Amino Acid Composition of Coastal Bermuda Grass Fractions

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The proteins and polypeptides of Coastal Bermuda grass were fractionated according to extractability, solubility, and molecular size. The fraction containing predominantly cytoplasmic proteins was less contaminated with non-protein aromatic compounds, higher in lysine and cystine, and lower in hydrophobic amino acids than the fractions containing predominantly chloroplastic proteins. Oxidative degradation of amino acids by endogeneous species from the chloroplasts and added sodium dodecylsulfate (SDS) appeared to be an important mechanism in lowering recoveries of amino acid nitrogen. Addition of sodium dodecylsulfate to the acid hydrolysis gave higher values of cystine recovered as cysteic acid than those reported as obtained by performate oxidation.

Previously, Coastal Bermuda grass was investigated as a new protein source because of its high productivity (Fishman and Burdick, 1977). The proteins in Coastal Bermuda grass were characterized by the extractability, solubility, and molecular size distribution of buffer and detergent (sodium dodecylsulfate, SDS) soluble constituents. Five major fractions were obtained, two insoluble and three soluble. A sixth minor fraction (cut 4) was obtained by the SDS extraction. Here, we report the amino acid composition of the fractions obtained from the borate–boric acid (pH 8.14) extraction of Coastal Bermuda grass at 0 and 1% SDS in the presence of 5 mmol of sodium metabisulfite. In addition to providing nutritional data on these fractions, we believe this work points out possible sources of error peculiar to the amino acid analysis of forages, (i.e., plant material consisting mostly of leaves).

EXPERIMENTAL SECTION

Materials. Coastal Bermuda grass (*Cynodon dactylon* (L.) Pers.) was cultivated, prepared, fractionated, and characterized as described elsewhere (Fishman and Burdick, 1977). Figure 1 is a flow sheet for the fractionation procedure.

Hydrolysis. Samples were hydrolyzed in 6 N HCl by refluxing at 108 °C under nitrogen. The HCl was saturated with nitrogen prior to sample addition. The ratio was 6 mL of HCl/mg of crude protein in the sample. After 24 h under reflux, samples were allowed to cool, dried by rotary evaporation, redissolved in distilled water, and redried by rotary evaporation. Each of the hydrolysates was dissolved in 15 mL of citrate diluting buffer (pH 2.2) and filtered with 1.0 μm and 0.45 μm millipore cellotate membranes. This hydrolysis method was tested on serum albumin and recoveries of Kjeldahl nitrogen as amino acid nitrogen were 95%.

Samples to be analyzed for tryptophan were hydrolyzed in base (Hugli and Moore, 1972).

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Amino Acid Analysis. Amino acid analyses were by ion-exchange chromatography (Spackman et al., 1958) as outlined in the instruction manual for the amino acid analyzer, a Beckman Model 121 (Beckman Inst. Co., 1970). Data collection and computation were reported elsewhere (Fishman et al., 1973) but are reported as grams of amino acid/16 g of recovered nitrogen from ion-exchange analyses.

The standard protein hydrolysate procedure of Spackman et al. (1958) was modified to separate aspartic acid from hydroxyproline which otherwise would have eluted together. The acid-neutrals column was equilibrated with 0.2 N citrate buffer, pH 2.77, and eluted with the same buffer for 35 min after sample injection, followed by elution with 0.2 N citrate buffer, pH 3.25. Hydroxyproline was eluted 76 min after injection, aspartic acid emerged 12 min later. This procedure will not resolve many of the acid-neutrals which are eluted after aspartic acid.

The data for nitrogen recovery (Table I) and amino acid composition (Table II) were analyzed statistically. Analysis of variance (ANOVA) indicated significant difference at the ($p \leq 0.01$) level with fraction and percent SDS in the extractant for the dependent variables measured. Hence, these variables were ranked in decreasing order of value and tested for significance of difference at the ($p \leq 0.05$) level (see Tables I and II) by the Duncan multiple range test (Duncan, 1955; Harter, 1960).

