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Effects of Alfalfa Leaf Juice and Chloroplast-Free Juice pH Values and Freezing upon the Recovery of White Protein Concentrate

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Juice extracted from alfalfa was frozen at −25 °C and stored for different periods of time. After the juice was adjusted to pH 8.0, 8.5, and 9.0 and pH 6.0 as control, the green proteins were separated by heat treatment. Soluble or white proteins were coagulated in the resulting chloroplast-free juice at two different pH values, 3.5 and 4.0. The results show that greater amounts of dry matter and nitrogen are recuperated in the white protein concentrate at alkaline pH values than at the natural pH value, 6.0, and the amount of concentrate recovered is also greater when soluble proteins are coagulated at pH 3.5 instead of 4.0, although the resulting concentrate has less protein. With regard to the effect of freezing, it was possible to conclude that freezing of the juice was not a desirable storage method because it resulted in the coagulation and subsequent loss of most of the soluble proteins.

The separation of two protein fractions, insoluble or green protein from soluble or white protein, is necessary to prepare chlorophyll-free protein concentrates. The most common method described in the literature is the separation of green proteins by heat treatment, followed by acid precipitation of the white proteins in the resulting chloroplast-free juice.

The main variables affecting heat treatment are temperature, heating time, and juice pH. Although the first two have been sufficiently studied, with regard to the optimum juice pH there is some disagreement in the literature, previously described in detail (Hernández et al., 1988) on whether alkaline or natural pH is more suitable.

The optimum pH value for coagulating the soluble proteins in chloroplast-free juice was studied by Miller et

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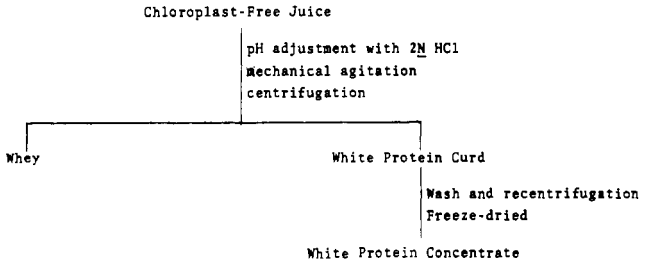

Figure 1. Obtention process scheme of white protein concentrate.

al. (1975). They found that decreasing pH from 4.0 to 3.5 increases the amount of concentrate obtained but decreases its protein content. In the literature there are not more data on this factor, and pH values of 3.5 or 4.0 are used indiscriminately by other authors.

We studied the effects of whole juice alkaline values (8.0, 8.5, 9.0) in comparison to those of natural pH (6.0) and of two different chloroplast-free juice pH values (3.5 and 4.0) on white concentrate recuperation and its protein content.

Freezing alfalfa juice for storage causes coagulation of the so-called "freezing curd", which is constitutes 50% of the dry matter and 60% of the nitrogen present in the juice. The formation of freezing curd has been proven to depend solely on the primary freezing rate, regardless of the length of time the juice remains frozen in storage (Hernández et al., 1988). So, in addition, we also examine the effect of freezing on the protein content and amount of white concentrate recuperated.

EXPERIMENTAL SECTION

Preparation of the chloroplast-free juice containing the soluble white proteins has been previously described in detail (Hernández et al., 1988). Briefly, alfalfa was harvested and then simultaneously pulped and pressed with IBP equipment. The juice was distributed into small containers and frozen at −25 °C until use. Each sample, when required, was thawed at room temperature for 18 h before use. The frozen storage time was recorded for each sample. After thawing, the freezing curd that formed was separated from thawed juice by filtering and sieving, and the juice pH was adjusted to the chosen value by means of a 2 N sodium hydroxide solution. Four different pH values were tested in assays (6.0, 8.0, 8.5, 9.0). Afterward, the green proteins were coagulated at 60 °C for 1 min by means of a thermostatically controlled glass column and then separated from the resulting chloroplast-free juice by centrifugation at 10 °C and 4396*g* for 20 min. In the present work, the chloroplast-free juice was stored at 4 °C and used within 24 h.

