Nutritive Quality of Leaf Proteins Coagulated at Different pH

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Summary Leaf proteins obtained by coagulation at different pH were examined for their chemical composition and nutritional quality. Green juice was extracted from alfalfa, red clover, Italian ryegrass and oats and leaf protein was coagulated by heating the juice after adjusting the pH to 4 or 8–8.5, or without any adjustment of the pH (about pH 6). The mild alkaline juice from Italian ryegrass and oats did not cause the satisfactory coagulation but it was achieved with the addition of Ca salt to the juice before heating.

There were no important differences in the amino acid compositions of the leaf proteins coagulated at different pH. Crude ash, Ca and Mg contents increased with an increase in pH of coagulation and the protein coagulated at pH 8 had remarkably high contents of crude ash, Ca, Mg, Na and P in each crop. The leaf protein coagulated at pH 6, on the contrary, had a high content of true protein and low contents of nitrogen free extracts and nucleic acid as compared with those at pH 4 and 8.

The pH of coagulation of leaf protein from alfalfa and red clover had no effect on the nutritional quality of the respective proteins. In Italian ryegrass and oats, however, the leaf protein coagulated at pH 8 was found to be nutritionally inferior to those coagulated at a lower pH.

The data presented in this work support that in general, the leaf protein produced by heating the green juice without any adjustment of pH may be suitable for protein resources because of its desirable properties, *i.e.* high true protein content and good nutritional quality.

Key Words leaf protein, protein digestibility, protein efficiency ratio, thermal coagulation, Ca, pH

Significant developments have been made in the research on the manufacturing process to obtain and edible protein from the green juice separated by the fractionation of green crops, but a heat treatment of green juice is one of the most

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common methods. Subba Rau and Singh (1) showed that alfalfa leaf protein coagulated at pH 4–4.5 was nutritionally inferior to that coagulated by heating at 5.9–6.2. Livingston *et al.* (2) reported that the thermal coagulation of protein at pH 6 gave as much as a four fold increase in saponin over that in the protein coagulated at pH 8. It was further demonstrated by Hegsted and Linkswiler (3) that alfalfa leaf protein containing low saponin had a higher nutritive quality than that containing high saponin. In addition, Enochian *et al.* (4) has stated that the adjustment of green juice to pH 8–8.5 reduced proteolytic activity, stabilizes carotene and xanthophyll during processing and increases the firmness of coagulated curd. These results suggest that the thermal coagulation of leaf protein under mild alkaline conditions has several advantages over the coagulation of protein at pH 4–4.5. However, these results and observations were based on the experiments conducted with the use of alfalfa as a green crop. The purpose of this study was to investigate the effects of pH coagulation on the nutritional quality of leaf protein with the use of several crops. Protein quality was evaluated by chemical analyses and biological tests.

EXPERIMENTAL

Sample preparation. Alfalfa, red clover, Italian ryegrass and oats were chosen for this study. Each crop was harvested before flowering or before earing. Freshly harvested crops were pulped with approximately equal amounts of water in weight and the green juice was expressed by a hand-press through muslin. The green juice was divided into three portions and three leaf-protein samples were prepared from each crop as follows:

1. pH 4-coagulated protein: prepared by heating the juice at 85°C after adjusting the pH of the juice to 4–4.5 with 10% HCl
2. pH 6-coagulated protein: prepared by heating at 85°C without any adjustment of pH of the green juice.
3. pH 8-coagulated protein: prepared by heating the juice at 85°C after adjusting the pH of the juice to 8–8.5 with 10% NaOH.

The green juice from Italian ryegrass and oats did not coagulate satisfactorily on heating at pH 8–8.5, but the coagulation of the alkaline juice was achieved with the addition of CaCl_2 followed by heating. Thus, when the pH 8-coagulated protein was prepared from Italian ryegrass and oats, CaCl_2 (Ca, 2.1–2.7 g) was added in the green juice (1 liter) and then the pH of the juice was readjusted to 8–8.5 before heating. Leaf protein coagulum was separated from the juice and most of the coagulum was washed with acetone repeatedly and the remainder being freeze-dried.

