Nitrogenous Constituents in Annual Ryegrass

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Annual ryegrass (AR) with 27.1% protein ($N \times 6.25$) was buffer extracted at pH 8.05. Two insoluble fractions, R_I and R_{II}, and three major soluble fractions were obtained. Fractions R_I and R_{II} were reextracted at pH 8.05 with buffer containing 1% detergent, and each gave two insoluble and four soluble fractions. By this method, 85% of total N (N_T) was extracted, and 67% of N_T was solubilized. AR gave a white, salt-soluble protein fraction. It had an average molecular weight (M_{A_1}) as determined by gel chromatography of 28 700, and N_T was 7.3%. R_I gave a green protein fraction, M_{A_1} was 16 900, and N_T was 17.1%. R_{II} gave a green protein fraction with an M_{A_1} of 18 100, and N_T was 22.0%. Kinetic studies on the salt-soluble protein fractions indicated that those from AR and R_I were degraded whereas the protein from R_{II} underwent aggregation. Amino acid compositions were obtained for the fractions from the buffer extraction and compared with the amino composition of the unfractionated grass.

Forage is the largest and most ubiquitous protein source in the world, but it cannot be consumed directly by humans and other monogastric animals because of its high fiber content (Kohler et al., 1978). The extraction of protein from forage is well justified because of anticipated worldwide protein shortages (Kinsella, 1970), its well-balanced amino acid composition (Gerloff et al., 1965), and its positive effects in animal feeding trials, both human and nonhuman (Woodham, 1971; Singh, 1971).

In spite of its potential usefulness, and more than 40 years of effort involving various technologies to process protein from leaves, leaf protein extracts have yet to gain wide acceptance as a food and feed (Bray and Humphries, 1978). Leaf protein is difficult to process because of the complexity of the leaf extract. Amino acids, the important nutrient, are found free and in the form of proteins, polypeptides, and various complexes between proteins and other compounds (e.g., protein–chlorophyll complexes). To complicate matters further, the highly compartmentalized plant cell contains many compounds which may drastically alter the structures of protein or protein-like compounds if allowed contact with them. For example, phenolics will react with proteins (Loomis and Battaile, 1966), and proteolytic enzymes will catalyze their breakdown (Peoples et al., 1979; Finley et al., 1980).

The complexities of plant extracts dictate that no one chemical or physical measurement will uniquely determine the processing characteristics of plant proteins individually or as a group. Recently, a laboratory method was developed to measure the potential of forage as a source of protein extracts and was applied to Coastal Bermuda grass (Fishman, 1976; Fishman and Burdick, 1977). To complement that study, we now report the application of the method applied to annual ryegrass (*Lolium multiflorum* Lam.). Ryegrass is a cool-season grass which fixes CO_2 via the phosphoglyceric acid, C-3, photosynthetic pathway, whereas Coastal Bermuda grass is a warm-season grass which fixes CO_2 via the dicarboxylic acid C-4 pathway; hence, these two grasses differ greatly anatomically (Chen et al., 1971). Ryegrass can be over-seeded on Coastal Bermuda grass to extend the growing season. The results herein can be compared with the results from our previous study on Coastal Bermuda grass (Fishman and Burdick, 1977).

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EXPERIMENTAL SECTION

Annual Ryegrass. Annual or Italian ryegrass (*Lolium multiflorum* Lam.) was greenhouse grown (Athens, GA) in a 100 × 70 × 20 cm galvanized metal flat with holes in the bottom for drainage. Fertilizer was applied every 2 weeks at the rate of 28 kg of N, 9.4 kg of P_2O_5, and 19.7 kg of K_2O per hectare (ha). The flat was watered to saturation every second day. The greenhouse temperature ranged from a low of 24 °C at night to a high of 32 °C in the day. The relative humidity in the greenhouse was 60%, and the plants were exposed to natural sunlight through glass with no supplemental lighting. Ryegrass seed was planted in mid-September 1977. One month's regrowth was harvested in mid-November. Within 2 h of cutting, the ryegrass was loosely packed in a 2-L freeze-drying jar and dried under vacuum at room temperature. The dried ryegrass was ground to pass a 420-μm screen and then ground to face powder consistency in a ball mill for 48 h. Ryegrass powder was stored in a desiccator at room temperature until time of extraction. Micro-Kjeldahl analysis (McKenzie and Wallace, 1954) revealed that the ryegrass contained 27.10 ± 0.68% crude protein on a dry weight basis.

