NITROGEN 15 ABUNDANCE IN PROTEIN FRACTIONS OF BEANS FERTILIZED WITH $(^{15}NH_4)_2SO_4$

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ABSTRACT: Studies evaluating the protein nutritive value of beans labelled with ^{15}N, using nitrogen balance and the quantitation of faecal and urinary endogenous nitrogen, determined by isotopic dilution, have been extensively used. The objective of this research was to verify if the isotopic labelling of raw, freeze dried beans (*Phaseolus vulgaris* L., cultivar Piratã 1) with 1.394 atoms% ^{15}N, resulted in the same abundance of the whole flour and of the protein fractions extracted from the beans with 0.5 mol L^{-1} NaCl. The isotopic abundance found in the whole bean flour, in the protein extract, in the globulin and albumin fractions were respectively: 1.394 ± 0.011; 1.403 ± 0.012; 1.399 ± 0.007 and 1.399 ± 0.028 atoms % of ^{15}N, presenting no difference (P > 0.05). However, a difference was found (P < 0.05) between the above mentioned abundances and the isotopic abundance found in the nitrogen of the proteins in the extraction residue, which was 0.969 ± 0.084. Since the abundances did not differ, the protein nutritive indexes, such as digestibility and biological value, determined from the nitrogen balance and corrected for isotopic dilution, would not be affected by extracting the proteins from the beans with 0.5 mol L^{-1} NaCl. If working with the nitrogen balance of the residual proteins after extraction and even with the whole flours, these indexes could present incorrect values, since the isotopic labelling of the residual proteins was less than that of the protein fractions.
Key words: *Phaseolus vulgaris* L., stable isotopes, isotopic label, nutritive value, experimental nutrition

ABUNDÂNCIA DE ^{15}N EM FRAÇÕES PROTÉICAS DE FEIJÃO FERTILIZADO COM $(^{15}NH_4)_2SO_4$

RESUMO: Estudos para determinação do valor nutritivo da proteína de feijões marcados com ^{15}N, utilizando balanço de nitrogênio e quantificação de nitrogênio endógeno fecal e urinário, determinados por diluição isotópica, têm sido muito utilizados. O objetivo deste trabalho foi verificar se a abundância de ^{15}N feijão cru e liofilizado (*Phaseolus vulgaris* L., cultivar Piratã 1), de 1,394 átomos % de ^{15}N, era a mesma, na farinha integral do feijão, no extrato protéico e nas frações protéicas extraídas do feijão com NaCl 0,5 mol L^{-1}. As abundâncias encontradas na farinha integral, extrato protéico e frações protéicas globulina e albumina foram, respectivamente: 1.394 ± 0.011; 1.403 ± 0.012; 1.399 ± 0.007 e 1.399 ± 0.028 átomos % de ^{15}N, não apresentando diferença (P > 0,05). Entretanto, foi encontrada diferença (P < 0,05) entre as referidas abundâncias e aquela encontrada nas proteínas que permaneceram no resíduo da extração, que foi 0,969 ± 0,084 átomos de ^{15}N. Uma vez que as marcações isotópicas não diferiram, pôde-se inferir que os índices de valor nutritivo de proteínas, como a digestibilidade ou valor biológico, determinados por balanço de nitrogênio e corrigidos por diluição isotópica, não serão afetados pela extração das proteínas do feijão com NaCl 0,5 mol L^{-1}. No caso das proteínas residuais ou mesmo de farinhas integrais, estes índices podem apresentar valores incorretos, visto que a abundância de ^{15}N das proteínas residuais foi menor.
Palavras-chave: *Phaseolus vulgaris* L., isótopos estáveis, marcação isotópica, valor nutritivo, nutrição experimental