Chemical analysis. Nitrogen, crude fat, crude fiber, crude ash and moisture were determined by the official methods (5). Crude protein was estimated as the amount of N $\times$ 6.25. Atomic absorption spectrophotometry (Nippon Jarrell-ash, AA-80) was used for determining Ca, Mg, K and Na. Each leaf protein was dry ashed at 500°C for determining these minerals (6). For total phosphorus, samples

were wet digested with a mixture of nitric and sulfuric acids until the mixture became colorless. The modified Allen's method (7) was used for P determination. Nucleic acid in the leaf protein was determined using the following procedure. Acetone-washed leaf protein was extracted successively, by ethyl ether, cold 5% perchloric acid and hot (70°C) 5% perchloric acid. P determined from the extract with hot perchloric acid was multiplied by a factor of 11 and this value was taken as the nucleic acid, including RNA and DNA (8).

The amino acid composition of leaf proteins was determined by ion exchange chromatography with an automated amino acid analyzer (Nihondenshi, JLC-6AH). The hydrolysis of leaf protein was carried out as described in the previous paper (9). Cyst(e)ine and tryptophan were not determined.

Biological test. Male rats of the Wistar strain weighing approximately 45 g were used for the assessment of acetone-washed leaf proteins. The protein quality of leaf proteins was estimated by the protein efficiency ratio (PER) and apparent protein digestibility. The basal protein-free diet was composed of 73.8% corn starch, 10.0% cane sugar, 10.0% corn oil, 4.0% mineral mix (10), 1.0% vitamin mix (9), 1.0% cellulose powder and 0.2% L-methionine. Each leaf protein was added to the basal diet in place of an equal weight of corn starch to provide about 8% protein. Each diet group included five rats and diets were fed *ad libitum* for a 14-day period. The apparent digestibility was determined by analyzing the nitrogen intake and fecal nitrogen during the last three days of the period.

RESULTS AND DISCUSSION

Leaf protein preparation

Ca and Mg contents in the green juice from each crop are shown in Table 1. There were larger amounts of Ca and Mg in the green juice from legume crops (alfalfa and red clover) than in the original green juice from grasses (Italian ryegrass and oats). In particular, the Ca content in the green juice from legume crops is more than double that in the juice from grasses. Moreover, it was observed that satisfactory coagulation was not caused by heat treatment in the original green juice adjusted to pH 8–8.5 from both grasses while the satisfactory coagulation of green juice from legume crops was achieved under the same conditions. The effect of addition of CaCl_2 to the pH adjusted (8.0–8.5) juice on the leaf protein coagulation was tested and the results are shown in Table 1. The addition of Ca caused the coagulation of the green juice from the grasses, but the green juices from the grass and legume crops differed widely in the Ca requirement for effective coagulation. The satisfactory coagulation was caused at a high Ca concentration (261 mg/100 g juice) in the juice from Italian ryegrass while at a low Ca concentration (88 mg/100 g juice) in that from red clover. Accordingly, it may be conceivable that there are some other factors which affect the firmness of the leaf protein curd, such as the amount of conjugated lipids of leaf proteins, though the Ca concentration appears to be main factor which causes the formation of coagulum on heat treatment under

Table 1. Effect of Ca concentration (mg/100 g juice) on the coagulation of green juice at pH 8–8.5.

Green juice	Estimated value		Amount of added Ca	Total amount of Ca	Coagulum ^a
	Ca	Mg			
Alfalfa	142	24	—	142	++
Red clover	88	24	—	88	++
Italian ryegrass	41	11	—	41	—
"	41	11	55	96	—
"	41	11	110	151	(+)
"	41	11	165	206	+
"	41	11	220	261	++
Oats	31	14	—	31	—
"	31	14	55	86	+
"	31	14	110	141	++

^a Green juice was tested for the formation of coagulum at pH 8–8.5 by heating to 85°C for 10 min. —, coagulation was not observed, or a finely dispersed coagulum which was difficult to separate by filtration; (+), partial coagulation; +, soft coagulum; ++, firm coagulum.

the mild alkaline condition.

Chemical composition

The chemical and mineral composition of freeze-dried leaf protein is shown in Table 2. Alfalfa leaf protein coagulated at pH 4 contained a lesser amount of crude protein than the other two proteins. On the contrary, the pH 8-coagulated proteins from red clover, Italian ryegrass and oats gave the lowest value of crude protein, largely due to the co-precipitation of carbohydrates (N-free extract, NFE) and minerals. This tendency was marked for the grass crops, Italian ryegrass and oats, and the greatest differences in crude protein and crude ash were observed between pH 8 and the other pH levels for both grasses. The eighth column in Table 2 indicates that the Ca contents in the pH 8-coagulated proteins from grass crops were significantly higher than those in the pH 8-coagulated proteins from legume crops. Therefore, the remarkably high content of crude ash in the pH 8-coagulated proteins from the grasses was attributed to the co-precipitation of the added Ca salt in the green juice.

