ARCHIVOS LATINOAMERICANOS DE NUTRICION
Organo Oficial de la Sociedad Latinoamericana de Nutrición

Vol. 45 N° 3, 1995

Effect of germination on the protein content and on the level of specific activity of lipoxygenase-1 in seedlings of three soybean cultivars

Bordingnon J.R. [1], *Ida E.L.* [2], *Oliveira M.C.N.* [1], *y Mandarino J.M.G.* [1]

SUMMARY. The effect of germination on the protein content and in the level of specific activity of lipoxygenase-1 on seedlings of Santa Rosa, FT-2 and Davis soybean cultivars were studied. Soybean seeds of the three cultivars were germinated from 0 to 72 hours in a seed germinator at 30 °C temperature, 100% relative humidity, and absence of light. The seeds were then frozen in liquid nitrogen, freeze-dryed and milled. The lipoxygenase-1 was extracted with 0,02M thris-HCI buffer at pH=8,2 and 13% saccharose for 30 minutes at 4 °C without shaking. The highest levels of specific activity of lipoxygenase-1 per miligram of flour (dry basis) in the Santa Rosa, FT-2 and Davis cultivars were observed between 18 and 36, 18 and 24, and 0 and 12 hours, respectively. The polinomial quadratic model best fits the regression analysis of the variable levels of specific lipoxygenase activity for cultivar Davis (R^2=0,9197). Although for cultivars Santa Rosa (R^2=0,9041)., and FT-2 (R^2=0,7486) the 4th degree polinomial model has shown the best fit. The protein content in the three cultivars studied reached the maximum values at 48h germination. The germination induced a relative increase of the protein content but caused a reduction of the level of specific lipoxigenase-1 activity.

RESUMEN. Efecto de la germinación sobre el contenido de proteína y sobre los niveles de actividad específica de lipoxigenasa-1 en plantines de tres cultivares de soya. En este trabajo se estudió el efecto de la germinación sobre el contenido de proteína y sobre los niveles de actividad específica de lipoxigenasa-1 en plantines de los cultivares de soya Santa Rosa, Davis y FT-2. Las semillas de soya de los tres cultivares germinaron entre 0 y 72h a 30 °C, 100% de humedad relativa y ausencia de luz. Las semillas se congelaron en N líquido, posteriormente fueron liofilizadas y molidas. La lipoxigenasa-1 se extrajo con buffer 0,02M tris-HCl pH 8,2 y sacarosa al 13%, durante 30 min a 4 °C, sin agitación. Los niveles más elevados de actividad específica por mg de harina, expresados en materia seca, para los cultivares Santa Rosa, FT-2 y Davis se observaron entre 18-36, 18-24, 0 y 12 horas, respectivamente. En el análisis de regresión de la variable nivel de actividad específica de lipoxigenasa, para el cultivar Davis, el mejor ajuste se obtuvo con el modelo polinomial cuadrático (R^2=0,9197). El modelo que mejor se ajustó para los cultivares Santa Rosa (R^2=0,9041) y FT-2 (R^2=0,7486) fue el polinomial de cuarto grado. En los tres genotipos estudiados el tenor de proteínas alcanzó el máximo valor a las 48h después del inicio de la germinación. La germinación indujo un aumento del contenido proteico y causó reducción del nivel de actividad específica de la lipoxigenasa-1.

INTRODUCTION

The soybean seed is a rich and economical source of high quality protein. Although its common use for human consumption, has been of low acceptability mainly due to the presence of some indesirable compounds which affect flavour. Among these, trypsin inhibitors, phytic acid and lipoxygenase are the major ones.

The lipoxygenases (E.C. 1.13.11.12) catalize the hydroperoxidation of the free polyunsaturated fatty acids and their derivates which contain the cis, cis-1,4-pentadien system, originating the corresponding monohydroperoxides. These hydroperoxides (9- or 13-cis, trans) are decomposed into aldehydes, ketones, acids and other secundary compounds which are involved in the formation of the undesired flavour characteristic of foods produced from leguminous plants (1). The unpleasant flavour of soybean is known as «beany» flavour and has been described as a limiting factor for its use as food source in the Occident (2).

