# Improved Separation of Green and Soluble Leaf Proteins by pH Shift

Carmen Merodio, Mercedes Martín, and Bartolomé Sabater*

The effects of pH, temperature, and detergent on the solubility of chlorophyll and protein were determined in spinach leaf juice. After precipitation, white protein dissolved better than thylakoid fragments when the pH changed. Making use of this characteristic, a method was proposed for the preparation of protein free of chlorophyll (≤0.1%) in which the juice was successively treated at an alkaline and an acid pH and centrifuged at a low rate (2000*g*). With this method, yields in white protein ranging from 46 to 78% were obtained with different plants. The appropriate pH values used were specific for each plant.

Although leaf protein concentrates have been incorporated into diets for human consumption (Heat, 1977; Pirie, 1980), their green color has prevented acceptance by many consumers and food producers. The thermal precipitation of chloroplastic material is not highly specific and usually yields as white protein less than a third of the protein present in crude leaf juice (Pirie, 1980). Ultracentrifugation allows a highly selective precipitation of green material (de Fremery et al., 1973), resulting in white protein yields of 60–65%. However, high centrifugal forces are not available for large-scale preparation.

The selection of appropriate plants and pH (Lundborg, 1980) and the use of flocculents (Knuckles et al., 1980) or calcium salts (Gwiazda and Saio, 1981), combined with the use of very high centrifugal forces (10000–30000*g*), have sometimes increased the yield of white protein.

The objective of this work was to determine the effects of pH, temperature, and detergent on the solubility of protein and chlorophyll in the juice of spinach leaf and on the solubilization of the precipitated protein and chlorophyll and also to determine the recovery of white soluble protein from different plants by use of successive pH treatments and low centrifugation (2000*g*) of the juices.

## EXPERIMENTAL SECTION

The leaves of the different plants used were obtained from either the local market or farmers.

A total of 100–500 g of wet leaves was chopped in a Waring Blendor, pressed, filtered through two gauzes, and centrifuged for 10 min at 2000*g* at room temperature, the supernatant being the leaf juice. After different treatments of the juice, insoluble fractions were removed by centrifugation for 10 min at 2000*g*. The effects of temperature and pH treatments were studied on 2-mL aliquots in a water bath. Final preparations were made from 100–500 mL of juice. pH was changed by addition of 0.1 M HCl or NaOH.

Protein was determined according to Lowry et al. (1951) after precipitation with 10% trichloroacetic acid (García et al., 1983). Chlorophyll was determined according to Arnon (1949).

## RESULTS AND DISCUSSION

Figure 1 shows the effect of pH on the solubility of the chlorophyll and protein in the juice of spinach leaves. The minimum solubility of protein was reached at pH 4, which is near the range reported for other plants (Betschart and Kinsella, 1973). Chlorophyll showed a wider range of low solubility than protein (pH 3.6–4). Within the range of pH examined, a higher percentage of chlorophyll than protein precipitated based on the total amount of each in the fresh juice (about 14.5 mg/mL protein and 650 μg/mL chlorophyll). The minimum value of the chlorophyll/protein ratio in the supernatant (about 0.003) was obtained at pH about 3.6. The recovery of protein in the supernatant of pH 3.6 was around 45%; however, it still was light green.

The effect of temperature on the precipitation of green and soluble protein was studied in the presence and in the absence of the commercial (Henkel SA) detergent Mistol (about 10% alkylarylsulfonate, 20% alcoholsulfonate). Figure 2 shows the percentages of chlorophyll and protein remaining in the supernatant after different temperature treatments of the juice of spinach leaves at pH 6.5. As a general rule, a higher percentage of the chlorophyll precipitated at lower temperatures than of protein. These results agree with those reported for other plants (Heat, 1977; Pirie, 1980). Mistol partially protected against the precipitation of both chlorophyll and protein. At pH 8 (Figure 3), Mistol only protected against chlorophyll and protein precipitation below 60 and 70°C, respectively.

Regardless of the treatments, it was not possible to recover free of chlorophyll (≤0.1% of the protein value) more than 30% of the original protein in the juice.

It is possible that the protein and the chlorophyll precipitated by moderate temperature treatments at alkaline pH were different from those precipitated at acid pH. Moreover, advantage can be taken if the protein and the chlorophyll precipitated at a given pH do not resuspend to the same extent when the pH changes. As Figure 4 shows, the last situation seems to be the case. According to Figure 1, some 75% of the protein precipitated at pH 4. By treatment of the precipitate with a solution of pH higher than 4, a substantial amount (some 30% of the precipitated protein was resuspended (Figure 4). However, only 3–9% of the total chlorophyll precipitated at pH 4 was resuspended at the higher pH. In Figure 4, material resuspension was made in the presence of Mistol, but similar results were found when the precipitate was treated in the absence of detergent. These results suggest that the precipitated white protein of cytoplasm and of chloroplast stroma is more easily resuspended than the precipitated thylakoid fragments containing chlorophyll and protein.