Extraction and Fractionation of Forage. Ryegrass powder, 5 g, was extracted with 100 mL of 0.2 M borate–boric acid buffer, containing 5 mmol of sodium metabisulfite, at pH 8.05 (buffer A). Extraction was for 1 h in a beaker equipped with a magnetic stirrer. The ryegrass extracted was fractionated as outlined in Figure 1. Each of the lyophilized residues (i.e., 0.75 g of R_I and 0.60 g of R_{II}) from the fractionation was extracted forth with 50 mL of buffer A also containing 2% (w/v) of sodium dodecyl sulfate ($NaDodSO_4$), and the extracts were fractionated by the procedure outlined in Figure 1. The three kinds of starting materials (i.e., powdered ryegrass, R_I, and R_{II}) were extracted in triplicate. Fractionation of supernatants into four cuts was by preparative gel chromatography on a 2.5 × 85 cm column packed with Sephadex G-15, as described previously (Fishman and Burdick, 1977). In the case of the powdered ryegrass, a 50-mL aliquot of the supernatant was pumped onto the column, whereas in the case of the supernatants from R_I and R_{II} extractions, all the supernatant (usually 34–38 mL) was pumped onto the column. The effluent was monitored as it emerged from the column by its ultraviolet absorbance at 254 nm. Figure 2 contains typical chromatograms for the three kinds of starting materials. Fractionation of the effluent was timed such that cut 1 (void volume peak) contained all the un-

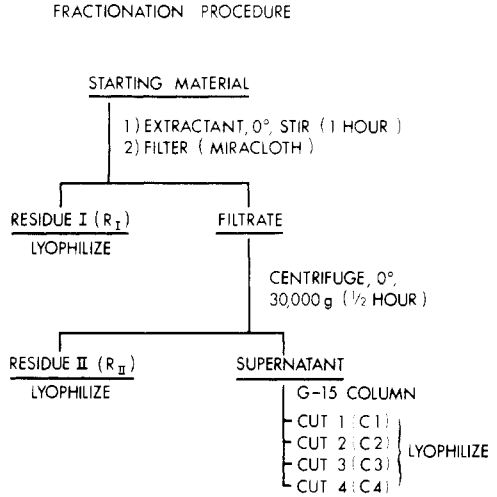

Figure 1. Fractionation scheme for the preparation of ryegrass fractions.

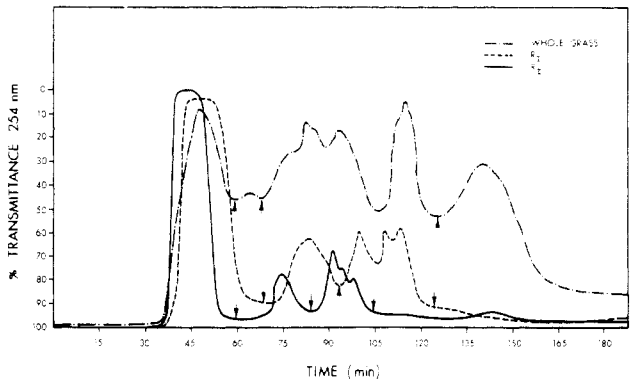

Figure 2. Chromatograms showing preparative separations on Sephadex G-15 of supernatants from whole ryegrass, residue I (R_I) and residue II (R_{II}).

resolved proteins in excess of 2000 daltons, cut 2-peak 2 contained the highest molecular weight polypeptides resolved on the column, cut 3 contained all the remaining polypeptides resolved by the column, and cut 4 contained amino acids which were retarded by adsorption as indicated by their emergence from the column after the bed volume. Crude protein was measured by micro-Kjeldahl analysis of each fraction.

Analytical Gel Chromatography. Cuts 1–3 were freeze-dried, dissolved in 0.8 N NaCl, and filtered through a 0.40-μm Nucleopore membrane filter. Analytical gel chromatography was performed on an automated gel filtration apparatus (Fishman and Burdick, 1977) with one modification. The peristaltic pump was replaced with a Model CMP-2V piston pump (LDC, Riviera Beach, FL). Separation was with four serially arranged gel columns of 6-mm inner diameter. Gels in order of packing were as follows: (1) 157 mm of Sephadex G-10 and G-25, (2) 195 mm of Sephadex G-50 and G-75 also mixed to give equal hydrated volumes, (3) 170 mm of Bio-Gel A 0.5 m and, (4) 153 mm of Bio-Gel A 5 m. Up to five 0.2-mL samples were injected automatically at intervals of 240 min. Protein concentrations ($N \times 6.25$) in the samples ranged from 1.0 to 5.0 mg/mL. Flow rates were between 0.096 and 0.11 mL/min. Absorbance of column effluent was measured with a Uvicord III, UV monitor (LKB, Silver Spring, MD) at 206 and 254 nm simultaneously. Data reduction and molecular weight calculations are described elsewhere (Fishman, 1976).