Even small amounts of lipoxygenase are able to cause the oxidation of the fatty acids and to form the compounds that

1 EMBRAPA-CNPSo -CxP 1061 -CEP 86.001-970- Londrina - PR-Brazil
2 Dept. Tec. Alim. Med. UEL - CxP 6001-CEP 86.051-970, Londrina - PR Brazil

originate the undesirable flavours (3). The soybean lipoxygenases are divided in four isozymes denominated lipoxygenase L_1, L_2, L_{3a}, and L_{3b}, (4). These last two isozymes, having similar properties, are considered identical for analysis purposes and are simply denominated as L_3.

Several processes have been developed to remove or inactivate the soybean lipoxygenases in the attempts to improve its use for human consumption. These processes, however, are usually expensive and not completely suitable since insolubility of proteins may occur and, for some processes, a «over cooked» or «over roasted» flavour may be enhanced (5,6).

The process of germinating the soybean seeds prior to their use for human consumption was also an attempt to overcome some disadvantages associated with the use of the seed «in natura», which are mainly the presence of trypsin inhibitors as well as the lipoxygenases activity (7,8). Depending on the cultivar, the germination process, in addition to the reduction of these compounds, improves the nutritional value of the soybean seed by increasing the protein and mineral contents as well as by decreasing the phytic acid content (9).

There are still some controversies in the literature concerning the main isozyme involved in the formation of n-hexanal. Fisher & Grosh, 1(10) found that lipoxygenase-1 is responsible for the formation of 90% of the 13-hydroperoxide at pH 7,0, and that lipoxygenase-2 induces the formation of only 30% of that compound at the same pH, Matoba et al (11) proved that the n-hexanal is solely a consequence of the 13-hydroperoxide breakdown.

The objetive of the present work was to investigate the effect of germination on the protein content and on the level of specific activity of lipoxygenase-1 in seedlings of Santa Rosa, FT-2 and Davis soybean cultivars.

MATERIAL AND METHODS

Cultivars

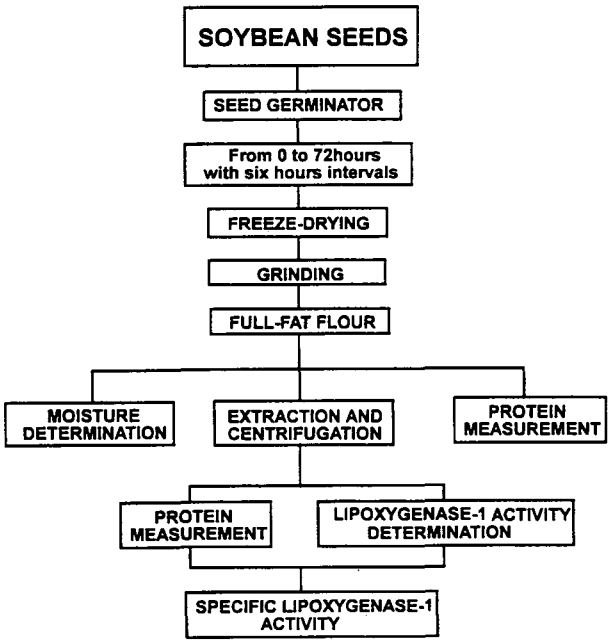
The three soybean cultivars used in this study were chosen based on the characteristics of their lipoxygenase-1 activity (low, intermediate, and high), according to Barros et al (12). The cultivars were: FT-2 (low L_1 activity), Davis (intermediate L_1 activity), and Santa Rosa (high L_1 activity).

The seeds of the three cultivars, harvested in the 1987/88 and 1988/89 growing seasons, were provided by the Germoplasm Bank of the National Soybean Research Center - CNPSo of the Brasilian Enterprise for Agriculture Research - EMBRAPA, located at Londrina Country, Paraná State, South Brazil.

Analytical Methods

Seed germination: The steps for seed germination and analytical determinations are shown in Fig. 1. The seeds of the three cultivars were germinated from 0 to 72 hours with six hours intervals. For each germination time a sample of 150 non surface sterilized seeds were previously hand selected and divided into three 50-seed subsamples. The subsamples were arranged on top of two sheets of germination paper which had been previously moinstened with distilled water. A third moistened sheet was used to cover the seeds. Each subsamples was then rolled up and randomly transfered to a 30 °C temperature and 100% relative humidity seed germinator, and kept in the dark for the different periods of time.
After each germination period the rolls were retreived from the germinator and dismounted. The seeds from each were then frozen in liquid nitrogen and transfered to opaque paper bags which were immediately vaccum sealed in plastic bags and stored at - 18°C for further freeze-drying.