INTRODUCTION

Radioactive carbon determinations in pods and seeds found by archaeologists in Aztec and Inca tombs, showed that the cultivation of beans must have been domesticated approximately 7,000 years ago in Central America, the center of its origin, and in the southern regions of the USA, Mexico and the north of South America (Kaplan, 1965). Beans were introduced into Europe in the XVI Century, and since then, have been an important crop in various parts of the world. In Brazil, beans are the protein base of the population's diet. The protein content of the various species of *Phaseolus* varies from 18 to 35%. Given the high daily consumption of this legume, due to economic and cultural problems, it is clear that it is responsible for supplying a significant part of the protein, calories and other nutrients in the world population diet. However, the biological and nutritional value of the bean protein is limited by various factors: limiting amounts of sulphur amino acids, low digestibility and low biological availability of the limiting amino acids, and also by the presence of toxic proteins and other anti-nutritional factors, especially when raw or badly processed.

Beans, despite their importance as a food and the problems connected with its use, have been inadequately studied, for example, in experimental nutrition studies using isotopic labelling with ^{15}N, in order to study the protein metabolism and nutritive index measurements. This research aimed to determine if the ^{15}N abundance obtained in raw, freeze dried beans (*Phaseolus vulgaris* L., cultivar Piratã 1) by fertilization with $(^{15}NH_4)_2SO_4$, was the same as that obtained in the protein fractions obtained by extraction with 0.5 mol L^{-1} NaCl.

MATERIAL AND METHODS

Whole raw, freeze dried beans were isotopically labelled with 1.394 atoms% of ^{15}N, by soil and leaf fertilization with $(^{15}NH_4)_2SO_4$ containing 10 atoms% ^{15}N in excess. The beans were cultivated in pot incubators in Campinas, SP, Brazil. Beans were harvested in the dry state, then frozen and freeze dried, aiming to preserve the raw material and also to help in the grinding and extraction processes of the proteins, and subsequent analyses.

Sample preparation

Beans were ground to a 70 mesh flour and the proteins extracted from this flour using 0.5 mol L^{-1} NaCl (Whitaker & Sgarbieri, 1981). The flour plus 1% polyvinylpolypyrrolidone (Sigma) were mixed with a 0.5 mol L^{-1} NaCl solution in the proportion of 1:6 (w/v). Initially the mixture was submitted to ultrasonic vibration (Bronwill ultrasonic vibrator, model Biosonik IV, with the fine spindle) for 4 minutes, and then agitated for 2 hours with the pH adjusted to 7.0 (Oliveira et al., 1987). The suspension was then centrifuged at 16,300 $\times$ g for 30 min at 4°C (Sorvall centrifuge model RC_2-B) and the supernatant dialysed, also at pH 7.0 (MW<8,000). The dialysed extract was frozen and freeze dried (VIRTIS freeze dryer model 10-146Mr-BA), thus producing a "bean protein extract". When a second centrifugation of the dialysed extract was carried out at 16,300 $\times$ g for 30 min at 4°C, two fractions were separated according to their solubility: one insoluble in water called "globulin fraction" and the other, a water soluble fraction called "albumin fraction", both being frozen and subsequently freeze dried. Under these conditions and at pH 7.0, Oliveira et al. (1987) showed that the globulin fraction obtained was relatively free of albumin, but that the albumin fraction was contaminated with some partially soluble globulins. Protein fractionation was carried out as shown in Figure 1. The extraction was carried out in triplicate, and a pool of each protein fraction collected in order to obtain greater amounts.

Analytical methods

Nitrogen was determined by the semi-micro Kjeldahl method (Cunnif, 1995) and protein as crude protein (N x 5.4) (Mossé, 1990). Nitrogen 15 was determined in the whole flour and protein fractions from the titres obtained in the nitrogen determinations (Bremner, 1965; International Atomic Energy Agency, 1972). The available *in vitro* methionine liberated into the supernatant as a result of protein hydrolysis by pepsin and pancreatin (respectively incubating at 37°C for 2 hours at pH = 7.0 and 24 hours at pH = 8.3) either in the free form or in soluble peptides, was determined as the control, using a modification of the colorimetric method of McCarthy & Sullivan (1941), as proposed by Genovese & Lajolo (1993). To a 1.0 mL sample of the hydrolysate, 0.25 mL of 5 mol L^{-1} NaCl, 25 µL of 10% sodium nitroprussiate and 0.5 mL of 3% glycine was added, the medium being acidified by addition of 0.5 mL 85% phosphoric acid, and reading the absorbance at 520 nm after 5 min of reaction.