The resuspension of the precipitated white and green protein is dependent on the time used in each pH treatment. To obtain the highest recovery of protein with the lowest chlorophyll contamination, time of treatment and other factors to include the exact pH for each treatment, the use of more than two pHs, the use of detergent, temperature, etc. must be studied. We intended to obtain protein free of chlorophyll (≤0.1%) by treating leaf juices

Departamento Fisiología Vegetal. Facultad Ciencias, Universidad de Alcalé de Henares, Apartado 20, Alcalé de Henares, Madrid, Spain.

0021-8561/83/1431-0957$01.50/0 

Table I. Recovery of White Protein after Successive pH Treatments of the Juice of Different Plant Leaves[a]

| plant | alkaline pH | acid pH | Mistol | initial protein, g/L | protein recovery, % | final chlor/prot × $10^3$ |
|---|---|---|---|---|---|---|
| *Brassica napus* L. | 8.0 | 5.2-6.0 | yes | 10.5 | 52.4 ± 0.28 | 1.0 |
| *Brassica oleracea* L. | 8.0 | 5.2-5.5 | yes | 7.5 | 56.1 ± 6.1 | 0.77 |
| *Lactuca sativa* L. | 8.0 | 5.1-5.4 | yes | 5.0 | 68.0 ± 0.38 | 0.96 |
| *Medicago sativa* L. | 9.0 | 5.7-6.6 | no | 23.0 | 46.3 ± 0.5 | 0.74 |
| *Raphanus sativus* L. | 8.0 | 5.4-6.0 | yes | 12.8 | 78.5 ± 4.6 | 0.24 |
| *Vicia faba* L. | 9.0 | 6.2-7.0 | no | 17.7 | 52.5 ± 0.3 | 0.4 |
| *Solanum tuberosum* L. | 8.5 | 5.6-6.0 | no | 11.8 | 50.1 ± 1.3 | 1.0 |

[a] Each pH treatment was maintained for 10 min at 35 °C. Recovery values for protein in the supernatants are based on their values in the respective fresh juice. Indicated values are the mean ± SE of at least three independent experiments.

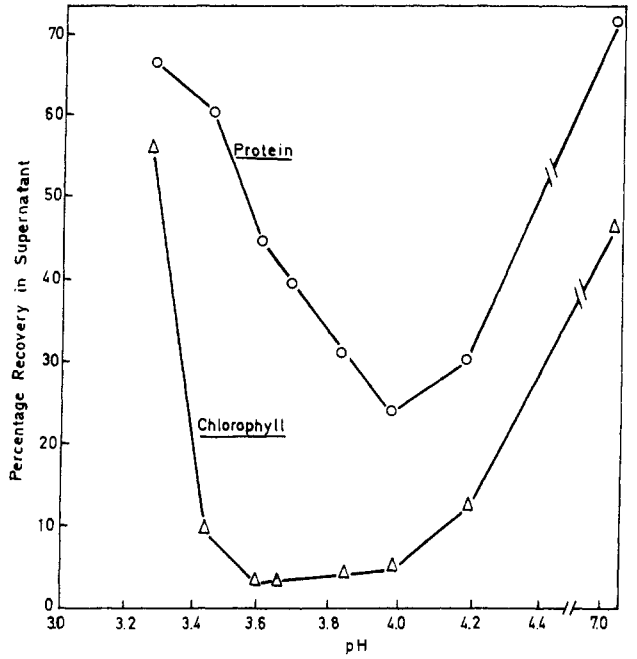


**Figure 1.** Effect of pH on the solubility of protein and chlorophyll in the juice of spinach leaves. pH treatments were performed at 25 °C.

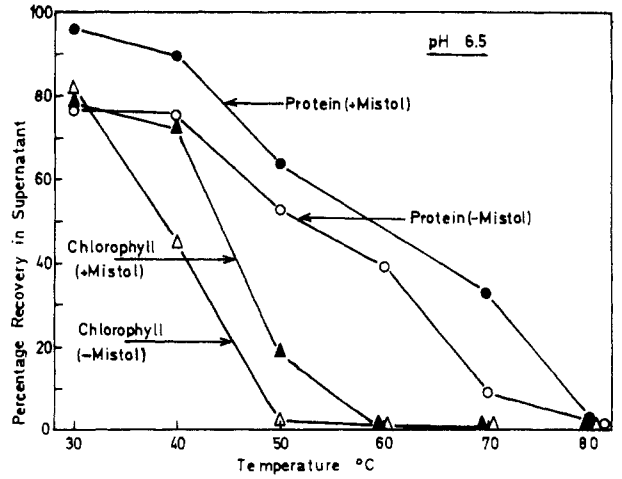


**Figure 2.** Effect of temperature on the solubility of protein and chlorophyll in the juice of spinach leaves at pH 6.5. When added, Mistol was present at 5 mg/mL.