RESULTS AND DISCUSSION

Three major steps are necessary to prepare proteins, polypeptides, and free amino acids in lyophilized, ground plant material for amino acid analysis by ion-exchange chromatography. These are solvation, solution, and hydrolysis of chemical bonds (e.g., peptide and ester linkages). During initiation of these three steps, proteins, polypeptides, and amino acids are liberated from within the plant cells due to partial degradation of the cell walls and membranes. Further solvation, solution, and hydrolysis are required to liberate the amino acids from the free polypeptides and proteins, including those located in the cell walls and membranes. Several mechanisms can cause the amino acid composition of the acid hydrolysate to differ from that of the intact plant. These include incomplete liberation of amino acids and their residues from

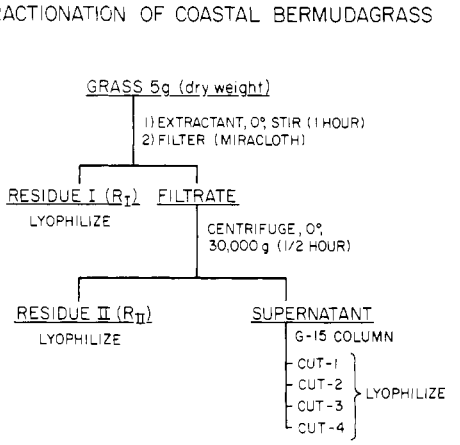

Figure 1. Flow diagram for the preparation of fractions from Coastal Bermuda grass.

the plant matrix, incomplete hydrolysis of peptide bonds, breakdown of amino acids to ammonia, and reactions with other endogenous species present, such as amino sugars, carbohydrates, and phenolics (Blackburn, 1968; Loomis and Battaile, 1966). The results will be discussed with regard to the steps for liberation of free amino acids and interfering mechanisms.

Percent Kjeldahl Nitrogen Recovered as Amino Acids and as Ammonia. Table I contains the percentages of Kjeldahl nitrogen recovered as amino acids (% N_{AA}) and as ammonia (% N_{AM}) from the various fractions at 0 and 1% SDS. Corresponding values for unfractionated Coastal Bermuda grass hydrolyzed with and without SDS are included as controls.

A comparison of % N_{AA} and % N_{AM} for unfractionated Coastal hydrolyzed without SDS (UC) and with SDS in 1:1 w/w ratio (UCD) showed no significant effect of SDS. Residue I extracted without SDS (R_I) had a significantly lower value of % N_{AA} than UC or the same fraction extracted with SDS (R_{ID}). Residue II extracted without SDS (R_{II}) and with (R_{IID}) have significantly lower values of % N_{AA} than UC or UCD, respectively. Further, % N_{AA} is significantly lower in R_{II} than R_{IID}. In each comparison, the fraction likely to contain more intact chloroplasts (Fishman and Burdick, 1977) had the lower value of % N_{AA}. Therefore, we concluded that intact chloroplasts hinder the liberation of amino acids.

Cut 1 from grass extracted without SDS (C1) and with SDS (C1D) did not differ significantly in values of % N_{AA} or % N_{AM}. In comparison with C1, C1D contained more chloroplastic proteins, had a somewhat lower average molecular weight (13 000 against 20 000), and was one-fourth as soluble in 0.8 N NaCl (Fishman and Burdick, 1977). Therefore, incomplete hydrolysis of peptide bonds, destruction of amino acids or protein, and solubility do not appear to be important factors in determining the value of % N_{AA} or % N_{AM} in UC or UCD.