Amino Acid Analysis. Samples were hydrolyzed by 6 N HCl refluxing at 108 °C under nitrogen. The ratio of HCl to crude protein was 6 mL to 1 mg. Amino acids were analyzed by ion exchange on a Beckman Model 121 analyzer. Data were collected and computed as reported elsewhere (Fishman et al., 1973) but are reported as grams of amino acid per 16 g of recovered nitrogen from ion-exchange chromatography. The method was tested on serum albumin and gave 95% recovery of Kjeldahl nitrogen as amino acid nitrogen.

Table I. Distribution of 100 Grams of Crude Protein (N_T) from Annual Ryegrass and Fractions[a]

fraction	starting material: whole grass[b]	R_I[c]	R_{II}[c]
residue I (R_I)	30.6 ± 3.3	23.5 ± 7.8	25.1 ± 8.2
residue II (R_{II})	30.4 ± 2.7	3.4 ± 0.4	5.4 ± 1.6
cut 1	7.3 ± 0.5	56.1 ± 7.1	72.5 ± 3.3
cut 2	0.7 ± 0.3	4.5 ± 0.7	1.5 ± 2.2
cut 3	14.2 ± 0.9	5.4 ± 1.3	1.7 ± 0.4
cut 4	0.4 ± 0.1	2.4 ± 0.2	1.7 ± 0.4
% recovery	84.6 ± 2.1	95.2 ± 6.7	107.9 ± 4.4

[a] Average of triplicate analyses. [b] Extracted with buffer A. [c] Extracted with buffer B.

RESULTS AND DISCUSSION

Nitrogen Distribution among Fractions and Their Protein Content. Fractionation of the whole grass extract revealed that about 22.6% (i.e., cuts 1–4) of the nitrogen was readily soluble (Table I), whereas 29.5% was soluble in the case of Coastal Bermuda grass (Fishman and Burdick, 1977). Cut 1 (7.3%) consisted predominantly of cytoplasmic proteins, whereas the remainder (cuts 2–4) consisted predominantly of cytoplasmic polypeptides and amino acids. For Coastal, about 10.8% of the nitrogen was cut 1. About 30.6% of the starting nitrogen was insoluble and nonextractable by buffer (i.e., R_I), and 30.4% was insoluble but extractable (i.e., R_{II}) compared to 46.9 and 15.7%, respectively, of the same fractions in Coastal.

Extraction of green lyophilized R_I with buffer B revealed that about 68.4% of the nitrogen could be solubilized with buffer containing 2% w/v detergent (Table I). About 56.1% of the nitrogen in R_I was fractionated into cut 1. Unlike the cream color of cut 1 extracted from whole grass, the color of cut 1 from R_I was brownish green (green protein), indicating the presence of a significant amount of plant pigments which were not readily extracted from the plant matrix by buffer A. Moreover, cuts 2–4 from R_I were cream colored, possibly containing polypeptides from fragmented membrane proteins. The insoluble R_I and R_{II} fractions remaining after extraction with buffer B were no longer green but cream colored and probably contained large proportions of lignin, structural carbohydrates, and bound protein.

Extraction of green, lyophilized R_{II} with buffer B solubilized 77.4% of its nitrogen (Table I). Most of the nitrogen solubilized was due to protein or high molecular weight polypeptides, as indicated by the high N_T value of cut 1. Fractions extracted from R_{II} were very similar in appearance to comparable fractions extracted from R_I.

Table II shows the composite material balance of extracted nitrogen as calculated from the three kinds of starting materials. All but about 14.8% of the nitrogen (i.e., the N_T of R_I) was extractable, 67.1% of the nitrogen was soluble, and 46.5% of the nitrogen was in the form of proteins or high molecular weight polypeptides compared to values of 30.8, 58.6, and 38.0, respectively, for Coastal Bermuda grass. Thus, ryegrass has a somewhat lower percentage of readily soluble nitrogen than Coastal Bermuda grass but a significantly higher percentage of extractable, buffer-insoluble nitrogen. In terms of

Table II. Maximum Extractability and Solubility of 100 Grams of Crude Protein (N_T) from Ryegrass

fraction	% total nitrogen (N_T)
residue I (R_I)	14.8
residue II (R_{II})	2.7
cut 1	46.5
cut 2	2.5
cut 3	16.4
cut 4	1.7
% recovery	84.6
% soluble (cuts 1-4)	67.1
% insoluble (R_I and R_{II})	17.5