All of the experiments reported here were performed in the following manner. A weighed sample of chloroplast-free juice was adjusted to the desired pH (3.5 or 4.0) by the addition of 2 N HCl. This pH was maintained during the 60-min equilibration period when the sample temperature never rose above 10 °C. The sample was centrifuged (4396*g* for 20 min at 10 °C), and the supernatant was decanted and saved. Distilled water, adjusted to the same precipitation pH, was added to the precipitate in the same centrifuge tubes at the curd/water ratio of 1/20. The sample was centrifuged again in the same manner, and the washing water was decanted and rejected (Figure 1). The whole amount of white curd and representative portions of chloroplast-free juice and supernatant (whey) were freeze-dried and analyzed for total nitrogen following the semimicro-Kjeldahl method using a factor of 6.25 for protein conversion.

Table I. Recovery of Dry Matter[a] and Nitrogen[b] in the White Protein Concentrate Obtained from the Chloroplast-Free Juice by Coagulation at pH 3.5

A. Data Table

pH, whole juice	rec dry matter, %	av	rec N, %	av	% N (dry wt)	av
6.0	2.70		6.68		10.31	
	2.36	2.89	6.13	6.98	9.98	9.93
	3.61		8.12		9.51	
8.0	2.86		6.34		9.49	
	3.73	3.25	7.08	6.68	8.45	9.23
	3.16		6.61		9.76	
8.5	4.66		11.54		9.62	
	4.72	4.43	9.92	9.86	9.08	9.32
	3.92		8.12		9.27	
9.0	3.75		8.76		9.71	
	5.23	4.09	11.17	8.85	9.10	9.45
	3.30		6.61		9.53	

B. ANOVA Table of the Recovery Dry Matter

source of variation	DF[c]	SS[c]	MS[c]	*F*
freezing storage time	2	0.40	0.20	0.35
whole juice pH	3	4.17	1.39	2.44
error	6	3.43	0.57	
total	11	8.00		

$F_{2/6}(0.5\%) = 14.54$; $F_{3/6}(0.5\%) = 12.92$

[a] Expressed as a percent of total dry matter in whole juice. [b] Expressed as a percent of total nitrogen in whole juice. [c] Key: DF = degrees of freedom; SS = sum of squares; MS = mean square.

Color of the white concentrates was measured with a Hunter color difference meter, which determines color in terms of the *a*, *b*, and *L* parameters (greenness, yellowness, and lightness, respectively), where 0 = black and 100 = white (Caux, 1980; Pomeranz and Meloan, 1982). The meter was calibrated with a standard white (C2-19913) whose values were $a = -0.77$, $b = -0.88$, and $L = 91.91$. Four reading were made on each sample.

RESULTS AND DISCUSSION

To eliminate the influences of the different juices, because each juice had specific dry matter and nitrogen percentages, the results are expressed in terms of dry matter and nitrogen recuperated in white protein concentrate in relation to their initial quantity in the juice. The results of all the fractioned samples at each whole juice pH value are shown for the chloroplast-free juice pH values 3.5 and 4.0 in Tables I and II, respectively. The samples are arranged in order of increasing freezing storage time for each whole juice pH.

A variance statistic analysis was carried out with a classification with two parameters without replication to check on whether the dry matter recuperated as white concentrate was affected significantly by freezing storage time and/or whole juice pH (Pollard, 1979; Stoodley et al., 1980). From the results of the analyses (Tables I and II) we deduced that the storage time had no significant influence on the amount of dry matter recuperated. However, we demonstrated that green concentrate recovery decreased with an increase in storage time (Hernández et al., 1988). This difference in behavior between white and green proteins may be due to the less hydrophobic nature of the white proteins in comparison to the green proteins and the fact that the former were not obtained by heat treatment but by acid treatment.