Cut 2 extracted without SDS (C2) was significantly lower in % N_{AA} than either UC or C1, but the values of % N_{AM} were not significantly different for these three fractions. The anomalously low value of % N_{AA} for C2 suggests the presence of nitrogen in a non-ninhydrin positive form. Cut 2 extracted with SDS (C2D) was not significantly different from C2 in % N_{AA} but was significantly higher in % N_{AM}. Apparently, SDS promoted the degradation to ammonia of the non-ninhydrin positive portion of cut 2, perhaps by extracting a non-nitrogeneous oxidizing agent.

Cut 3 extracted without SDS (C3) was significantly lower in % N_{AA} and significantly higher in % N_{AM} than either C1 or UC. Moreover, C3 was not significantly different from C2 in % N_{AA}, but significantly higher in % N_{AM}. Therefore, the low value of % N_{AA} for C3 may be attributed to the presence of nitrogen in a non-ninhydrin positive form and because of its relatively low average molecular weight (453.6) underwent considerable degradation during hydrolysis. We do not exclude the possibility that the high value of % N_{AM} also may indicate a relatively high extent of amino acid degradation. In agreement with the foregoing speculations, cut 3 extracted with SDS (C3D) was not significantly different from C3 in % N_{AA} or % N_{AM}.

Only the detergent extraction of Coastal gave enough cut 4 (C4D) for analysis. This fraction was significantly lower in % N_{AA} and higher in % N_{AM} than all other fractions. Further comment on this fraction will be reserved for the discussion of its amino acid composition.

Comparisons of Amino Acid Composition. Table II contains amino acid compositions for unfractionated Coastal and for the fractions extracted with and without SDS. A comparison of UC, UCD, R_I, and R_{ID} show that overall patterns are quite similar. Therefore, R_I or R_{ID} should not differ much from UC as a protein source for ruminants. The significant differences were in the values of threonine and serine which were much lower and cysteic acid which was higher in UCD than UC, R_I, or R_{ID}. Blackburn reported that serine and threonine can undergo oxidative degradation to NH_3 during hydrolysis, and that cystine can be oxidized to cysteic acid (Blackburn, 1968). Accordingly, our results may indicate that SDS promoted oxidation when present during the acid hydrolysis of forages. Moreover the half-cystine value in UCD (obtained as cysteic acid, see Table II) was comparable to or higher than values obtained for other sources of leaf protein (Byers, 1971) by the performate oxidation of cystine to cysteic acid. Previously (Wilkinson, et al., 1968) reported

Table I. Percentage Nitrogen Recovered[a] as Amino Acids and Ammonia for Coastal Bermuda Grass and Fractions

fraction	0% sodium dodecylsulfate			1% sodium dodecylsulfate		
	% N_{AA}[b]	% N_{AM}[c]	total[d]	% N_{AA}[b]	% N_{AM}[c]	total[d]
unfractionated grass (UC and UCD)	71.3a[e]	9.98de	81.3ab	71.4a	11.3d	82.7ab
residue I (R_I and R_{ID})	56.0b	6.83ef	62.9c	69.1a	8.41de	77.5b
residue II (R_{II} and R_{IID})	30.5c	2.96f	33.4g	49.2b	12.3d	61.5cd
cut 1 (C1 and C1D)	76.8a	7.94de	84.7a	76.6a	7.51def	84.1a
cut 2 (C2 and C2D)	35.8c[f]	8.49de[f]	44.3f[f]	34.1c[f]	19.1c[f]	53.2e[f]
cut 3 (C3 and C3D)	36.9c[f]	27.0b[f]	63.9c[f]	33.0c	23.1bc	56.1de
cut 4 (C4D)				13.1d	31.6a	44.7f

[a] (Amino acid or NH_3 nitrogen by ion exchange/Kjeldahl nitrogen of the fraction) × 100. [b] Standard error based on 33 measurements ± 1.87. [c] Standard error based on 33 measurements ± 1.01. [d] Standard error based on 33 measurements ± 1.21. [e] Means with same standard error are ranked by Duncan multiple range test. Ranked means with no letters in common are significantly different at the ($p \leq 0.05$) level. [f] These numbers are means of two measurements, remaining means of 3.