Statistical analysis

Data were analyzed by ANOVA, followed by Duncan's new multiple-range test to evaluate differences of the means (Duncan, 1955).

RESULTS AND DISCUSSION

The raw freeze dried bean flour presented a crude protein content of 20.6 ± 0.04%, which is in agreement with that found by Marquez & Lajolo (1981), who reported a value of 21.1% crude protein for the same cultivar. Verifying the protein percentages in the protein fractions of the cultivar under study, separated according to their solubility characteristics, the values were 51.6% for the albumins and 58.1% for the globulins. For the protein extract and residue these protein values were 56.6% and 6.4% respectively. The values of grams of methionine liberated per 100 g protein during the *in vitro* hydrolysis of the raw flour and its protein fractions showed that the highest values were found for the protein extract and the globulin fraction, 0.41 g Met/100g, and the lowest for the whole flour, 0.30 g Met/100g protein. The albumin fraction showed an intermediate value, 0.37 g Met/100 g protein. These values are in accordance with those reported by Pompeu & Roston (1988) who have found a level of bioavailable methionine of 0.39 g Met/100g protein.

Table 1 - Isotopic ^{15}N abundance in whole, raw, freeze dried bean flour (*Phaseolus vulgaris* L., cultivar Piratã 1) and their protein fractions. Beans fertilized with $(^{15}NH_4)_2SO_4$.

Material	Atoms% ^{15}N
Whole flour	1.394 (0.011)[1] a
Protein extract	1.403 (0.012) a
Globulin fraction	1.399 (0.070) a
Albumin fraction	1.399 (0.028) a
Residue	0.969 (0.084) b

[1]The numbers in brackets correspond to the standard deviations of the results with respect to the mean presented (at least n=3). a, b Values with the same notation did not differ for Duncan's multiple-range test ($P > 0.05$).