Table III. Percentage Crude Protein in Ryegrass Fractions[a]

fraction	starting material: whole grass	residue I (R_I)	residue II (R_{II})
residue I (R_I)	21.7 ± 0.5	5.2 ± 1.0	7.0 ± 2.1
residue II (R_{II})	25.6 ± 2.3	5.0 ± 0.9	3.3 ± 0.7
cut 1	47.2 ± 0.3	44.6 ± 1.4	49.9 ± 0.4
cut 2	9.2 ± 2.7	3.4 ± 0.8	1.3 ± 0.4
cut 3	12.6 ± 0.6	5.4 ± 0.3	2.2 ± 0.4
cut 4	11.8 ± 6.1	0.6 ± 0.1	0.3 ± 0.1
cuts 1-4	15.8 ± 0.6	9.6 ± 1.1	8.69 ± 0.7

[a] Average of triplicate analyses.

"chloroplastic" (green) proteins to white, the ratio was 5.4:1 for Annual ryegrass compared to 2.6:1 for Coastal Bermuda grass (Fishman, 1980; Fishman and Burdick, 1977).

The crude protein content ($N \times 6.25$) for each fraction of ryegrass is given in Table III. For buffer-extracted, unfractionated ryegrass, all fractions had crude protein values equal or greater than those of comparable fractions in Coastal. This last result is consistent with a higher overall crude protein content in ryegrass compared to Bermuda grass (i.e., 27.1% against 13.8% on a dry weight basis).

Molecular Weight Determination by Analytical Gel Chromatography. The gel chromatograms in Figure 3 show that cut 1 contains a broad distribution of proteins and polypeptides, whereas cuts 2 and 3 are relatively more narrow in distributions of polypeptides. The chromatograms in Figure 3 were obtained by detection at 206 nm, a wavelength chosen for its high sensitivity to the peptide linkage common to all proteins and polypeptides. Chromatograms, also, were measured at 254 nm, a wavelength sensitive to conjugated double bonds such as those present in phenolics and aromatic amino acid residues.

The first moment apparent molecular weight [M_{A_1} (Fishman, 1976)] for cut 1 from whole grass was determined to be about 13 times greater at 206 than at 254 nm (Table IV). In contrast, cut 1 from residues I and II had lower molecular weight averages when monitored at 206 than at 254 nm. Possibly, cut 1 extracted from whole grass contained low molecular weight soluble fragments from lignin or lignin precursors (i.e., highly aromatic structures) which would account for the much smaller molecular

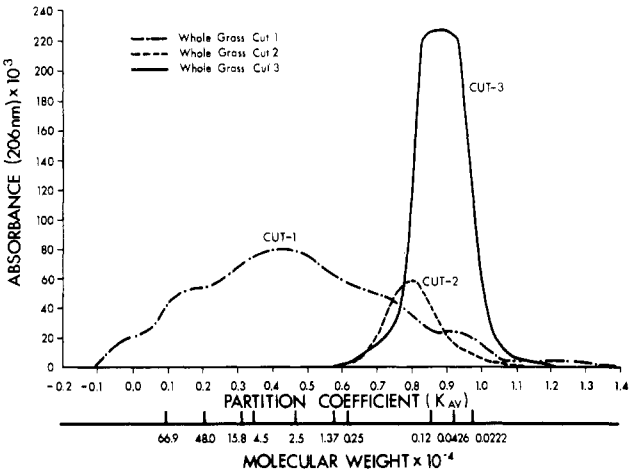

Figure 3. Analytical gel chromatograms of soluble fractions from whole ryegrass. Each cut was run separately on a four-column chromatograph. Extractant contains buffer A (see the text).

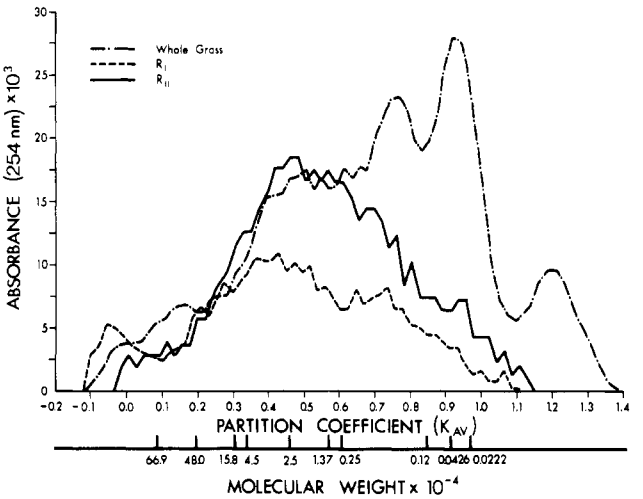

Figure 4. Analytical gel chromatograms of cut 1 from whole ryegrass, residue I (R_I) and residue II (R_{II}). Wavelength of detection was 254 nm. Chromatograms have not been normalized for concentration (0.5–0.1 mg of N × 6.25/mL).