Table II. Amino Acid Composition of Coastal Bermuda Grass and Fractions

	0% sodium dodecylsulfate, g of aa/16 g of N						1% sodium dodecylsulfate, g of aa/16 g of N						
	unfractionated grass (UC)	residue I (R_I)	residue II (R_{II})	cut 1 (C1)	cut 2[a] (C2)	cut 3[a] (C3)	unfractionated grass (UCD)	residue I (R_{ID})	residue II (R_{IID})	cut 1 (C1D)	cut 2[a] (C2D)	cut 3 (C3D)	cut 4 (C4D)	standard error[b]
lysine	6.72c[c]	6.92bc	7.03bc	8.71a	6.91bc	0.95e	6.88bc	7.12bc	6.31c	7.72b	4.83d	1.08e	1.13e	0.28
histidine	2.41a	2.46a	2.58a	2.30a	0.58c	1.36b	2.39a	2.56a	2.14a	2.53a	0.58c	1.31b	0.24c	0.13
NH_4Cl	7.60ef	6.67ef	5.41f	5.73ef	11.7d	24.9b	8.40e	6.64ef	12.2d	5.46f	21.9c	25.4b	44.4a	0.82
arginine	6.03b	6.64a	6.71a	6.44ab	4.03c	1.34e	6.49ab	6.33ab	5.99b	6.70a	2.76d	0.99e	1.06e	0.19
cysteic acid[c]	0.19c	0.28c	0.19c	0.12c	0.59bc	1.02bc	2.75a	0.18c	1.45b	0.19c	3.17a	1.01bc	0.34c	0.37
hydroxyproline	Tr	ND[e]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
aspartic acid	14.1cde	11.8def	11.5def	11.0ef	22.0b	26.9a	14.6cd	11.5def	11.2def	10.6f	15.8c	27.1a	2.54g	1.04
threonine	5.28ab	5.33ab	5.33ab	5.50a	4.44bc	2.58e	3.75cd	5.40ab	4.70ab	5.15ab	3.20de	2.75e	0.81f	0.28
serine	5.11a	5.50a	5.50a	5.60a	3.99b	3.18b	2.80b	5.23a	5.05a	5.59a	2.99b	3.41b	0.79c	0.38
glutamic acid	11.6c	11.6c	11.6c	13.9a	11.1c	4.64c	9.77d	11.7c	9.71d	12.8b	9.46d	4.85e	1.92f	0.35
proline	5.91ab	5.73ab	6.45ab	4.64ab	6.04ab	3.96ab	4.89ab	5.92ab	6.44ab	4.69ab	6.61a	5.40ab	0.99c	0.56
glycine	5.61a	5.36ab	5.78a	5.44ab	3.68c	1.94d	5.33ab	5.93a	4.83b	5.59a	3.24c	1.87d	5.56a	0.23
alanine	8.78c	8.05c	7.71cd	7.34d	17.9a	14.2b	8.72cd	7.81cd	7.61cd	7.56cd	14.4b	14.9b	1.77e	0.42
cystine (half)[d]	0.29bc	0.18bc	0.43bc	1.34a	0.98a	0.0c	0.08c	0.42bc	0.0c	0.71b	0.0c	0.0c	0.30bc	0.14
valine	6.34b	6.58b	6.68ab	6.96a	3.85d	2.49e	6.12b	6.44b	5.76c	6.81db	3.18c	2.67e	0.55f	0.11
methionine	1.97abc	1.77abc	1.87abc	2.81a	1.74abc	0.41d	2.21ab	2.13ab	1.36bcd	2.69a	0.40d	0.40d	0.64cd	0.40
isoleucine	5.11d	5.49bc	5.57ab	5.13d	2.64e	1.98f	5.12d	5.29cd	4.70e	5.80a	2.15f	2.08f	0.74g	0.08
leucine	9.14d	10.0a	10.3a	8.92d	2.77f	2.90f	9.23cd	9.67b	7.74e	10.3a	2.30g	3.02f	0.79h	0.12
tyrosine	3.79de	3.96cd	4.39bc	4.37bc	0.27g	0.66g	3.43ef	3.99cd	3.15f	4.58b	0.14g	0.60g	13.5a	0.16
phenylalanine	5.80c	5.94bc	6.64a	5.07d	0.34g	1.81f	5.32d	6.31ab	4.01e	6.30ab	0.25g	1.62f	3.84e	0.15
tryptophan	0.17	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.02

