Soluble Protein from Alfalfa Juice by Membrane Filtration

Benny E. Knuckles,* Donald de Fremery, E. M. Bickoff, and George O. Kohler

After removing the green chloroplastic protein from fresh alfalfa juice, ultrafiltration and/or diafiltration was used to prepare a soluble, cream-colored protein concentrate. Ultrafiltration was useful in concentrating the protein by removing 80 to 90% of the water and nonprotein components. Continuous diafiltration removed additional quantities of nonprotein components yielding a clear, stable protein solution. The purified protein, when precipitated at 25° and pH 4.8, redissolved on adjustment of pH (<3.5 or >7.0). Freeze drying of the purified protein solution having a pH of 7 yielded a product of about 93% protein which could be readily redissolved in water.

The Pro-Xan process was developed to separate a portion of the protein and carotenoid pigments from alfalfa juice as a dry product that is essentially devoid of fiber (Knuckles *et al.*, 1970, 1972; Kohler and Bickoff, 1970; Lazar *et al.*, 1971; Miller *et al.*, 1972; Spencer *et al.*, 1970, 1971). The use of this product, Pro-Xan, as a food is limited to situations where its color and taste are not undesirable.

The Pro-Xan process was subsequently modified to yield both a feed supplement and a protein isolate suitable for human consumption (Edwards *et al.*, 1973; de Fremery *et al.*, 1973). The feed supplement, Pro-Xan II, contains the green pigment. This product differs from Pro-Xan by having a lower content of protein and a higher content of fat and carotenoid pigments. The off-white product intended for human consumption is prepared by heat coagulation. Since this heat coagulated product is insoluble, its use in food preparations may be limited. In order to prepare a protein product which retained its native solubility characteristics, we considered the membrane processes, ultrafiltration and diafiltration.

Membrane filtration has been used to purify protein from cheese whey (Porter and Michaels, 1971a; Horton *et al.*, 1972), skim milk (Peri *et al.*, 1973), and cotton seed whey (Lawhon *et al.*, 1973). It has also been used to fractionate protein mixtures (Blatt, 1971). In our experiments, membrane filtration was effective in removing pigments, off-flavors, and other low molecular weight impurities. The effectiveness of ultrafiltration and diafiltration for removing these components to yield a soluble cream colored protein isolate is described in this paper.

EXPERIMENTAL SECTION

Preparation of Chloroplast-Free Juice (Centrate). Freshly expressed alfalfa juice contains suspended chloroplasts and chloroplastic fragments. In our experiments, these particles are normally removed by centrifugation. The word "centrate," as used in this paper, refers to the clear brown solution from which these particles have been removed by centrifugation, in much the same way that filtrate refers to the clear solution from which filtrable particles have been removed by filtration. The word "centrate" is in common usage in the centrifuge industry.

On a laboratory scale, juice was extracted from fresh alfalfa using a meat grinder and a hydraulic press. This operation was carried out at 2–5°. The green chloroplastic material was removed from the juice by mild heat treatment followed by centrifugation in a manner similar to that described by de Fremery *et al.* (1973). In this case, 500 ml of green juice was heated to 56–60°, held for 20 sec before cooling, and then centrifuged in a Sorvall RC 2 B super speed centrifuge at 28,000*g*. The clear brown centrate, containing soluble protein, was held in an ice-water bath until used. Centrate used in some experiments was prepared in the Western Regional Research Laboratory pilot plant by the method of Edwards *et al.* (1973).

In most experiments sodium metabisulfite at a 0.1% SO_2 equivalent (based on fresh weight of alfalfa) was added to the alfalfa prior to extraction of juice.

Ultrafiltration and Diafiltration Procedure. In ultrafiltration, the protein solution was added to the container surrounding the hollow fiber membranes. Vacuum was applied to the membrane bundle as the solution was stirred by magnetic stirrer. The pressure difference (600 mm) across the membranes caused the water to pass through, carrying with it molecules whose molecular weight was less than the molecular weight cut-off of the membrane. As this water passed through the membranes, additional centrate was continuously and automatically added so that the liquid level in the unit remained constant. In this way, solutions of increased protein concentration could be obtained. The permeation rate (rate of liquid passage through the membrane, expressed in terms of gallons per square foot per 24-hr day) was measured at selected times during runs. When the volume was reduced to a desired point, aliquots of permeate and concentrate were taken for analysis.