Since the freezing storage time has no influence on concentrate recovery, a data reclassification was performed with replication and considering two parameters: whole

Table II. Recovery of Dry Matter[a] and Nitrogen[b] in the White Protein Concentrate Obtained from the Chloroplast-Free Juice by Coagulation at pH 4.0

A. Data Table

pH, whole juice	rec dry matter, %	av	rec N, %	av	% N (dry wt)	av
6.0	1.91		4.71		10.93	
	1.90	1.95	4.28	4.55	10.10	10.52
	2.03		4.65		10.52	
8.0	2.36		5.61		10.22	
	2.54	2.39	5.25	5.29	9.48	10.05
	2.28		5.01		10.45	
8.5	2.97		7.74		10.79	
	3.06	2.78	6.92	6.65	10.18	10.52
	2.32		5.30		10.58	
9.0	2.89		7.11		10.33	
	2.89	2.69	6.80	6.37	10.49	10.48
	2.29		5.21		10.63	

B. ANOVA Table of the Recovery Dry Matter

source of variation	DF[c]	SS[c]	MS[c]	F
freezing storage time	2	0.52	0.26	10.40
whole juice pH	3	1.44	0.48	19.20
error	6	0.15	0.025	
total	11	2.11		

$F_{2/6}(0.5\%) = 14.54$; $F_{3/6}(0.5\%) = 12.92$

[a] Expressed as a percent of total dry matter in whole juice. [b] Expressed as a percent of total nitrogen in whole juice. [c] Key: DF = degrees of freedom; SS = sum of squares; MS = mean square.

Table III. Analysis of Variance of Recovery Dry Matter in White Protein Concentrate

A. Data Table

chloroplast-free juice pH	whole juice pH 6.0	8.0	8.5	9.0
3.5	2.70	2.86	4.66	3.75
	2.36	3.73	4.72	5.23
	3.61	3.16	3.92	3.30
4.0	1.91	2.36	2.97	2.89
	1.90	2.54	3.06	2.89
	2.03	2.28	2.32	2.29

B. ANOVA Table

source of variation	DF[a]	SS[a]	MS[a]	F
chloroplast-free juice pH	1	8.91	8.91	31.82
whole juice pH	3	5.25	1.75	6.25
interaction, two pH	3	0.36	0.12	0.43
cells	7	14.52		
error	16	4.50	0.28	
total	23	19.02		

$F_{1/16}(0.1\%) = 16.12$; $F_{3/16}(1\%) = 5.29$

[a] Key: DF = degrees of freedom; SS = sum of squares; MS = mean square.

juice and chloroplast-free juice pH. From the results of the analysis (Table III) it can be deduced that the influence of the chloroplast-free juice pH on white concentrate recovery is highly significant at the 0.1% level, whereas whole juice pH is significant at the 1.0% level. Also, it is possible to deduce that there is no interaction between the two parameters since the influence of each pH manifests itself independently of the other.

When the mean recuperation values are considered (Tables I and II), it can be seen that more dry matter and nitrogen are recuperated in the white concentrate at alkaline pH levels than at the natural pH, 6.0; maximum recuperation, unaffected by the pH of chloroplast-free juice, is at pH 8.5. This observation confirms those of Betschart and Kinsella (1973) and Hood and Brunner (1975) with regard to the greater solubility of soluble proteins and the decrease in proteolytic degradations in juice at alkaline pH levels. We find pH 8.5 to be the optimum, and it is the same pH recommended by other investigators using Superfloc for green protein separation followed by acid coagulation of the soluble proteins (Bray and Humphries, 1979; Fiorentini and Galoppini, 1980; Fiorentini et al., 1980). The lower yield obtained from juice at pH 9.0 in comparison to pH 8.5 may be due to undesirable protein reactions occurring in very alkaline media and thwart posterior protein recuperation (Nashef et al., 1977; Finot, 1983; Whitaker and Feeney, 1983; Friedman et al., 1984).