weight at 254 than at 206 nm. If it is assumed that cut 1 did contain soluble lignin fragments or precursors, the chromatograms detected at 254 nm (Figure 4) would indicate that some had a molecular weight as low as 500. Since cut 1 eluted at the void volume of a Sephadex G-15 column whose exclusion limit was 1500, some of these low molecular weight compounds may have been components of aggregates held together by noncovalent bonds when first isolated. The cut 1 chromatogram from whole ryegrass detected at 254 nm (Figure 4) shows much more solute eluting in the molecular weight range below 1500 than chromatograms detected at 206 nm (cf. Figures 4 and 5).

The average molecular weights, M_{A_1}, for cut 1 from R_I and R_{II} were somewhat lower than for cut 1 from whole grass (Table IV and Figure 5). Dissociation of proteins by

Table IV. First Moment Apparent Molecular Weight Averages ($M_{A_1} \times 10^{-3}$) for Ryegrass Fractions As Determined by Gel Chromatography[a]

fraction	whole grass: 206 nm	whole grass: 254 nm	residue I (R_I): 206 nm	residue I (R_I): 254 nm	residue II (R_{II}): 206 nm	residue II (R_{II}): 254 nm
cut 1	28.7 ± 2.8	2.2 ± 0.2	16.9 ± 1.9	25.9 ± 2.5	18.7 ± 3.9	24.3 ± 7.1
cut 2	1.3 ± 0.2	1.1 ± 0.2	1.4 ± 0.5	1.3 ± 0.5	1.5 ± 0.2	1.4 ± 0.2
cut 3	0.50 ± 0.16	0.60 ± 0.15	<0.22	<0.22	<0.22	<0.22

[a] Average of triplicate analyses.

Table V. Linear Least-Squares Parameters for Indicating Stability of Cut 1 Components

	starting material					
	whole grass		R_I		R_{II}	
	206 nm	254 nm	206 nm	254 nm	206 nm	254 nm
int ($\times 10^{-3}$)	25.5	2.05	20.5	28.9	18.0	14.1
slope	−16.1	−0.556	−16.4	−21.7	3.40	6.60
corr coeff	−0.97	−0.98	−0.94	−0.96	0.99	0.93
SE ($\times 10^{-3}$)	1.50	−0.043	2.31	2.31	0.078	0.53

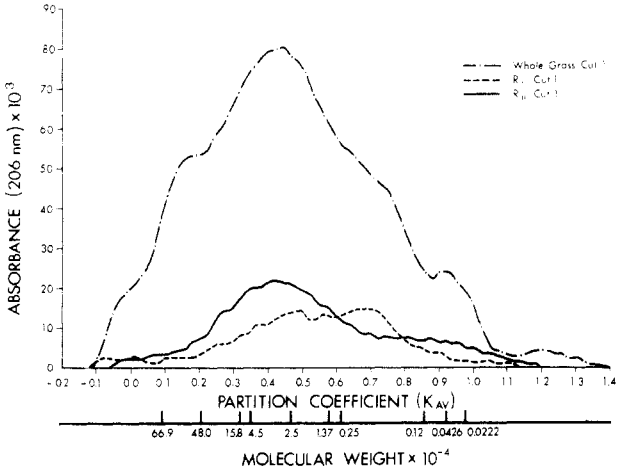

Figure 5. Analytical gel chromatograms of cut 1 from whole ryegrass, residue I (R_I) and residue II (R_{II}). Wavelength of detection was 206 nm. Chromatograms have not been normalized for concentration (0.5–0.1 mg of N × 6.25/mL).

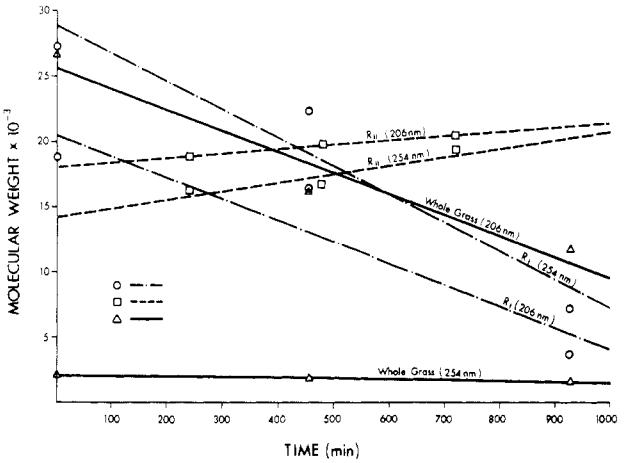

Figure 6. Changes in the first moment apparent molecular weights of cut 1 proteins as a function of time. Cut 1 fractions are from whole ryegrass, residue I (R_I), and residue II (R_{II}).