[a] Columns of numbers which are the means of two measurements, remaining are means of three measurements. [b] Based on 33 measurements except for tryptophan which is based on three measurements. [c] Rows are ranked by Duncan multiple range test: means with no letter in common are significantly different at the ($p \leq 0.05$) level. [d] Calculated as g of half-cystine/16 g of N. [e] ND is not determined.

that commercially dehydrated Coastal contained 20% hydroxyproline. With our modified procedure for hydroxyproline (see Experimental Section), we found only trace amounts in UC. Moreover, for all amino acids but cystine, our values were appreciably higher. In UC, much of the cystine was destroyed, but in UCD we found more cysteic acid than determined by Wilkinson et al. (1968) by performate oxidation. These discrepancies may have been caused by differences in treatment prior to hydrolysis. We determined tryptophan separately in UC by alkaline hydrolysis and found it to be lowest of all 18 standard amino acids, 0.171 ± 0.018 g/16 g of N.

As compared to R_{II}, R_{IID} was significantly higher in ammonia and cysteic acid and significantly lower in arginine, glutamic acid, glycine, valine, isoleucine, leucine, tyrosine, and phenylalanine. Thus, more oxidation appeared to have occurred during the hydrolysis of R_{IID} than R_{II}. In contrast, the amino acid composition of R_{II} is not significantly different from UC or R_I.

C1D compared to C1 was significantly higher in the hydrophobic amino acids (Tanford, 1962) isoleucine, leucine, and phenylalanine. The ability of SDS to solubilize hydrophobic proteins apparently aids their extraction from chloroplasts and other plant organelles, in addition to breaking chloroplasts by solubilizing the chlorophyll–protein membrane (Helenius and Simons, 1975). The higher hydrophobicity of C1D compared to C1 may partially explain its lower resolubility in 0.8 N NaCl (Fishman and Burdick, 1977).

Lysine, glutamic acid, and cystine values are significantly higher in C1 and C1D. Since the proteins in C1 were predominantly cytoplasmic, while approximately two-thirds of the proteins in C1D were chloroplastic, the lower values of lysine and cystine may be further evidence that the chloroplast is the site of protein destruction during extraction. Previously, we found that the absorbance of aromatic groups increased by a factor of 2.3 in C1D compared to C1 (Fishman and Burdick, 1977). The phenylalanine in C1D increased by a factor of 1.2 over C1, while tyrosine increased by 1.05 (the tyrosine value was not statistically significant at ($p \leq 0.05$ level)).

Since the tryptophan level in grass is low (see Table II), we conclude that contamination of the protein through side reactions with phenolics accounted for most of the increase in aromatic absorption. Moreover, the lower level of lysine in C1D compared to C1 may indicate reaction between the ϵ-amino group of lysine and phenolics.

As compared to R_{II}, C1 contained significantly more lysine, glutamic acid, and cystine and less phenylalanine. A similar comparison for C1D with R_{IID} indicated that in addition to lysine, glutamic acid, and cystine, C1D was significantly higher in arginine, glycine, valine, isoleucine, leucine, tyrosine, and phenylalanine. The last four named amino acids are hydrophobic and their increases probably arose from the solubilization of membrane proteins by SDS. In contrast, cysteic acid and ammonium chloride were significantly higher in R_{IID} than C1D. Of the four cuts separated by the gel column after SDS extraction of Coastal, C1D was least likely to contain detergent because of molecular weight considerations. R_{IID}, on the other hand, may have some residual SDS as a consequence of containing particulate matter in the form of chloroplasts and their fragments. We strongly suspect, therefore, R_{IID} underwent a greater degree of oxidation then C1D during acid hydrolysis.

