0273-2289/05/121–124/721–730/$30.00

Leaf Protein from Ammonia-Treated Dwarf Elephant Grass (*Pennisetum purpureum* Schum cv. Mott)

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Abstract

Proteins can be an excellent byproduct of the biorefining of lignocellulosic materials. In this work, extraction conditions for the white leaf proteins (cytoplasmic) of ammonia-treated dwarf elephant grass were established to obtain a protein juice suitable for the production of leaf protein concentrates. A calcium hydroxide solution was used as extracting agent, at several solid–liquid ratios, pHs, temperatures, and times. Extractions were carried out in Erlenmeyer flasks containing 5 g (dry basis) of forage with constant agitation (100 rpm). The soluble protein content was determined by the Lowry method. Optimal extraction conditions for the ammonia-treated forage were 12.60 pH, 1:10 solid–liquid ratio, 90°C, and 30 min extraction time, resulting in 52.65% extraction yield. The ammonia treatment significantly increased ($p<0.05$) the release of proteins from the fibrous matrix, facilitating their extraction.

Index Entries: Leaf proteins; ammonia; dwarf elephant grass; extraction.

Introduction

Currently, there is a large deficit of proteinaceous foods in the world for animal and human consumption, so that new sources should be found and developed. Leaves are a suitable source of protein in tropical countries, because the vegetation vigorously grows the whole year. Venezuela possesses a great reserve of plants whose leaves have a relatively high protein content; these include both grasses and legumes. One of the grasses most abundant in the western region of Venezuela *(1)* is dwarf elephant grass (*Pennisetum purpureum* Schum cv. Mott). The plant has many advantages including a protein content of 10–15% depending on the age of the plant *(2)*, perennial growth, high productivity, and a nutrition value that is generally higher that other grasses that grow in tropical areas, due to the low fiber

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Table 1
Productivity, Protein Content and Animal Yield of Major Forages Grown in the Western Side of Venezuela

Grass	Productivity (tons/yr)	Protein content (%)*	Animal yield (animals/hectare**/yr)
Panicum maximum (Guinea grass)	12–40	8–12	1.5–2.5
Brachiaria radicans (Tanner grass)	20–25	8–12	2–2.5
Brachiaria humidicola	18–25	4–8	1.5–2
Pennisetum purpureum Schum cv. Mott (Dwarf elephant grass)	40–50	10–15	3–5

*dry basis.
**1 hectare = 10,000 m^2.

structure of the leaf *(3–5)*. Up to five animals could be raised per hectare, which is greater that the other grasses (Table 1). Although the major grown legumes, *Leucaena leucocephala* and *Gliricidia sepium* have a higher protein content (20–25%), they do not comprise a significant volume of feed.

In spite of the great potential for leaf protein, the complexity of the lignocellulosic matrix and the fiber content limit its use for single-gutted animals. Protein extracted from alfalfa, a legume, can be substituted for soybean protein, the major protein source in animal feeding, based on the quality of amino acid profile *(6)*. However, extraction on an industrial scale *(7)* has failed because the yield of the extracted protein has been very low and the remaining fiber has a very low digestibility and poor nutritional value for ruminants. Urribarrí et al. *(8)* reported very low protein yields using alkaline extraction of proteins from dwarf elephant grass (11.7%) that was subjected only to milling, which indicates the need for physical–chemical treatments that increase the release of proteins from the grass. In general, the literature is very scarce on this topic.

Ammonia treatments such as AFEX seem to facilitate the extraction of protein from grasses under alkaline conditions *(9,10)*, and a protein juice is obtained. However, previous studies using this technique did not include either all of the major parameters that play a role in the solubilization of the protein or a wide range of conditions for the parameters studied. In addition, extraction conditions for white leaf proteins, which have higher nutritional value than green proteins, have not been proposed for ammonia-treated materials. Green leaf proteins refer to chloroplastic proteins, with the enzyme Rubisco considered as the major protein, whereas the rest are considered white leaf proteins and are mainly cytoplasmic proteins *(11)*. Both types of protein are present in approximately the same amounts.

Recently *(12,13)*, several materials including dwarf elephant grass have been subjected to ammonia reactor treatment such as pressurization and depressurization with ammonia (PDA) to increase the susceptibility to enzymatic hydrolysis. These treatments gave high sugar yields with low enzyme dosages in short times such that the proteins could be extracted prior to the enzymatic hydrolysis and thus become a byproduct of the overall biorefining process. The objective of this investigation was to carry out a thorough study to establish physical–chemical conditions of extraction that increase the release of white protein from the lignocellulosic matrix of the leaves of dwarf elephant grass. The protein juice could then be used directly as a liquid feed in swine diets or to produce a leaf protein concentrate as a component of a feed for single-gutted animals.