Diafiltration was carried out in a manner similar to that described for ultrafiltration. The centrate was added to the unit and stirred, but water was added continuously to replace the liquid passing through the membranes.

Both ultrafiltration and diafiltration were carried out at 0–2° to retard fermentation and proteolysis.

Pigment Measurement. Nonspecific pigment concentration was measured photometrically at 450 nm. The absorbance value obtained in a 1-cm cell was assumed to be the value for 1 ml of solution. From the dilution factor and the total volume, a number proportional to the pigment in the fraction could be calculated.

Gel Filtration Chromatography. The protein solutions (10 ml) were applied to a Bio-Gel P-60 (Bio-Rad Laboratories) column, 2.4 × 46.5 cm, and eluted at 40 ml/hr with a 0.05 *M* KCl–0.01 *M* borate buffer at pH 8.5. The eluent stream was monitored at 280 nm and recorded continuously.

Analytical Methods. Total solids were determined gravimetrically, drying in an oven at 110°. 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.

Acid Solubility of Protein. The centrate was brought to 25° and 0.1 *N* HCl was added slowly to the vigorously stirred solution. When the centrate was stabilized at the predetermined pH, the suspension was held for 0.5 hr.

Western Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Berkeley, California 94710.

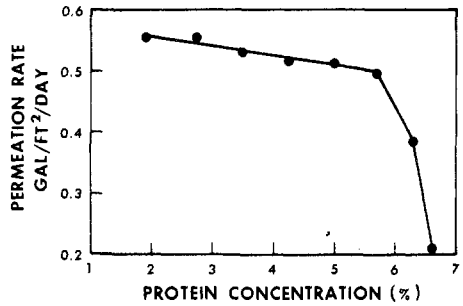

Figure 1. Effect of protein concentration on permeation rate. Gal/ft²/day may be converted to ml/cm²/hr by multiplying by 0.1698.

The suspension was then centrifuged at 0° and 17,000*g* for 20 min. The supernatant from this step was decanted, the pellet was resuspended in water at the same pH and centrifuged at 48,000*g*, and the combined supernatants were analyzed for protein nitrogen. By comparison with the amount of protein nitrogen in the starting material, the per cent of soluble protein was readily calculated.

The solubilities of the protein in the diafiltered centrate and the ultrafiltered concentrate were measured in the same manner. However, the ultrafiltered concentrate was diluted to roughly the original concentration with water for easier mixing and to eliminate possible concentration effects.

RESULTS AND DISCUSSION

Selection of Ultrafilter. The Bio Fiber 80 ultrafilter unit (Bio-Rad Laboratories), containing a hollow fiber type membrane, was used to evaluate a membrane of 30,000 mol wt cut-off. The Iopor ultrafilter unit (Dorr-Oliver, Inc.) was used in the evaluation of membranes having 100,000 (Dorr-Oliver, Inc., GP-100) and 300,000 (Amicon Corp., XM-300) mol wt cut-off. After diafiltering centrate with water until five times the sample volume was collected as permeate, the three membranes retained 90, 61, and 31%, respectively, of the protein in the centrate. The Bio Fiber 80 unit was used in the subsequent studies reported here because the other membranes passed unacceptably large amounts of protein.

Ultrafiltration. Ultrafiltration was found to be a convenient method for concentrating soluble protein contained in alfalfa juice. Centrate could be concentrated to 5.5% protein (corrected for nonprotein nitrogen) before the permeation rate began to decrease rapidly (Figure 1). This decrease in permeation rate is a result of a number of factors which are reported in the literature. Porter and Michaels (1971a) reported that permeation rate decreased with increased viscosity and protein-gel formation on the membrane (concentration polarization). Peri *et al.* (1973) showed that increases in the concentration of protein and lactose in skim milk decreased the permeation rate. Based on the observed decrease of permeation rate and the recommendation of Peri *et al.* (1973), it appears that the maximum protein concentration for most efficient processing is 5.5%.