Based on recovered nitrogen from amino acid analyses, the levels of most of the amino acids were higher in C2 than in C2D even though % N_{AA} was the same. If C2D did not contain significantly more ammonium chloride (% N_{AM}) than C2 would have had approximately the same level for most of the amino acids. Only cysteic acid was significantly lower in C2 compared to C2D, a further indication of greater oxidation in C2D, which may have been caused by some residual SDS in C2D from the extraction and endogenous oxidant released from the chloroplasts.

Previous data (Fishman and Burdick, 1977) showed that the ultraviolet absorption of compounds which absorb at 254 nm increased by a factor of 1.7 in C2 or C2D compared to C1, yet the levels of tyrosine or phenylalanine were lower in C2 or C2D compared to C1 by a factor of 15 to 30. The increase in 254 nm absorbance taken with the decreases in tyrosine and phenylalanine is evidence that C2 is more contaminated with nonprotein materials than C1.

By comparison, C3 and C3D were not significantly different in amino acid composition. Since the amount of tyrosine and phenylalanine were three to seven times greater in C1 than C3, but ultraviolet absorption at 254 nm was about equal, we concluded that C3 was more contaminated with nonprotein material than C1 but less so than C2.

Comparison of the soluble fractions, C1, C2, and C3, revealed that the amounts of most of the amino acids decreased with increasing cut number, i.e., decreasing molecular size. All but aspartic and cysteic acids had lower values in C3 than C1. Aspartic acid, alanine, cysteic acid, and ammonium chloride increased with cut number. The increases of cysteic acid and ammonium chloride were probably due to increased oxidation associated with decreasing molecular size during hydrolysis. The high values of aspartic acid in cut 3 may reflect its presence as a free amino acid in Coastal. The second and third highest amino acids in C3 were alanine and glutamic acid. All three are abundant free acids in Coastal (Barnett and Nayler, 1966). Earlier work in this laboratory on a nonprotein nitrogen fraction separated from Coastal and which essentially contained cuts 2 and 3 showed the same order of abundance, i.e., aspartic acid, alanine and glutamic acids (Evans et al, 1974).

Over 70% of the recovered nitrogen in C4D was in the form of ammonia. Compared to C1D, there was three times as much tyrosine and an equal amount of glycine and much smaller quantities of the remaining amino acids. The retention time of C4D on the G-15 preparative column indicated that it was retarded by adsorption. Nevertheless, the unbalanced amino acid composition and large quantities of ammonia and tyrosine indicated that this fraction probably contained polypeptides of less than 1800 daltons and/or free amino acids.

Amino Acid Material Balances. Figure 2 is grams of amino acids in each fraction per 100 g of the same amino acid in UC for the 0% SDS extraction. Figure 3 is similar data for the 1% SDS extraction. A comparison of Figure 2 and Figure 3 revealed that there was 60–70% recovery for most of the amino acids from the fractions extracted with 0% SDS compared to 80–90% recovery for the fractions extracted with 1% SDS. The exceptions are cystine and cysteic acid which gave 100 and 130% recovery, respectively, for the 0% SDS extraction and 150% recovery for cystine and 200% cysteic acid for the extraction at 1% SDS. Further, the 0% SDS extraction gave an ammonia recovery of about 87%, while the 1% SDS extraction yielded about 105% ammonia recovery. It might be inferred that SDS can improve amino acid recoveries from Coastal by enhanced disruption of chloroplasts and solubilization of hydrophobic proteins, if the protein can be rapidly removed from the other endogenous molecules in the chloroplast as SDS also promotes the oxidative