As can be seen from Table 2, in each crop, the pH 6-coagulated protein contained a higher amount of crude fat and a lower amount of NFE than the leaf proteins coagulated at pH 4 and 8, excluding NFE in alfalfa leaf protein. The amount of NFE in alfalfa leaf proteins coagulated at pH 6 and 8 was very low as compared with that of the pH 4-coagulated protein. Alfalfa may be different from the other crops in the type of NFE compounds of leaf protein.

It will be also noted from Table 2 that Ca, Mg and P contents increased with the

Table 2. Chemical and mineral composition of freeze-dried leaf proteins.

Leaf protein	pH of coagulation	(% on dry-weight basis)				(mg/100 g dry matter)				
		Crude protein	Ether extract	N-free extract	Crude fiber	Crude ash	Ca	Mg	Na	K
Alfalfa	4	55.04	10.31	30.91	0.39	3.35	540	100	30	1,060
	6	63.06	12.27	18.70	0.57	5.40	1,200	140	30	1,150
	8	62.65	9.51	18.84	0.40	8.60	2,360	250	180 ^b	1,200
Red clover	4	56.02	13.31	27.60	0.32	2.75	270	60	50	850
	6	57.19	16.00	22.84	0.13	3.84	600	110	60	1,020
	8	53.57	11.23	26.84	0.13	8.23	1,470	250	340 ^b	1,630
Italian ryegrass	4	54.14	17.06	24.88	0.26	3.66	260	30	70	590
	6	60.08	17.06	18.65	0.18	4.03	510	130	50	1,160
	8	40.46	12.22	27.44	0.18	19.70 ^a	6,600 ^b	200	230 ^b	1,160
Oats	4	53.45	20.10	23.55	0.22	2.68	130	30	180	620
	6	53.57	21.27	22.09	0.25	2.82	260	50	140	740
	8	46.54	13.66	26.01	0.24	13.55 ^a	4,330 ^b	200	260 ^b	730

^aCrude ash includes Ca salts originated from the added Ca in green juice. ^bA large portion of Ca and Na was originated from the added CaCl₂ and NaOH in the green juice.

Table 3. Chemical composition of acetone-washed leaf proteins (% on dry-weight basis).

Leaf protein	pH of coagulation	Crude protein	Ether extract	N-free extract	Crude fiber	Crude ash	Nucleic acid
Alfalfa	4	69.53	1.38	24.88	0.80	3.41	1.96
	6	76.01	0.99	15.81	0.65	6.54	1.51
	8	70.90	1.31	17.41	0.71	9.67	1.88
Red clover	4	65.16	2.04	29.88	0.37	2.55	1.92
	6	73.07	1.05	21.27	0.45	4.16	1.51
	8	67.99	1.22	21.76	0.44	8.68	1.70
Italian ryegrass	4	68.53	1.68	24.28	1.36	4.15	3.31
	6	70.73	1.11	20.94	2.22	5.00	2.21
	8	48.26	1.83	28.25	0.21	21.45 ^a	2.46
Oats	4	70.10	3.96	22.18	0.28	3.48	4.93
	6	69.85	3.98	21.87	0.32	3.98	2.07
	8	50.18	2.94	31.14	0.38	15.36 ^a	2.33

^a Crude ash includes Ca salt originated from the added Ca in green juice.

increasing pH of coagulation, excluding the P content of oats. Such a tendency was not observed in K and Na contents, but the highest content of Na was found in the pH 8-coagulated proteins. It is apparent that the mineral content of leaf proteins depends on the ion binding capacity of the proteins and on the amount of salts which are co-precipitated with the protein from the green juice as a result of the effect of pH on the solubility of salts.

The chemical composition of acetone-washed leaf proteins is presented in Table 3. It is observed that crude protein, NFE and crude ash contents in the acetone-washed leaf proteins varied with the pH of coagulation in a similar manner as those in the freeze-dried leaf proteins. It will be observed, however, by comparing figures in Table 2 with the corresponding figures in Table 3 that washing of the wet protein curd with acetone caused a great increase in the crude protein content of leaf protein preparation, while the same treatment caused only a small variation in NFE and crude ash contents. It can therefore be presumed that the acetone-washing treatment not only removed most of the crude fat but also some of the NFE and crude ash. Table 3 also shows that in each crop, the pH 4-coagulated protein contained a higher amount of nucleic acid while the pH 6-coagulated protein contained a lower amount.