Centrate contains about 0.16% nonprotein nitrogen and 0.17% protein nitrogen on an as-is basis. When the total volume of solution was reduced from 1000 to 200 ml during ultrafiltration, the concentrate contained about 5% protein. About 70% of the original solids passed through the membrane during this operation. When the total volume was reduced by a factor of 19, the concentrate was about 9.5% protein. More than 80% of the original solids had passed through the membrane at this point. Table I gives the protein and ash contents of ultrafilter fractions on a dry weight basis. (The fat and fiber values were less than 1% in all samples.) Fivefold concentration resulted in a product with nearly three times the protein content and only half the ash content of the starting material. When concentrated 19-fold, the protein content of the solution had increased to nearly 60%. At this point, more than 88% of the original nonprotein nitrogen had passed through the membrane. The 61.2% crude protein value for this product is greater than the 45–50% crude protein value reported for a cheese whey product after removing 90% of the water (Horton *et al.*, 1972). Horton *et al.* (1972) were able to obtain a cheese whey product of higher crude protein content (about 80%) by removing more than 95% of the water.

Table I. Composition of Ultrafiltered Fractions[a]

Fraction	Crude protein,[b] %	Protein,[c] %	Ash, %
Centrate	24.7	13.8	25.8
Ultrafilter concentrate			
5-fold concentration	48.8	39.8	12.8
19-fold concentration	61.2	59.1	8.4
Ultrafilter permeate	13.9	<1	26.0

[a] Dry weight basis. Average of two experiments. [b] Total nitrogen times 6.25. [c] Crude protein corrected for nonprotein nitrogen.

Table II. Composition of Retentates from Diafiltration of Centrate[a]

Material	Permeate ratio (sample: permeate)	Crude protein,[b] %	Protein,[c] %	Ash, %
Centrate		24.7	13.8	25.8
Retentate	1:4	72.5	68.8	7.6
Retentate	1:8	92.4	92.0	3.6
Retentate	1:10	93.4	93.4	1.6

[a] Dry weight basis. Average of two experiments. [b] Total nitrogen times 6.25. [c] Crude protein corrected for nonprotein nitrogen.

The amount of pigment remaining in the ultrafilter concentrate after a fivefold concentration was 40% of the original; the ash content was only 17% of the original. The comparison of pigment and ash retained in this concentrate indicates that the pigment moves through the membrane at a slower rate than other nonprotein components. Since all of the water, which contains pigments and off-flavors, cannot be removed by ultrafiltration, an additional purification step is necessary.

Diafiltration. Continuous diafiltration was a satisfactory method of removing salts and other nonprotein solids from the centrate. The composition of the retentate or nonpermeable fraction varied according to the amount of diafiltration (Table II). In order to obtain a product of less than 2% ash, it was necessary to diafilter until the permeate volume was 10 times that of the sample. Porter and Michaels (1971a) reported that diafiltration (5 times sample volume) removed 99% of low molecular weight contaminants. The difference in the volume required for purification by diafiltration can be explained by the work of Blatt (1971) who showed that in mixed systems the rejection coefficients are elevated resulting in fractional solute extraction in the filtrate. Therefore, our centrate must have nonprotein contaminants of a relatively high rejection coefficient (>0.6) requiring more diafiltration for purification.

When a redissolved acid-precipitated protein (Miller *et al.*, 1973) was diafiltered (10 times sample volume), 23% of the total solids were removed. More than 60% of these solids were of undetermined components, possibly carbo-