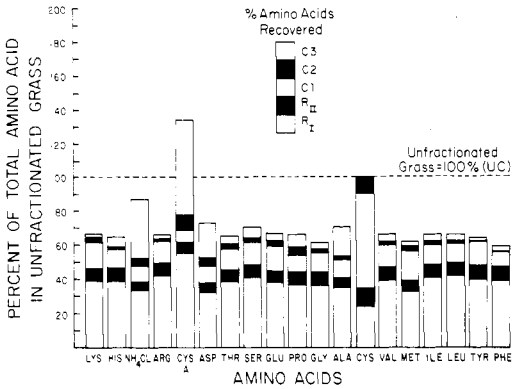

Figure 2. Material balance of amino acids extracted at 0% SDS based on unfractionated grass (UC).

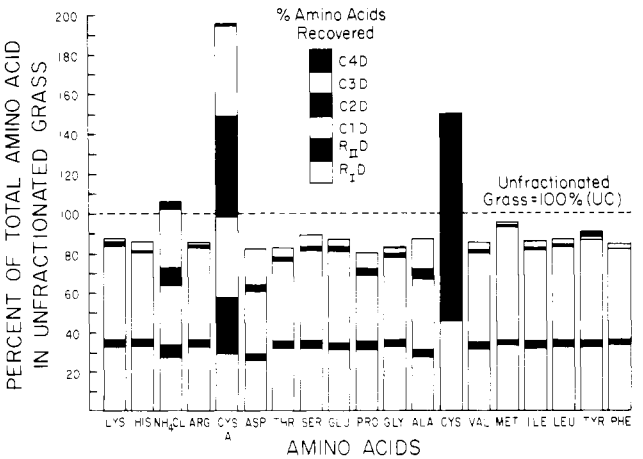

Figure 3. Material balance of amino acids extracted at 1% SDS based on unfractionated grass (UC). For cystine, the white block is R_{ID} and the dark block is C1D; for the rest, blocks are according to the legend in the figure.

degradation of proteins by the same endogeneous species. Moreover, the high recoveries of cysteic acid for the sum of the SDS extracted fractions and for UCD as compared to UC indicated that hydrolysis in the presence of SDS may be a less time-consuming alternative to performate oxidation for the determination of cystine in leaf protein.

CONCLUSIONS

The predominantly cytoplasmic protein fraction of Coastal (C1) contained more lysine, cystine, and soluble proteins and is less contaminated by aromatic impurities than the predominantly chloroplastic protein fractions R_{II} and C1D. These differences may be more marked in Coastal than in many other sources of leaf protein (e.g., alfalfa) because of the greater difficulty in disrupting the cells and chloroplasts. Moreover, each of these differences are important in that they could contribute to the lower nutritive quality of chloroplastic protein compared to cytoplasmic.

Comparisons of UC and UCD, C1D and R_{ID}, R_{II} and R_{IID}, and C2 and C2D revealed that the latter of each pair is higher in cysteic acid and NH_4Cl. We postulate that the oxidation of cystine to cysteic acid is promoted when cystine, an endogenous oxidizing agent arising from the chloroplast fraction (mol wt $\simeq$1800) and SDS are present during the hydrolysis reaction.

Further, if cystine is a limiting acid in Coastal in particular, and in leaf protein in general, destructive oxidation reactions with endogenous species released from the chloroplasts could be a cause. Moreover, the same oxidant may be partly responsible for the relatively low recoveries of Kjeldahl nitrogen as amino acids in Coastal, 71% as compared to 95% which we obtained for the pure protein serum albumin. Nevertheless, we believe that some portion of the unrecovered nitrogen represents forms of nitrogen in grass which cannot be obtained as the 18 standard amino acids, cysteic acid, or ammonia.

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