$NaDodSO_4$ extraction may account for the lower molecular weights of R_I and R_{II} proteins compared to whole grass proteins.

Within the precision of our measurements, the M_{A_1} of cut 2 is independent of starting material and wavelength of detection. Cut 3 from whole ryegrass appears to have a slightly higher M_{A_1} than cut 3 from R_I or R_{II}.

The lyophillized cut 1's from the three kinds of starting material (i.e., whole grass, R_I, and R_{II}) were dissolved in 0.8 M NaCl, and the first moment apparent molecular weights measured as a function of time, M_{A_1} showed a slow decrease for cut 1 proteins from whole grass and R_I but a slow increase for proteins from R_{II} (Figure 6; Table V). Whole grass and R_I proteins may degrade because of proteolytic enzymes in the extracts. The reason for the rather surprising increase in M_{A_1} for R_{II} proteins is unknown at present.

Earlier, molecular weight measurements by gel chromatography on Coastal Bermuda grass (Fishman and Burdick, 1977) gave results which were quantitatively similar for cuts 2 and 3 but only qualitatively similar for cut 1 when compared with comparable cuts from ryegrass. As with ryegrass, buffer-extracted protein from Bermuda grass measured higher in molecular weight when detection was at 206 than at 254 nm, whereas the reverse was true for the detergent-extracted protein. Generally, for comparable fractions, molecular weights for ryegrass were higher than those for Bermuda grass when measured at either 206 or 254 nm. The lone exception was the molecular weight for cut 1 from unfractionated rye measured at 254 nm which was lower than the cut 1 254-nm value from Coastal Bermuda grass.

Protein Purity. Previously, Fishman and Burdick (1977) found, for a series of standard proteins, that the parameter A_{206}/A_{254}, obtained from the ratio of peak areas in analytical gel chromatograms detected at 206 and 254 nm, ranged from 79.3 to 31. The alternating tetrapeptide of glycine and phenylalanine gave an A_{206}/A_{254} ratio of 13.

Table VI. Parameters for Comparing the Solubilities and Purities of Soluble Fractions from R_I and R_{II} with Those of the Same Fractions from Whole Grass

fractionation sources	A_{206}	A_{254}	A_{206}/A_{254}	F_S
cut 1				
whole grass	20.0 ± 0.4	6.9 ± 0.1	2.8 ± 0.3	
R_I[a]	2.5 ± 0.7	2.2 ± 0.1	1.2 ± 0.1	0.12 ± 0.04
R_{II}[b]	1.0 ± 0.2	1.1 ± 0.2	1.0 ± 0.1	0.05 ± 0.01
cut 2				
whole grass	24.6 ± 5.3	1.5 ± 0.5	16.9 ± 1.7	
R_I[a]	14.2 ± 1.2	0.5 ± 0.1	32.4 ± 5.0	0.6 ± 0.2
R_{II}[b]	19.0 ± 2.7	0.7 ± 0.1	25.6 ± 3.2	0.8 ± 0.3
cut 3				
whole grass	54 ± 11	3.3 ± 0.6	16.7 ± 4.0	
R_I[a]	75 ± 12	3.4 ± 0.1	21.6 ± 3.1	1.4 ± 0.5
R_{II}[b]	60 ± 11	5.4 ± 0.9	11.3 ± 2.2	1.1 ± 0.3

[a] Residue I. [b] Residue II.

Thus, A_{206}/A_{254} values for the various cut 1's from ryegrass which were between 2.8 and 1 (Table VI) indicate a higher presence of aromatic residues in cut 1 than would be expected from the amount of phenylalanine and tyrosine found by amino acid analysis (Table VII). Therefore, the conclusion from first moment molecular weight averages that cut 1 from whole grass was contaminated by highly aromatic non amino acid structures, can be extended to cut 1 from R_I and R_{II} as well. The A_{206}/A_{254} ratios for cuts 2 and 3 from Annual ryegrass are appreciably higher than for cut 1 and are much closer to the standard protein ratios than cut 1.

A comparison of A_{206}/A_{254} values reveals that ratios from cut 1 of ryegrass were slightly lower than those from Coastal Bermuda grass, whereas cut 2 and 3 ratios were appreciably higher. As was found for Annual ryegrass, cut 1 of Coastal Bermuda grass gave A_{206}/A_{254} ratios which were too low if the sole source of UV absorbence at 254 nm were aromatic amino acid residues.