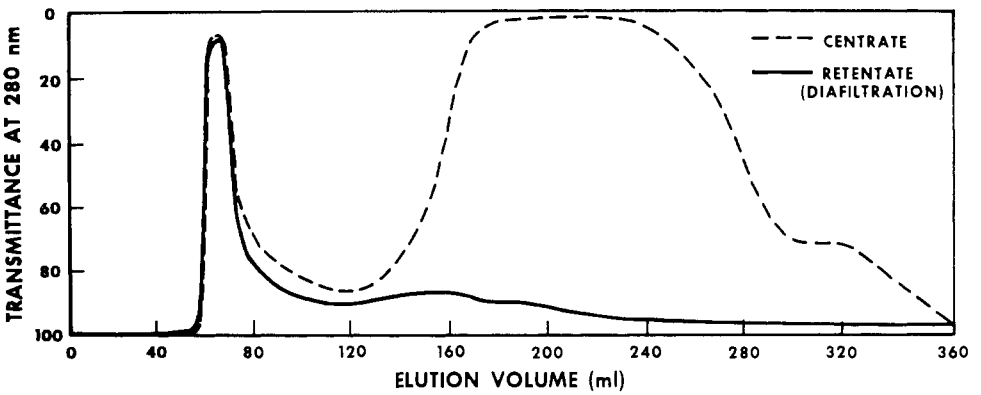

Figure 2. Gel filtration of centrate and retentate from diafiltration of centrate. The narrow peak represents protein; the broad peak represents nonprotein and highly pigmented components.

hydrates. The remainder of the solids were composed of ash and nonprotein nitrogen. The removal of these impurities increased the protein content of the dry product from 70.1 to 93.7%. It also reduced the ash content from 5.1 to 1.7%.

The pigment remaining with the protein after diafiltration (10 times sample volume) of either centrate or redissolved acid-precipitated protein was only 5% of the original. The removal of pigment from centrate by diafiltration is demonstrated in the gel filtration chromatograms shown in Figure 2. The elution of centrate from the Bio-Gel P-60 column showed two major areas of low transmittance. The first fraction to be eluted represents protein since an ultraviolet (uv) spectrum of the combined fraction showed a curve typical of protein. The second fraction was nonprotein and highly pigmented. These pigments tend to become adsorbed to the gel since it required more than one column volume (220 ml) of eluent to elute them. The pigmented fraction was not detected in the gel filtration chromatogram of the retentate obtained by diafiltration of centrate. The absence of this fraction confirms that diafiltration was effective in removing pigment from the centrate.

The purified centrate solution could be freeze dried to yield a bland tasting, cream colored, water-soluble powder. In those experiments in which sodium metabisulfite was omitted, the freeze-dried purified protein was noticeably darker. This darkening may be explained by the reports of Loomis (1969) and Jones and Lyttleton (1972) who observed that pigment formation in plant extracts may be caused by the binding of quinones to protein sulfhydryl groups, a reaction which is inhibited by sodium metabisulfite. The usefulness of sodium metabisulfite in reducing pigment formation has been demonstrated in prune juice by Stafford and Bolin (1972).

Solubility of Protein following Ultrafiltration and Diafiltration of Centrate. The solubility of alfalfa protein was altered by the removal of low molecular weight components either by ultrafiltration or diafiltration. The solubility of the protein in the ultrafilter concentrate and the retentate from diafiltration of centrate increased at pH levels below 4 (Figure 3). The increased solubility was most evident in the retentate from diafiltration where 95% of the protein was soluble at pH 3.5 and 100% soluble at pH 3 and below. The changes in solubility are consistent with a report which shows that solubility is a function of the nature and concentration of solutes present in the protein solution (Mahler and Cordes, 1966). The property of solubility in the pH range of 2–3.5 may allow the purified alfalfa protein to be incorporated into carbonated beverages. This proposed use is suggested by the laboratory studies of Holsinger *et al.* (1973) in which soft drinks were successfully fortified with cheese whey protein exhibiting solubility in this same pH range.

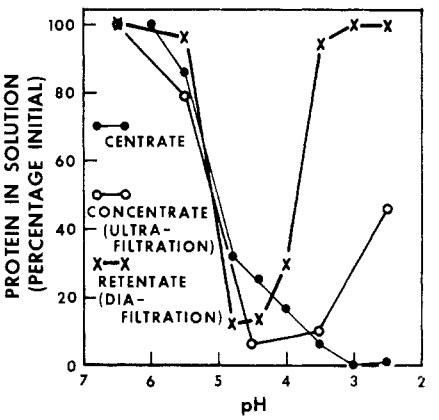

Figure 3. Effect of ultrafiltration and diafiltration on acid solubility of protein.