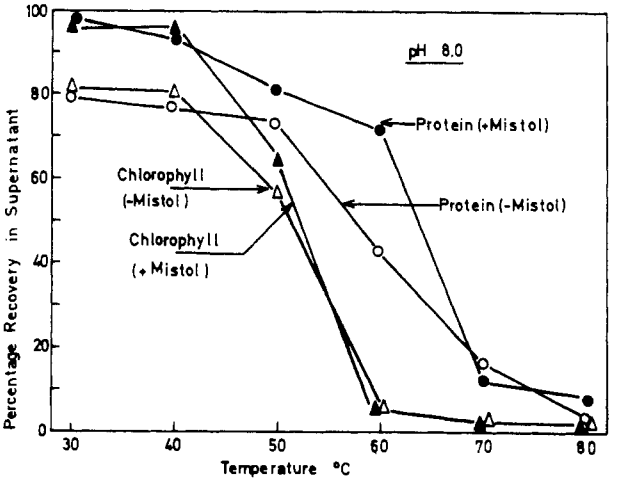


**Figure 3.** Effect of temperature on the solubility of protein and chlorophyll in the juice of spinach leaves at pH 8.0. When added, Mistol was present at 5 mg/mL.

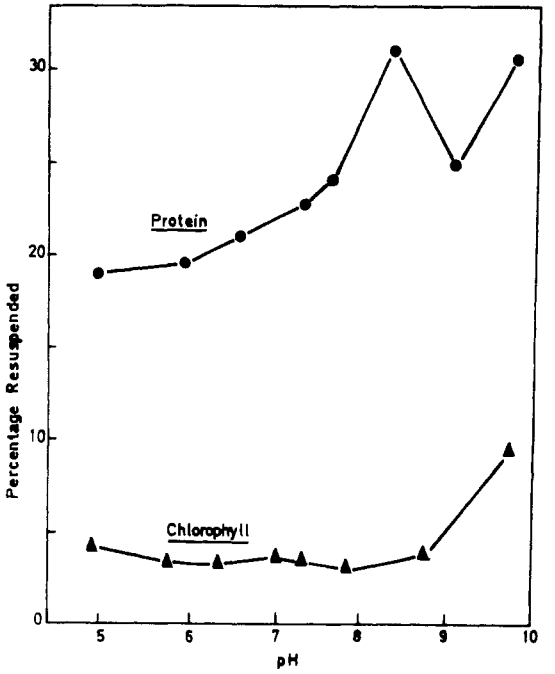


**Figure 4.** Solubilization of the acid-precipitated protein and chlorophyll. Resuspension solutions contained 5 mg/mL Mistol. Temperature was 25 °C.

successively to an alkaline and an acid pH. The first assays were carried out with the juice of spinach leaves.

Figure 5 shows the effect of the acid pH treatment on the recovery of protein and chlorophyll in the supernatuant of spinach leaf juice. In this assay, the original pH of the juice was first raised to pH 8 with NaOH. After 20 min, the pHs of different aliquots were lowered with HCl to different acid pHs and maintained for 10 min. Finally, the juices were centrifuged for 10 min at 2000*g*. According to Figure 5, after an acid treatment at pH 6.3 no detectable chlorophyll remained in the supernatant, which still retained 52% of the original protein. This recovery of chlorophyll-free protein was higher than the recovery ob-

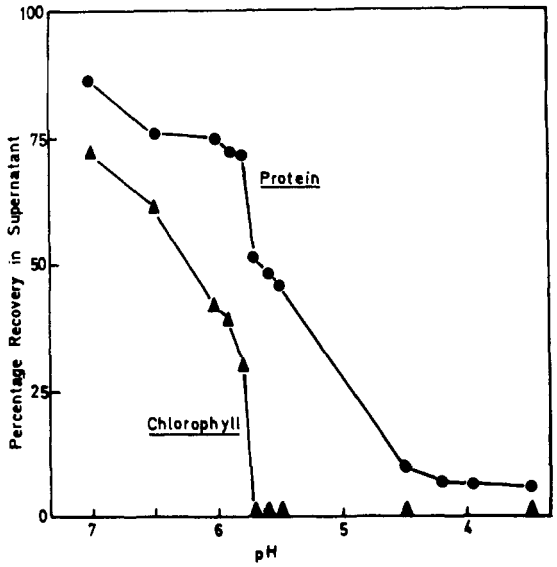


**Figure 5.** Effect of the acid pH treatment on the recovery of protein and chlorophyll from spinach leaves. Mistol was added at a final concentration of 2 mg/mL. Temperature was 30 °C.

tained (some 30%) with the thermal treatments (Figures 2 and 3).