Protein Solubility. The change in solubility of proteins in the various cuts from fractions extracted with NaDod-

Table VII. Amino Acid Composition of Annual Ryegrass and Fractions[a]

	unfractionated grass	residue I (R_I)	residue II (R_{II})	cut 1 (C1)	cut 2 (C2)	cut 3 (C3)	cut 4 (C4)	standard error[b]
lysine	6.81B[c]	6.77B	6.98B	8.19B	12.6A	0.86D	3.02C	0.52
histidine	2.39A	2.22A	2.71A	1.94A	1.84A	0.0A	2.45A	0.89
ammonia	3.03B	2.70B	1.62B	2.17B	3.39B	13.7A	11.0A	1.0
arginine	6.14AB	6.33AB	6.91A	5.97B	3.76C	0.15D	1.42D	0.53
cysteic acid[d]	1.63A	0.71B	0.17C	0.14C	0.40BC	0.30BC	0.68B	0.14
aspartic acid	10.6A	10.6A	10.0A	10.3A	12.7A	1.51C	4.66B	0.83
threonine	5.24A	5.25A	5.62A	5.99A	3.56B	0.36D	1.77C	0.27
serine	5.27A	5.35A	5.18A	5.58A	3.61B	0.76D	1.85C	0.16
glutamic acid	12.6B	11.7B	11.5B	12.8B	22.6A	5.75C	1.63C	0.89
proline	5.41B	4.88B	5.58B	5.06B	4.26B	14.2A	1.47C	0.97
glycine	5.90B	6.03B	6.22B	6.03B	7.23A	1.66C	6.08B	0.22
alanine	7.42A	6.97A	7.21A	7.29A	4.76B	7.52A	2.89C	0.46
cystine (half)	0.01C	0.0C	0.52C	1.35B	4.49A	0.0C	0.0C	0.16
valine	6.26A	6.26A	6.94A	7.25A	2.92B	1.02C	2.21BC	0.43
methionine	0.29B	2.21A	2.74A	2.67A	3.16A	0.52B	1.37A	0.58
isoleucine	5.15A	5.16A	5.73A	4.93A	2.63B	1.23C	2.79B	0.24
leucine	9.13A	9.11A	9.99A	8.24A	3.32B	1.34B	3.92B	1.1
tyrosine	0.67B	4.34BC	5.03B	4.84B	1.31BC	0.13C	9.60A	1.2
phenylalanine	5.99B	7.02A	7.84A	5.88B	1.62C	0.17D	2.46C	0.40
% N recovered as NH_3	11.0B	11.3B	6.9E	9.8C	8.7D	20.4A	9.6C	0.12
% N recovered as amino acids	60.0D	67.7C	76.2B	78.2A	41.9E	8.6F	8.7F	0.40
total N recovered	71.0D	79.0C	88.0A	88.0A	50.6E	29.0F	18.3G	0.46

[a] The means of two measurements. [b] Based on 16 measurements. [c] Rows are ranked by Duncan's multiple range test: means with no letter in common are significantly different at the $p \leq 0.05$ level. [d] Calculated as grams of half-cystine per 16 g of N.

SO_4 as compared to cuts extracted without $NaDodSO_4$ was measured by analytical gel chromatography (Fishman and Burdick, 1977). The sequence of events—insoluble in buffer, soluble in $NaDodSO_4$, insoluble in salt solutions—strongly suggest that most of the proteins in R_I and R_{II} were insoluble in aqueous solvents because of their inherent hydrophobicity and not because of preparation or extraction method. Most of the detergent soluble proteins of Coastal Bermuda grass were insoluble in salt solutions.

About 40% of the cut 2 polypeptides from R_I and 20% of those from R_{II} were insoluble also, as shown by the data in Table VI. These results are consistent with the hypothesis that the cut 2 fractions from R_I and R_{II} contained polypeptide fragments from structural proteins and chlorophyll–protein complexes.

The data in Table VI indicate that cut 3 polypeptides from R_I and R_{II} were completely soluble in the salt solution, as were cut 3 polypeptides from Coastal Bermuda grass. The fraction of soluble proteins (F_S) in R_I is about 0.12 of the total and about 0.05 in R_{II} (Table VI). The solubility change is quantitated by the parameter F_S, which is defined as the A_{206} for any given cut from R_I or R_{II}, divided by the A_{206} for the same cut obtained from the whole grass. Thus as compared to cut 1 from whole grass, the cut 1 fractions from R_I and R_{II} were, respectively, 0.12 and 0.05 times as soluble (Table VI).