The protein precipitated from centrate by adjustment of pH to 4.5 at 25° was only about 20% soluble in water at pH 7.5. Under these same conditions, solubility of protein precipitated from ultrafilter concentrates or retentates from diafiltration was much greater than 20%. In ultrafiltered products of 11.4 and 8.4% ash, the solubility of protein at pH 7.5 was about 35 and 55%, respectively. Diafiltered products of 5.1 and 1.7% ash had protein solubilities at pH 7 of about 69 and 100%, respectively. This greater solubility appeared to correlate inversely with ash content of the dried product. Since ash contributes to the ionic strength of the centrate, these results can be related to the work of Betschart (1974) who showed that alfalfa protein concentrates had decreasing solubility at increasing ionic strength.

When incubated at pH 6.1 and 25°, protein in the retentate from diafiltration remained soluble for long periods. No precipitation or pH change occurred during a 1-week period. However, the protein remaining in solution decreased by 3.8% in 4 hr and 10% in 24 hr. A corresponding increase in nonprotein nitrogen indicated that proteolysis had occurred. Although no direct comparison can be made with the proteolytic activity in whole alfalfa juice (de Fremery *et al.*, 1972), it appears that the activities in the retentate from diafiltration and whole alfalfa juice are similar.

Although the results in this study were obtained primarily on a hollow fiber unit of limited capacity, the same experimental procedure should be applicable to any ultrafilter unit. We plan to continue these experiments with pilot plant scale equipment.

ACKNOWLEDGMENT

The authors wish to express their thanks to Raymond Miller for the preparation of soluble acid-precipitated pro-

tein and to Richard Edwards for centrate prepared in the pilot plant.

LITERATURE CITED

Betschart, A. A., *J. Food Sci.*, **39**, 1110 (1974).
Blatt, W. F., *J. Agr. Food Chem.* **19**, 589 (1971).
de Fremery, D., Bickoff, E. M., Kohler, G. O., *J. Agr. Food Chem.* **20**, 1155 (1972).
de Fremery, D., Miller, R. E., Edwards, R. H., Knuckles, B. E., Bickoff, E. M., Kohler, G. O., *J. Agr. Food Chem.* **21**, 886 (1973).
Edwards, R. H., Miller, R. E., de Fremery, D., Knuckles, B. E., Bickoff, E. M., Kohler, G. O., presented at the 166th National Meeting of the American Chemical Society, Chicago, Ill., Aug 26-31, 1973.
Holsinger, V. H., Posati, L. P., DeVilbiss, E. D., Pallansch, M. J., *Food Technol.* **27**, 59 (1973).
Horton, B. S., Goldsmith, R. L., Zall, R. R., *Food Technol.* **26**, 30 (1972).
Jones, W. T., Lyttleton, J. W., *Phytochemistry* **11**, 1595 (1972).
Knuckles, B. E., Bickoff, E. M., Kohler, G. O., *J. Agr. Food Chem.* **20**, 1055 (1972).
Knuckles, B. E., Spencer, R. R., Lazar, M. E., Bickoff, E. M., Kohler, G. O., *J. Agr. Food Chem.* **18**, 1086 (1970).
Kohler, G. O., Bickoff, E. M., SOS/70, 3rd International Congress of Food Science and Technology, Washington, D.C., Aug 9-14, 1970.
Lawhon, J. T., Lin, S. H. C., Cater, C. M., Mattil, K. F., *Food Technol.* **27**, 26 (1973).
Lazar, M. E., Spencer, R. R., Knuckles, B. E., Bickoff, E. M., *J. Agr. Food Chem.* **19**, 944 (1971).
Loomis, W. D., *Methods Enzymol.* **13**, 555 (1969).
Mahler, H. R., Cordes, E. H., "Biological Chemistry," Harper and Row, New York, N.Y., 1966, p 57.
Miller, R. E., de Fremery, D., Bickoff, E. M., Kohler, G. O., presented at the American Chemical Society Pacific Conference on Chemistry and Spectroscopy, San Diego, Calif., Nov 1-3, 1973.
Miller, R. E., Edwards, R. H., Lazar, M. E., Bickoff, E. M., Kohler, G. O., *J. Agr. Food Chem.* **20**, 1151 (1972).
Peri, C., Pompei, C., Rossi, F., *J. Food Sci.* **38**, 135 (1973).
Porter, M. C., Michaels, A. S., *Chem. Technol.* **1**, 56 (1971a).
Porter, M. C., Michaels, A. S., *Chem. Technol.* **1**, 248 (1971b).
Spencer, R. R., Bickoff, E. M., Kohler, G. O., Witt, S. C., Knuckles, B. E., Mottola, A. C., *Trans. Amer. Soc. Agr. Eng.* **13**, 198 (1970).
Spencer, R. R., Mottola, A. C., Bickoff, E. M., Clark, J. P., Kohler, G. O., *J. Agr. Food Chem.* **19**, 504 (1971).
Stafford, A. E., Bolin, H. R., *Food Eng.* **44**, 128 (1972).

