60
56				55	62*
58		56*	56*	55	
60	66*	55	54	53	
62		55	54	50	
64	61*	50	51		

[a] Samples marked with an asterisk had a suspended solids content exceeding 0.8% (vol) after 40-sec test in Gyro-Tester. For analysis, heated juice samples were centrifuged at 37,000 × *g* for 15 min at 1°. All samples were treated at pH 8.3–8.6.

Table IV. Effect of pH on Supernatant Protein Concentration after Steam Injection Heating (Heated for 20 Sec)

	Supernatant protein concentration, % total juice protein[a]		
Temp, °C	pH 8.0	pH 7.0	pH 5.9
56			46
58		55	46
60	57*	54	42
62	54	53	
64	51		

[a] Samples marked with an asterisk had suspended solids content exceeding 0.8% (vol) after 40-sec test in Gyro-Tester. For analysis, heated juice samples were centrifuged at 37,000 × *g* for 15 min at 1°.

The higher yield of soluble protein in acceptable samples following steam injection compared to slug flow heating (50–55 *vs.* 35–44%) suggests that direct steam injection is a superior method of heating juice prior to centrifugal separation. Since the concentration of soluble protein in an ultracentrifugal supernatant was 62% of the total juice protein, the 54–55% protein concentration in the samples heated for 10–20 sec at 60–62° represents a recovery of 87–89% of the soluble white protein. At a pH of 8.3–8.6, it appears that 56° is too low a temperature, 58° is marginal, 60–62° is optimal, and 64° is too high a temperature. It is interesting to note that at least 10 sec of heating must occur before the treatment can be considered successful; the two samples brought just to temperature and then rapidly cooled did not receive adequate heat treatment, even though one of those samples was heated to 64°.

The experiment reported in Table III was carried out at pH 8.3–8.6. Since pH has a major influence on the coagulability of proteins (see also Tables I and II), the pH of the juice was varied in the next experiment. Alfalfa juice, adjusted to pH levels of 5.9, 7.0, or 8.0, was heated by direct steam injection for 20 sec at temperatures of 56–64°. The supernatant protein concentrations are shown in Table IV. The level of 0.8% (vol) for suspended solids was again selected as a limit of acceptability. The results show quite clearly that lowering the pH decreases the amount of soluble protein remaining in the supernatant. When this experiment was repeated identically except that the holding time was decreased to 10 sec, results were very similar except for a slightly increased yield of soluble protein. The adjustment of pH, then, becomes equally

important to the selection of time and temperature of heat exposure for the optimum separability of the green chloroplastic proteins from the soluble white proteins.

The experiments reported in this paper have confirmed and extended the observations of others that controlled heating allows the centrifugal sedimentation of the green chloroplastic proteins while having only minimal effect on the soluble white proteins. The effects of pH, temperature, and time of exposure have been studied extensively; increasing the pH reduces the denaturation of the soluble proteins, whereas increasing either the temperature or the time of exposure has the opposite effect. By increasing the severity of the pH–temperature–time treatment, the clarity of the centrifugal supernatant can be improved markedly but only at the cost of a decreased level of soluble white protein. The results of these experiments are currently being applied in the development of a process which can separate the white from the green proteins of freshly expressed alfalfa juice on a continuous basis.

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Optical Resolution of DL-Lysine by Preferential Crystallization Procedure

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To develop a practical method for the production of L-lysine, the optical resolution of synthetic DL-lysine by preferential crystallization procedure was studied. As the properties of free DL-lysine were unsuitable for this resolution method, DL-lysine was converted to various aromatic sulfonates and the suitability of each was investigated. As a result, DL-lysine-*p*-aminobenzenesulfonate was found to be resolved in a good yield by preparing a supersaturated aqueous solution of the racemic modification and seeding with crystals of the desired isomer. The mother liquor was used repeatedly for the resolution of the opposite antipode. The unused D isomer could be easily racemized by heating in an autoclave. A general method was also established to distinguish whether or not resolution of a given racemic modification was possible. However, it is not yet possible to predict what kind of sulfonates are resolvable.

L-Lysine, one of the essential amino acids, is an important substance in pharmaceutical and nutritional fields, and its market has been expanding rapidly in recent years. Today, most commercial L-lysine is produced by a fermentation method, very seldom by a synthetic method because this procedure yields racemic lysine. Also, a satisfactory resolution method suitable for industrial application has not appeared. Up until now, several fairly practical studies of the optical resolution of DL-lysine have been reported. Namely, two diastereoisomeric salts of DL-lysine with L-glutamic acid (Emmick, 1951; Rogers, 1953) and of aminocaprolactam, a synthetic intermediate of DL-lysine, with L-pyrrolidone carboxylic acid (Brenner and Rickenbacher, 1958; Nelemans *et al.*, 1963) were resolved by fractional crystallization. Also the acyl derivatives of DL-lysine, ε-benzoyl-*N*-acetyl-DL-lysine, and ε-benzoyl-DL-lysine were hydrolyzed asymmetrically by mold aminoacylase (Chibata *et al.*, 1956; Yamada *et al.*, 1956) and bacterial ε-lysine acylase (Ishikawa *et al.*, 1962), respectively. However, these methods are unsatisfactory in competition with a fermentation method. Therefore, it is very much desired to establish an advantageous resolution method for DL-lysine. In these circumstances, we have studied a practical method for the optical resolution of DL-lysine.

Although a number of methods for the optical resolution of DL-amino acids have been reported, most of them employ chemical or enzymatic procedures and reports on a preferential crystallization procedure are relatively few (Greenstein and Winitz, 1961). If successfully applied, a

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