In Tables I and II, chloroplast-free juice pH, independent of whole juice pH, is seen to influence dry matter and nitrogen recuperation. Between 1.3 and 1.6 times more material is recuperated in white protein concentrate at pH 3.5 than at pH 4.0, coinciding with Miller et al.'s (1975) results that indicate that an impurity low in nitrogen and insoluble in acid medium begins to precipitate at pH 3.5. Nitrogen percentages in the concentrate are seen to increase by 6, 9, 13, and 11% at the respective juice pH's of 6.0, 8.0, 8.5, and 9.0, when the soluble proteins are coagulated at pH 3.5 instead of 4.0. Miller et al. (1975) found an increase of 7% with juice at natural pH.

Table IV shows data on the white protein concentrate yielded by fresh juices from bibliographic sources as well as the mean values obtained at pH 6.0 and 8.5 for whole juice and then at pH 4.0 for chloroplast-free juice in this paper. Note that, for juices that have been freezing, the recuperation of dry matter (1.95%, 2.78%) and of nitrogen (4.55%, 6.65%) decreases by 67–71% at pH 6.0 and 51–53% at pH 8.5, in comparison with the yield from fresh juice. This fact indicates that as a result of freezing approximately 50–70% of the total soluble proteins in foliar juice coagulate while the rest remain in solution when the juice is thawed. Since soluble chloroplastic proteins represent between 50 and 65% of the total soluble foliar proteins (Costes, 1981), depending on plant species, we can confirm the hypothesis that most of the chloroplasts separate into the so-called "freezing curd" as a result of freezing (Hernandez et al., 1988).

The color parameters of the white concentrates are shown in Table V. The maximum L values correspond to concentrates obtained with whole juice at pH 6.0, and L values were similar in concentrates obtained at alkaline pH values. If we consider the low a parameter values, we can conclude that the concentrates do not contain chlo-

Table IV. Comparison between Bibliographical Data for Fresh Juice and Data Obtained for Freezing Juice

treatment		rec white conc, %		
green conc	white conc	dry matter	N	reference
heat (pH 6.0)	heat	7.38	22.71	Edwards et al. (1975)
centrifugation	heat	7.96	28.80	Gastineau and Mathan (1982)
centrifugation	acid (pH 4.0)	6.00	14.66	Felicioli (1977)
superfloc (pH 8.5)	acid (pH 4.0)	5.80	12.77	Fiorentini and Galoppini (1980)
heat (pH 6.0)	acid (pH 4.0)	1.95	4.55	Table II
heat (pH 8.5)	acid (pH 4.0)	2.78	6.65	Table II

Table V. Color Parameters *L*, *a*, and *b* of the White Protein Concentrates Obtained at Different pH Values

whole juice pH	chloroplast-free juice pH					
	3.5			4.0		
	L	*a*	*b*	*L*	*a*	*b*
6.0	68.85	0.37	18.17	66.33	−0.04	18.13
8.0	59.74	0.55	16.51	59.34	0.31	15.81
8.5	59.36	0.87	16.73	62.29	−0.25	17.06
9.0	59.22	−0.32	17.93	58.18	0.22	16.63

rophyll. However, *b* parameter values are elevated in all concentrates, indicating that they contain carotenoids. There are no visible color differences between concentrates, and all are yellowish white.

With regard to solubility, significant differences in nitrogen solubility between white protein concentrates obtained at different pH only arose at solubility pH 2.0 (Hernández et al., 1987).

CONCLUSIONS

1. When the object of the plant fractionating process is white concentrate production, freezing is not an advisable storage method because many soluble proteins are retained in the resulting freezing curd and lost.

2. More dry matter and nitrogen were recuperated in white concentrate from whole juice at alkaline pH levels than at the natural pH, 6.0. The maximum recuperation was obtained with pH 8.5.

3. Regardless of whole juice pH, between 1.4 and 1.6 times more dry matter and nitrogen are recuperated in white protein concentrates coagulated from chloroplast-free juice at pH 3.5 than are recuperated at pH 4.0. Nevertheless, when the soluble proteins are coagulated at pH 4.0, protein content increases in the resulting concentrate. This increase varies at different whole juice pH levels and is greatest at pH 8.5.

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