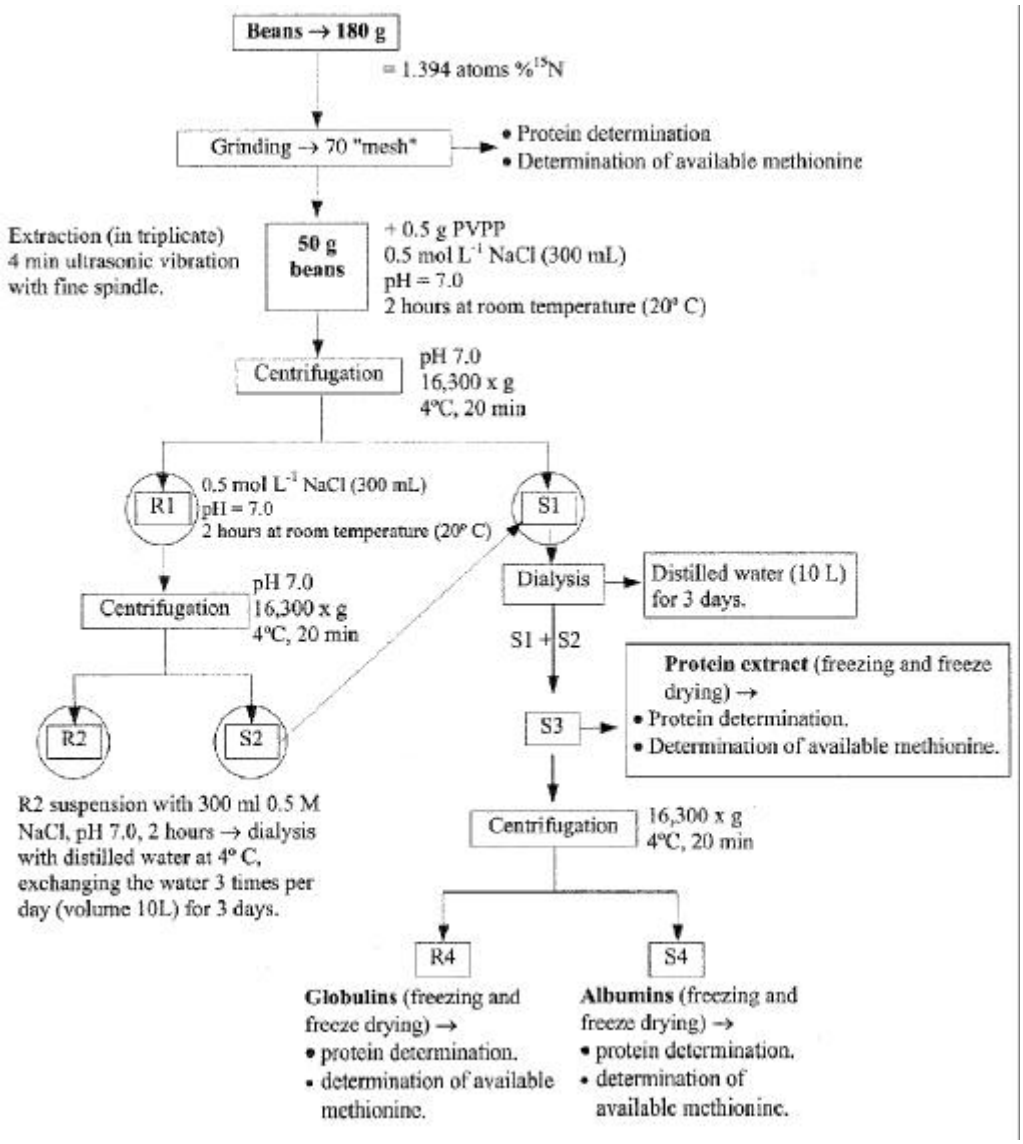

Figure 1 - Fractionation of proteins extracted from raw freeze dried beans (*Phaseolus vulgaris* L., cultivar Piratã 1) using 0.5 mol L^{-1} NaCl.

The isotopic abundances are shown in Table 1. The results for the whole flour, the protein extract and the isolated albumin and globulin fractions showed no difference ($P > 0.05$). However differences were found ($P < 0.05$) between the above cited abundances and the abundance of the nitrogen from the proteins of the extraction residue, which was 0.969 ± 0.084 atoms % ^{15}N.

The results of biological experiments, in which the raw material used as the only protein source of a balanced diet to be used in nutritional assays is labelled with ^{15}N, should be viewed with caution, because the protein constituents of such raw materials can present non-homogenous abundances, at least in the case of this bean. Thus using the whole flour of the raw material as the only protein source of the diet, an isotopic dilution will occur in the faeces (Oliveira & Sgarbieri, 1986; Marquez & Lajolo, 1991), which does not depend exclusively on the secretion and excretion of endogenous or corporal nitrogen by the animal used in the experiment. Those proteins which generally remain in the extraction residue, in the case of the present study, about 30% of the total protein of the beans, which present a relatively less intense abundance of ^{15}N as compared to the extract and fractions obtained by differential solubility, are the main cause of this isotopic dilution. Thus, in this particular case the isotopic dilutions tend to present estimates of the secretions and excretions of the experimental animals superior to those really coming from the body, which could influence the calculations of the protein nutritive indexes. On the other hand, when the amounts of these residual proteins are small, for practical purposes, this influence can be neglected.

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