Amino acid composition

The amino acid contents of various acetone-washed leaf proteins are given in

Table 4. Amino acid composition of leaf proteins coagulated at different pH.
(g amino acid per 100 g recovered amino acids)

Amino acid	Alfalfa leaf protein			Red clover leaf protein			Italian ryegrass leaf protein			Oats leaf protein		
	pH 4	pH 6	pH 8	pH 4	pH 6	pH 8	pH 4	pH 6	pH 8	pH 4	pH 6	pH 8
Lys	6.53	6.24	6.79	6.36	6.58	6.48	6.38	6.79	6.88	6.39	6.14	6.78
His	2.52	2.57	2.51	2.44	2.58	2.55	2.43	2.31	2.39	2.52	2.56	2.71
Arg	6.39	6.55	6.66	6.41	6.59	6.40	6.67	6.47	6.44	6.33	6.41	6.49
Asp	10.31	10.08	10.42	10.42	10.49	10.69	9.83	9.36	9.55	9.65	9.43	9.45
Thr	5.29	5.28	5.42	5.45	5.28	5.38	5.11	4.97	4.95	5.18	5.56	4.99
Ser	4.63	4.42	4.56	4.84	4.70	4.83	4.73	4.65	4.71	4.61	4.49	4.44
Glu	11.11	11.06	10.99	11.99	11.63	11.94	11.59	11.02	11.39	11.59	11.20	11.62
Pro	5.59	6.01	5.88	4.25	4.67	4.71	4.78	4.88	5.03	4.51	4.66	4.66
Gly	5.43	5.39	5.42	5.68	5.52	5.49	6.08	5.95	6.00	6.11	5.89	5.97
Ala	6.49	6.12	6.25	6.15	6.13	6.31	6.91	6.94	6.93	6.72	6.75	7.00
Val	6.78	6.77	6.74	7.20	7.20	7.02	6.91	6.84	6.75	6.93	6.96	6.71
Met	1.94	2.07	2.04	1.77	1.75	1.86	2.15	2.29	2.20	1.94	2.15	2.09
Ile	6.07	6.15	5.65	5.93	5.98	5.79	5.77	6.03	5.92	6.11	6.17	6.21
Leu	9.83	9.97	9.78	9.81	9.73	9.54	9.56	10.10	9.80	9.75	9.90	9.73
Tyr	4.68	4.70	4.57	4.89	4.80	4.78	4.52	4.64	4.59	4.72	4.73	4.52
Phe	6.41	6.62	6.32	6.41	6.37	6.23	6.58	6.76	6.47	6.94	7.00	6.63
Yield of N%	84.96	87.00	84.09	77.50	81.10	79.58	79.59	89.42	87.93	83.72	91.60	88.88
True protein (%) ^a	59.07	66.13	59.62	50.50	59.26	54.11	54.54	63.26	42.44	58.69	63.98	44.60

^a Crude protein content (%) in dry matter $\times$ yield of nitrogen per cent.

Table 4. No major or significant differences were found among the leaf proteins coagulated at different pH. Furthermore, as pointed out by many workers (11, 12), there was a similarity in the amino acid composition of the various leaf proteins obtained from different crops.

The second row from the bottom in Table 4 shows the yield of nitrogen per cent which was obtained by the summation of amino acid nitrogen as % of total nitrogen in each leaf protein and is a useful check for the contamination of non-protein nitrogen compounds in a protein material. The yield for the pH 4-coagulated protein was lower than those for the proteins coagulated at pH 6 and 8, excluding alfalfa. Such lower yields for the pH 4-coagulated protein may be associated with a larger amount of nucleic acid and other non-protein nitrogen compounds in them. The value calculated by multiplying the crude protein content by the yield of nitrogen per cent was tentatively taken as the true protein value and the values are presented on the last row of Table 4. It is noted that the pH 6-coagulated protein

Table 5. Diet intake, weight gain, digestibility and PER of male rats fed on a diet containing leaf protein coagulated at different pH.