Materials and Methods

Material

Dwarf elephant grass (*Pennisetum purpureum* Schum cv. Mott) was used *(1)*. Eight-wk-old dwarf elephant grass was harvested from a farm in Santa Bárbara del Zulia (Zulia State, Venezuela). The grass was partially dried in a forced convection oven at 48°C for 48 h, milled to a 1 mm particle size *(9)*, and stored in plastic bags at 8°C until use.

Chemical Analyses

Moisture content *(14)*, crude protein *(15)*, and cellulose, hemicellulose, and lignin *(16)* were determined in the raw and ammonia-treated materials. The soluble protein (true protein) present in the extracts was determined by the Lowry modified method *(17)* in an UV-visible Cary 50 spectrophotometer (Mulgrave, Victoria, Australia) at 742 nm. Bovine serum albumin was used as a standard for the calibration curve. Interferences were eliminated by adding desoxicholate and TCA to precipitate the proteins, which were solubilized later to determine their concentration. The concentration of proteins that could not precipitate with the TCA was also quantified in the supernatant.

Ammonia Treatment

A representative sample of 300 g of partially dry dwarf elephant grass (13.2% moisture) was processed in a 2 kg biomass PDA pilot plant. A 28% ammonium hydroxide solution was used as the source of ammonia. The processing conditions were 0.75 kg ammonia/kg dry matter, 70% moisture content (wb), 150 psi and 90°C for 5 min, which are considered as suitable for the production of sugars by enzymatic hydrolysis *(11)* and high digestible feeds for ruminants *(18)*. A temperature of 90°C is not supposed to affect the concentration of amino acids in proteins extracted from ammonia-treated grasses *(9)*. Non-treated dwarf elephant grass was used as a control. After processing, the grass was aerated under a hood for 24 h

to eliminate the ammonia remaining in the biomass and to allow moisture to reach equilibrium.

Protein Extraction

A calcium hydroxide solution was used as the extracting agent. All the extractions were carried out in triplicate in 250 mL Erlenmeyer flasks with 5 g of dry matter. The extracting agent was conditioned in a water bath to the desired temperature, subsequently poured on the Erlenmeyer flasks with constant agitation (100 rpm) and incubated at the desired temperature for a certain period of time according to the experimental design. The content of the Erlenmeyer flasks were vacuum-filtered through synthetic cloth and the recovered solids were washed with 10 mL of distilled water. The pH of the overall extract was measured. The extracts were stored at 4°C for 24 h and then centrifuged (10,000*g*) for 30 min in a Centra centrifuge NTMP4R (International Equipment Company, Nedham Heights, MA, USA), to separate the fiber and the green protein present in the protein extract. The white (cytoplasmic) protein remained in the extract *(19)*.

The soluble protein present in the extracts was determined in duplicate. The extraction yield was calculated as the quotient between the grams of soluble protein present in the extract and the grams of initial crude protein expressed as percentage.

Selection of the Optimal Extraction Conditions of the White Proteins of Dwarf Elephant Grass

- *Extraction time:* Extractions with a solution of calcium hydroxide pH 10 *(9)*, at 60°C and a 1:10 solid/liquid ratio for 5, 10, 20, and 30 min were carried out.
- *pH of the extracting solution:* Extractions with solutions of calcium hydroxide pH 10, 11, 12, and 12.60 (saturated solution) at 60°C and a 1:10 solid/liquid ratio for the period of time selected from the previous experiment were carried out.
- *Temperature and solid/liquid ratio:* A 5×2 factorial experiment for temperature (30, 45, 60, 75, and 90°C) and solid/liquid ratio (1:10 and 1:15) was carried out for the period of time and pH selected from previous experiments.

The protein extraction yield was determined in each of the aforementioned experiments and conditions with the highest yields were considered as optimal.

Statistical Analysis

The results of the extractions were analyzed using GLM procedures of SAS *(20)*. A variance analysis was applied in each of the studies. The effect of the main variables was evaluated, as well as interactions in the factorial design.

Table 2
Cellulose, Hemicellulose, Lignin, Crude Protein and Moisture Contents of Ammonia-Treated and Non-Treated Dwarf Elephant Grass

Sample	Cellulose* (%)	Hemicellulose* (%)	Lignin* (%)	Crude protein* (%)	Moisture (%)
Treated	32.79	20.94	4.01	11.68	10.24
Non-treated	33.72	18.80	3.72	12.26	13.20

*% calculated in dry weight basis.