With leaf juices of some other plants, different alkaline and acid pHs were assayed to obtain chlorophyll-free (≤0.1% with respect to protein) protein preparations by successive treatments at an alkaline pH and an acid pH. Table I shows the results of protein yields, final chlorophyll/protein ratios, and other information such as the pH values selected to optimize the yield of protein. From Table I it seems that the use of successive pH treatments is a good method to obtain a high yield of leaf protein essentially free of chlorophyll. The values of the appropriate pHs depended on the plant, and probably, a study of other factors, e.g., temperature and time, should improve the yields. The addition of Mistol improved by some 10–25% the yield in white protein from juices of some plants. Mistol is not included in Table I for plants in which it did not improve the yield in white protein more than 10%. Together with the high protein yield, the main interest of the method is based on the use of simple and rapid techniques and low centrifugal forces. The white protein of the supernatants may almost quantitatively (around 95%) be recovered by thermal denaturation at 90–100 °C.

Reasons other than the low resuspension of the precipitated thylakoid fragments may also contribute to the high protein yields of Table I. Batley and Bray (1978) found that the treatment of the juices at moderate alkaline pH increases the content of cytoplasmic white protein.

LITERATURE CITED

Arnon, D. I. *Plant Physiol.* **1949**, *24*, 1–15.
Batley, W. R., Jr.; Bray, W. J. U.S. Patent 4 130 553, Dec 19, 1978.
Betschart, A.; Kinsella, J. E. *J. Agric. Food Chem.* **1973**, *21*, 60–65.
de Fremery, D.; Miller, R. E.; Edwards, R. H.; Knuckles, B. E.; Bickoff, E. M.; Kohler, G. O. *J. Agric. Food Chem.* **1973**, *21*, 886–889.
García, S.; Martín, M.; Sabater, B. *Physiol. Plant.* **1983**, *57*, 260–266.
Gwiazda, S.; Saio, K. *Agric. Biol. Chem.* **1981**, *45*, 2659–2660.
Heat, S. B. In "Plant Protein"; Norton, G., Ed.; Butterworth: London, 1977.
Knuckles, B. E.; Edwards, H. E.; Kohler, G. O.; Whitney, L. F. *J. Agric. Food Chem.* **1980**, *28*, 32–36.
Lowry, O. H.; Rosebrough, N. H.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265–275.
Lundborg, T. *Physiol. Plant.* **1980**, *48*, 186–192.
Pirie, N. W. In "Applied Protein Chemistry"; Grant, R. A., Ed; Applied Science Publishers, Ltd.: London, 1980.

Received for review November 8, 1982. Revised manuscript received March 2, 1983. Accepted April 28, 1983. Presented in part at the Xth Congreso Sociedad Española de Bioquimica, Santander, Spain, Sept 23–25, 1982. This investigation was supported in part by a grant from Caja de Ahorros y Monte de Piedad de Madrid.

# Studies on Linseed Proteins

K. T. Madhusudhan and Narendra Singh*

A simple method of dehulling and demucilaging of linseed was developed. Solubility of the proteins of the linseed meal in water, 0.5 and 1.0 M NaCl, and 2% sodium hexametaphosphate solutions was determined in the pH range 2–12. The solubility was minimum around pH 3–6 and maximum around pH 8.0. The solubility minimum shifted to pH 0.5–4.5 in 1 M NaCl and to 0.6–5.3 in 2% sodium hexametaphosphate. The total proteins were characterized by techniques of gel filtration, ion-exchange chromatography, electrophoresis, and ultracentrifugation. The presence of at least three components was observed. The meal extracts showed proteolytic and trypsin inhibitor activities but no hemagglutinating activity. Amylase and amylase inhibitor activities were not detected.

In India, linseed (*Linum usitatissimum*) is one of the five major oilseeds and an important commercial oilseed crop. The deoiled meal obtained after expelling oil contains 25–35% protein. However, due to the presence of several antinutritional factors, viz., mucilage, phytic acid (Peterson, 1958), cyanogenic glycosides (Conn, 1969), antipyridoxine factor (Kratzer, 1947; Klosterman, 1974), allergens (Spies, 1974), and goitrogens (Care, 1954), its use is limited to livestock feed. Earlier work done in this laboratory has given information on these factors and also suggested simple detoxification techniques to render linseed cake fit for poultry feed (Mandokhot and Singh, 1979; Singh, 1979). The current investigation aims at more detailed information, including the nature of changes in

Protein Technology Discipline, Central Food Technological Research Institute, Mysore 570013, India.

0021-8561/83/1431-0959$01.50/0 