Received for review July 19, 1974. Accepted November 11, 1974. Presented at the 167th National Meeting of the American Chemical Society, Los Angeles, Calif., March 31-April 5, 1974. Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

Effect of Auxins and Herbicides on Enhancement of Protein Synthesis in Fungi

Michael Szabo, Pasquale V. Scarpino, and Charles Rogers*

A number of test compounds were investigated as potential compounds for improving the quantity of fungal protein that can be synthesized from organic wastes. Zinc sulfate was also evaluated in combination with selected test compounds. Where the cost of converting organic wastes to microbial protein may be marginal, the data suggest that low concentrations of test compounds, especially when used in conjunction with $ZnSO_4$, may improve the economic feasibility of fermentation processes to produce protein.

In 1880, Van Sachs postulated that plants are regulated by hormones. In 1934, Kogl and Erxteben isolated the growth regulating substance of plants, the auxin indoleacetic acid (IAA). Some of the same physiological effects of IAA on plants, which could also exert important influences on microorganisms, are cell elongation, root initiation, and induced synthesis of new RNA and protein.

Introduction of the synthesis of new RNA and protein by the exogenous application of IAA has been demonstrated in a variety of tissues, *e.g.*, Rhoeo leaves and bean endocarp (Sacher, 1967), yeast cells (Shimoda *et al.*, 1967), and green pea stem sections (DeHertogh, 1965). With the use of specific inhibitors, this synthesis activity of IAA was proven to be associated with IAA auxin-induced cell wall plasticity and extension. Puromycin, actinomycin D, chloramphenicol, and 8-azaguanine are four inhibitors frequently used in studies on the inhibition of the biosynthesis of RNA and protein.

In studies with herbicides, Ries *et al.* (1967) demonstrated an increase in nitrate reductase activity when suboptimal levels of the herbicide simazine were applied to plants grown with nitrate, but not to plants grown with ammonia as the sole source of nitrogen. An increase in total protein per plant was also confirmed. Rye plants receiving 0.5 to 0.8 μmol of simazine contained up to 45% more water-extractable protein than the controls without simazine. Atrazine, diuron, and terbacil caused similar effects.

Although most of the studies concerned with growth regulators have dealt specifically with plants, there is also some information that describes the action of this group of chemicals on the bacterial and fungal microflor of the soil.

Sikka *et al.* (1965) determined the effect of several concentrations of atrazine on the mycelial growth in liquid media of four common soil fungi: *Trichoderma, Fusarium, Penicillium,* and *Geotrichum.* The herbicide stimulated the growth of the four fungi at concentrations ranging from 1 to 10 ppm. Addition of 10 ppm increased the weight of the mycelium by almost 100%. In general, mycelial growth increased only up to a point with higher concentrations of the herbicides. We conclude from the work of other investigators that auxins and selected herbicides may be effective agents in stimulating the metabolism of nitrogenous compounds in fungi and, thus, in producing fungi with high protein content.

The production of protein from various organic wastes has already been demonstrated with the use of both fungi and bacteria (Callihan and Dunlap, 1971; Rogers and Scarpino, 1972). Dailey (1972) has also reported that one could induce up to an 11.6% increase in protein in *Aspergillus niger* with the use of 10 ppm of IAA, with glucose as

Solid & Hazardous Waste Research Laboratory, National Environmental Research Center, Environmental Protection Agency, Cincinnati, Ohio 45268 (M.S., C.R.), and Department of Civil and Environmental Engineering, University of Cincinnati, Cincinnati, Ohio 45268 (P.S.).