Leaf protein	pH of coagulation	Diet intake for 14d. (g)	Weight gain for 14d. (g)	Digestibility of protein (%)	PER
Alfalfa	4	115.3±0.7	39.3±2.0	80.2±1.2	3.83±0.19
	6	115.8±0.3	39.5±1.1	79.7±1.5	3.83±0.11
	8	115.7±0.5	38.9±0.6	81.2±1.4	3.78±0.07
Red clover	4	115.2±0.6	33.0±1.2	74.2±2.0	3.46±0.09
	6	115.4±0.6	34.1±2.5	76.3±1.5	3.56±0.23
	8	115.8±0.2	34.4±1.2	78.1±0.6	3.58±0.11
Italian ryegrass	4	115.5±0.4	40.0±1.2	78.3±1.6	3.92±0.12
	6	115.5±0.4	38.0±1.4	77.3±1.6	3.72±0.13*
	4	115.7±0.2	33.2±1.7	76.1±0.7	3.50±0.18
	8	115.5±0.5	28.7±2.4*	71.3±1.7*	3.00±0.25*
Oats	4	115.4±0.5	34.3±0.8	80.8±2.1	3.76±0.09
	6	115.2±0.8	34.6±1.8	79.7±1.6	3.62±0.18
	8	115.6±0.2	33.3±0.5	71.8±3.4*	3.60±0.06

PER, g weight gain/g protein intake. Values are the means±SD. * Significantly different from the value of the corresponding leaf protein coagulated at lower pH ($p < 0.05$).

had the highest value of true protein in each crop.

Biological test

The growth data, apparent digestibilities and PER values obtained with the acetone-washed leaf proteins are shown in Table 5. In the case of legume crops, alfalfa and red clover, there were no differences in the growth performance of rats fed on the leaf proteins coagulated at different pH from each crop. Livingston *et al.* (2) pointed out that the saponin content of alfalfa leaf protein prepared at an alkaline pH was lesser than that of leaf proteins prepared at a lower pH. The alfalfa leaf proteins used for our experiment were prepared by washing the wet leaf protein curd with acetone and accordingly, it is probable that the pH 4-coagulated protein contained a small amount of saponin as the pH 8-coagulated protein. In other words, it can be concluded that the reverse effect of saponin on the nutritional quality of the leaf protein washed with acetone was negligible. Therefore, the nutritional quality of acetone-washed leaf proteins made from the legume crops was not influenced by the pH of coagulation.

In Italian ryegrass, the PER value of the pH 6-coagulated protein was significantly lower than that of the pH 4-coagulated protein but the difference

Table 6. Diet intake, amount of feces and crude ash content in feces in male rats fed on a diet containing leaf protein coagulated at different pH.

Leaf protein	pH of coagulation	Diet intake for 3d. (g)	Feces for 3d. (g)	Crude ash in feces (%)
Red clover	4	25.8 ± 0.2	1.56 ± 0.12	6.26 ± 0.20
	6	26.0 ± 0.1	1.34 ± 0.08	6.43 ± 0.81
	8	25.9 ± 0.1	1.42 ± 0.04	14.24 ± 0.94*
Italian ryegrass	4	25.9 ± 0.1	1.49 ± 0.05	7.21 ± 0.23
	8	25.8 ± 0.3	2.81 ± 0.14*	33.82 ± 1.07*
Oats	4	29.8 ± 0.3	1.29 ± 0.10	7.27 ± 1.01
	6	29.4 ± 0.8	1.37 ± 0.06	8.03 ± 0.22
	8	29.7 ± 0.2	2.22 ± 0.22*	27.49 ± 0.84*

Values are the means ± SD. * Significantly different from the value of the corresponding leaf protein coagulated at lower pH ($p < 0.01$).

between the values of the two proteins was very small. On the other hand, significant differences in body weight gain, apparent digestibility and PER value between the leaf proteins coagulated at pH 4 and 8 were observed. The pH 8-coagulated protein made from oats was significantly different in apparent digestibility from the proteins coagulated at pH 4 and 6. It seems reasonable to presume that the depression of the nutritional quality of the pH 8-coagulated proteins was due to the remarkable high content of crude ash in it. Table 6 shows the quantity of feces voided and crude ash content in feces of rats fed on various leaf proteins made from red clover, Italian ryegrass and oats. It is indicated that the quantity and ash content of feces of rats fed on the pH 8-coagulated proteins made from Italian ryegrass and oats were remarkably larger than those of rats fed other leaf proteins, while the feeding of the pH 8-coagulated protein from red clover caused only a higher ash content in feces. These results suggest that the addition of a large amount of Ca salt into the green juice to induce the coagulation of leaf protein under the mild alkaline condition caused the depression of nutritional quality of the coagulated protein.

The data presented in this work support that in general, the leaf protein produced by heating the green juice without any adjustment of pH may be suitable for protein resources because of a high true protein content and good nutritional quality.

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