Table 3
Protein Extraction from Ammonia-Treated Dwarf Elephant Grass with a Calcium Hydroxide Solution at pH 10, 60°C, 1:10 Solid/Liquid Ratio for Several Extraction Times

Extraction time (min)	Protein extraction yield (%)
5	33.71 c
10	33.98 c
20	34.44 b
30	34.90 a
45	34.88 a
60	34.34 b

Different letters indicate significant differences ($p<0.05$).

Results and Discussion

The chemical characterization of ammonia-treated and non-treated dwarf elephant grass is shown in Table 2. The cellulose, hemicellulose, and lignin contents for the non-treated grass are within the range reported by Flores et al. *(3)* for this type of grass and the crude protein content is within the limits reported by Clavero and Ferrer *(2)* for an 8-wk-old dwarf elephant grass. The ammonia treatment solubilized 10.2% of the hemicellulose and 7.2% of the lignin. The slight increases in cellulose and protein are just apparent. Ammonia treatments carried out at higher pressures (300 psi) have solubilized hemicellulose up to 50% and lignin up to 30% *(11)*.

Selection of the Optimal Extraction Conditions of the White Proteins of Dwarf Elephant Grass

Time of Extraction

Experimental conditions applied to optimize the extraction time (pH 10, 60°C, 1:10 solid/liquid ratio) were those reported in the literature for protein extraction from AFEX-treated materials *(9)*. Table 3 shows that the protein extraction increases as the extraction time increases up to 30 min,

Table 4
Protein Extraction from Ammonia-Treated Dwarf Elephant Grass with a Calcium Hydroxide Solution at 60°C, 1:10 Solid/Liquid Ratio, for 30 min and Several pH Values

pH of the calcium hydroxide solution	Protein extraction yield (%)
10	33.71 d
11	34.61 c
12	37.61 b
12.60	45.92 a

Different letters indicate significant differences ($p<0.05$).

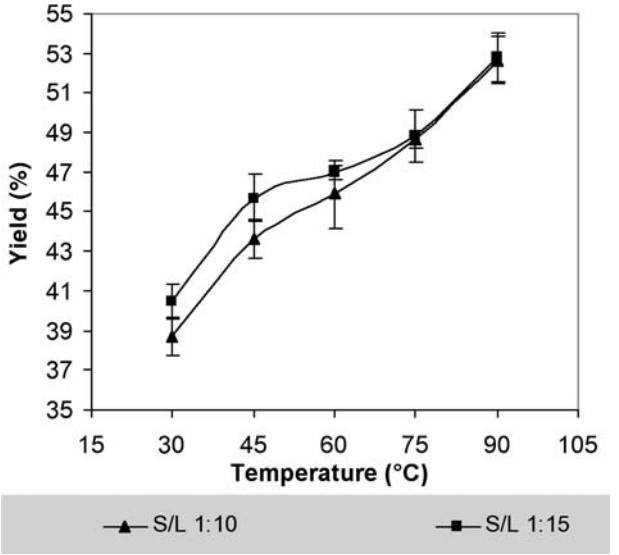

Fig. 1. Effect of temperature and solid/liquid ratio (S/L) on the protein extraction yield for ammonia-treated dwarf elephant grass. pH: 12.6. Time: 30 min.

where a maximum is reached. Protein extracted after this period of time decreases due to insolubilization. The statistical analysis revealed significant differences ($p<0.05$) among the times, although the differences were very small (3.5% between the highest and the smallest values); reproducibility of the soluble protein analysis was extremely high. An extraction time of 30 min was selected for the rest of experiments, although the extraction time will likely be 5 min on an industrial scale, improving agitation and temperature control, among other parameters. In addition, 96.5% of the extractable protein was already extracted at 5 min.

pH of the Extracting Solution

Table 4 shows that the protein yield increases as pH increases when protein is extracted at 60°C and 1:10 solid/liquid ratio for 30 min. The

Table 5
Effect of Temperature on the Protein Extraction Yield for Ammonia-Treated Dwarf Elephant Grass

Temperature (°C)	Yield (%)*
30	39.58 e
45	44.66 d
60	46.44 c
75	48.27 b
90	52.72 a

* Mean for two solid/liquid ratios and three replicates of each. Different letters indicate significant differences ($p<0.05$).

Table 6
Effect of the Solid/Liquid Ratio on the Protein Extraction Yield for Ammonia-Treated Dwarf Elephant Grass

Solid/liquid ratio	Yield (%)
1:10	45.91 a
1:15	46.76 a

Similar letters indicate no significant differences ($p>0.05$)

largest extraction yield was 45.92% ($p<0.05$) for pH 12.6, which was selected for the rest of experiments.