Amino Acid Composition. The amino acid composition, grams of amino acid per 16 g of N and percentages of Kjeldahl nitrogen recovered by amino acid analysis for unfractionated Annual ryegrass and fractions extracted with buffer A (i.e., borate–boric acid buffer without detergent) are given in Table VII. Analysis of variance (ANOVA) at the $p \leq 0.05$ level indicated that all amino acids but histidine and all nitrogen recoveries showed significance difference by fraction. Thus, each row of means in Table VII except those belonging to histidine was ranked in decreasing numerical value and tested for significance of difference by the multiple range test (Harter, 1960). In each row of Table VII, means with no letter in common are different at the $p \leq 0.05$ level.

Nitrogen recovered as amino acids (see Table VII) indicates that at least two-thirds of the Kjeldahl nitrogen in cut 1, R_I, and R_{II} are derived from amino acids. Amino acid analysis in conjunction with gel chromatography data confirms that proteins constitute a considerable portion of cut 1, R_I, and R_{II}. Cuts 2–4 give significantly lower percentages of nitrogen recovered as amino acids than R_I, R_{II}, or cut 1. Since cuts 2 and 3 are lower in molecular weight than cut 1 or the major portion of detergent-soluble extracts from R_I or R_{II}, the low recovery of nitrogen as amino acids in cuts 2 and 3 may result from a high percentage of volatile nitrogen in these fractions. Consistent with results from the fractions, the percentage of N recovered as amino acids from the unfractionated grass was higher than that from cuts 2–4 but lower than R_I, R_{II}, or cut 1.

For most amino acids, cuts 3 and 4 were lower in amino acid concentrations than all other fractions. With a few exceptions, the amino acid composition of unfractionated grass, R_I, R_{II}, C1, and C2 were similar. Notable exceptions were a higher value of cysteic acid and lower values of methionine and possibly tyrosine in the unfractionated grass as compared to the fractions. Furthermore, lysine and half-cystine were higher in cut 2 than in other fractions or in unfractionated grass.

Generally, the number of differences between amino acids in fractions is smaller for Annual ryegrass than Coastal Bermuda grass (Fishman and Evans, 1978). Comparison of the buffer-soluble fractions of Coastal, C1–C3, revealed that amounts of most of the amino acids decreased with molecular weight, whereas no such trend is apparent for comparable soluble fractions of ryegrass.

The amino acid compositions of unfractionated Annual ryegrass and Coastal Bermuda grass are remarkably similar. The differences, methionine and tyrosine lower and cystine higher in ryegrass than Bermuda grass, are related more to ease of hydrolysis than to actual composition. We concluded the latter because there are larger amounts of tyrosine and methionine in the fractions of ryegrass than

in the unfractionated grass and larger amounts of cysteic acid in the fractions of Coastal Bermuda grass compared to the unfractionated material.

CONCLUSION

When fractionated, buffer extracts of vacuum-dried ryegrass yield a leaf protein concentrate (cut 1) that is free of green pigmentation. Apparently, chloroplasts dehydrated in vacuo at room temperature or somewhat below become more resistant to disruption. Possibly, lyophilization mildly denatures the chlorophyll–protein complexes in chloroplasts (by exposing hydrophobic groups through conformational changes) and thus lowers the solubility of the complexes in aqueous solvents. This possibility may apply to other grasses also, because Coastal Bermuda grass behaved similarly (Fishman and Burdick, 1977). Furthermore, extractions by buffer of wet, freshly harvested ryegrass or Coastal Bermuda grass carried out in this laboratory gave a cut 1 which contained protein–chlorophyll complexes. This observation also supports the hypothesis that dehydrated chloroplasts withstand disruption better than hydrated ones.

The nitrogen distribution data in Table I and the solubility data of Table VI suggest that maximally, 7% of the crude protein in ryegrass is true protein with functional properties as compared to 11% for Coastal Bermuda grass. About 14.8% of the crude protein (R_I in Table IV) is resistant to dissolution and extraction, whereas about 30.8% of the crude protein in Coastal is insoluble and unextractable. This fraction might be a superior source of protein for ruminants. The insolubility and relatively large size of particles in this fraction may "naturally protect" it from degradation in the rumen, thus making it available for digestion in the abomasum and absorption from the lower digestive tract of the ruminant. About 35% of the crude protein in ryegrass is true but insoluble protein and could be a food supplement, whereas 29% of the crude protein in Coastal Bermuda grass could supplement food. Thus, ryegrass has slightly less true protein with functional property capability and slightly more true protein with food supplement capability than Coastal Bermuda grass.

The amino acid composition of both grasses are comparable and of high quality. If either is nutritionally limiting in a particular amino acid (e.g., tyrosine, methionine, or cystine in Annual ryegrass or cystine in Coastal Bermuda grass), there is likely to be degradation during processing or digestion rather than absence in the unprocessed grass.

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