Temperature and Solid/Liquid Ratio

Figure 1 shows that as the extraction temperature increases, the yield of extraction of the proteins from ammonia-treated dwarf elephant grass gradually increases, at optimal pH (12.6) and extraction time (30 min). The increase was significant for all the temperatures as Table 5 indicates ($p<0.05$). For temperatures between 30 and 60°C, the extraction of proteins was always higher at 1:15 solid/liquid ratio, although the differences were not significant ($p>0.05$, Table 6) likely due to the usual variability found in extraction studies of polymers. At higher temperatures, the solid/liquid ratio did not influence the extraction yield, which is convenient since a 1:10 ratio means lower volumes and therefore a lower cost of the extracting agent. Tables 5 and 6 present the statistical analysis for the main effects of temperature and solid/liquid ratio on the extraction yield. According to the results, a temperature of 90°C and a 1:10 solid/liquid ratio were considered as optimal for an extraction yield of 52.65% of white protein. It is not convenient to rise the temperature above 90°C because the protein will suffer deterioration of certain amino acids, mainly lysine *(21)*, decreasing the nutritional quality of the protein.

Effect of the Ammonia Treatment on the White Protein Extraction from Dwarf Elephant Grass

When the grass undergoes the ammonia treatment, the protein extraction yield increases from 11.68% for the same sample of the non-treated grass *(8)* to 52.65% (the present work), which represents about 4.5-fold increase, which suggests that the ammonia treatment defibrillates the grass, so that the protein is more exposed and is released to a greater degree in an alkaline environment from the fibrous matrix. These new proteins may have a better quality, since they could include not only proteins that are constituents of the cell wall themselves but proteins from membrane-bound organelles in the cytoplasm such as mitochondria which are reported to have better amino acid profiles *(11)*.

The optimal pH of the extracting solution was 12.6, the same value found for the non-treated grass *(8)*, indicating that the proteins extracted under both conditions (treated and untreated) reach the maximum solubility at the alkaline end of the pH, where there is a maximum electrostatic repulsion between the protein molecules by increasing their negative charges, which also increases their solubility as the protein molecules move away from their isoelectric point, which is around 4–4.5 *(22)*. The higher increase in protein extraction from pH 12 to pH 12.6 can only be explained by the presence of arginine, the amino acid with the highest pK_a (about 12), which looses the positive charge at the side chain group as pH increases above 12 contributing to the increase of negative charges in the proteins *(21)*.

The extraction of the proteins from the ammonia-treated grass can be carried out at a lower solid/liquid ratio (1:10) than the non-treated (1:15) *(8)*, indicating that proteins from the treated grass are more accessible and therefore more extractable that those from the non-treated grass. On the other hand, the use of a lower solid/liquid implies a smaller expense of reagents. A lower ratio can not be used because of fluidity problems.

The treated grass had a different behavior with respect to temperature because increasing the temperature in the non-treated grass caused denaturation of the proteins and the extraction yield decreased *(8)*, therefore 30°C was the optimal temperature. On the contrary, the highest yield in the treated grass was obtained at 90°C. Protein denaturation could also occur, although it was not evident because much more protein was extracted from the treated grass. It is important to indicate that some proteins become heat resistant when subjected to a previous mild heating *(23)*.

As true protein in grasses is usually about 80% of crude protein *(24)*, and the cytoplasmic protein is about 40–50% of the leaf protein *(16)*, 52.65% is considered a very high yield, near maximum.

In theory, it is possible to use the protein extracts in animal feeding, but it is generally necessary to concentrate them in order to use them efficiently in the preparation of the feeds. In addition, it has been demonstrated that the vegetable extracts usually present toxic compounds that decrease their

protein value *(25)*. Therefore, in order to use the extracted proteins in animal feeding, they should be separated from the extract, and precipitation by heating at an acid pH seems to be the most appropriate method.

Conclusions

The ammonia treatment increased the amount of white protein extracted while partially solubilizing hemicellulose and lignin. The extraction conditions for the proteins from dwarf elephant grass treated with ammonia that rendered the maximum yield, 52.65%, were pH 12.60, 90°C, 1:10 solid/liquid ratio for 30 min. The ammonia treatment increased 4.5 times the white protein extraction yield from dwarf elephant grass, requiring a higher temperature and a lower solid/liquid ratio than for the non-treated grass.

Acknowledgments

The authors thank Parque Tecnológico Universitario and CONDES (University of Zulia) for their economic support, and the Food Technology and Industrial Fermentations Laboratory at the Engineering Faculty, the Nutrition Laboratory at the Medicine Faculty, and the Experimental Center for Animal Production at the Veterinary Sciences Faculty of the University of Zulia for their invaluable help.

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