FIGURE 1
Seed germination procedure and analytical determinations



Freeze-drying: After freezing all samples were transfered inside of paper bags to a Freeze Dryer (Labconco model 8) for 27 to 36 hours until humidity was stabilized around 5%, to allow grinding.

Grinding: All samples were then ground for one minute in a cyclone Sample Mill and stored at-18° in sealed plastic flasks until use.

Lipoxygenase-1 extraction: The extraction condition and the effects of granulometry and freezing time on the lipxoygenase-1 activity were studied by Bordingon, (13).

The lipoxygenase-1 extraction was caried out using 300 mg of full-fat flour and 15ml of 2mM Tris-HCl (pH 8,2) buffer,

containing 13% of saccharose, at 4 °C for 30 minutes without shaking. The obtained extracts were centrifuged for 15 minutes at 11.000g at 4 °C. The supernatant was used for lipoxigenase-1 activity and soluble protein determination. Three extractions were done for each subsample.

Substrate preparation: Linoleic acid 10mM was prepared as described by Axelrod et al (4). According to the description, 70mg of linoleic acid and the same amount of Tween 20 were weighed, carefully homogeneised with 4ml of oxygen-free distilled water in a volumetric flask of 25 ml. Enough amount of 0.5N NaOH was added to produce an opticaly clear solution. The volume was completed with oxygen-free distilled water and the solution was homogenized by inversion avoiding excessive aeration. Aliquots of 1 and 2 ml were stored at -18 °C in ambar glass flaks previously sealed under a nitrogen atmosphere.

Lypoxygenase-1 activity measurement: The lipoxygenase-1 activity was determined using 100µl of substrate; (2,9-X) ml of 0.02M borate buffer (pH 9,0) and Xml of the extract. Reaction velocity was measured at 280 nm in a Varian 634 Spectrophotometer al 25 °C in 30 seconds intervals until seven minutes after the enzyme addition. The inclination angle formed by the relation between the optical density variation (ΔDO) and the time (minutes) was calculated. These values were used to calculate the lipoxygenase-1 activity.
One unit of activity was defined as the amount of enzyme (mg) necessary to produce a variation of 0.001 unit on the optical density, under the assay conditions. The activity determinations were accomplished in triplicate.
Specific lipoxygenase-1 activity (SA) was defined as the ratio between the specific activity of lipoxygenase-1/mg of soybean flour (on dry-basis), multiplied by 15:

$$SA = \frac{\Delta OD.min^{-1}.\ ml^{-1}}{P.F.\ 0.001} \times 15$$

where: $\Delta OD.min^{-1}.ml^{-1}$= optical density variation/minute/ml of extract;

15= total extract volume (ml);

P= soluble protein amount (mg);

F= flour amount (mg) in dry basis, used in the extraction.

Protein measurement: The protein content of the extracts was determined in triplicate according to the method of Lowry et al (14).
The nitrogen determination in the flours was made according to the micro-Kjeldahl method (15). The protein conversion factor used was 6.25.

Moisture determination: The moisture content of the germinated seeds was determined using a drying oven regulated at 105 °C (15)

Experimental design and statistical analysis: The experimental design used to evaluate the results obtained from the different time of germination of the soybean seeds (Santa Rosa, FT-2 and Davis) was the complete randomized design of the treatment combinations in factorial arrangement = 12 times x 3 cultivars, with 3 replications, totalizing 36 treatments. An exploratory analysis of the data was carried out for the lipoxygenase activity and protein through the Liliefors, (16) and Burr & Foster, apud Anderson & McLean, (17) tests. From these analyse it was possible to evaluate the residue distribution and variance homogeneity of the treatments studied.
These variables were also submited to variance (18) and to regression (19) analyse, while the Multiple Range Test used was Tukey (P=0.05).

RESULTS AND DISCUSSION

Germination of the soybean seeds: The germination of the soybean seeds was made without previous seed hydration to avoid lixiviation of some constituents, according to Giri et al, (20).
Maximum germination time used in this study was 72 hours, since prolonged germination could cause a pronounced growth of the radicle, increasing the probability of fungus growth (21) and color changes (9).
Since the radicle size can vary during the germination process, depending on the soybean cultivar (9, 22, 23), in this study the germinated seeds were classified according to the germination time. Soybean seeds not submited to germination were used as the experimental control.

Level of specific lipoxygenase-1 activity: the results of the specific activity per miligram of flour (dry basis) defined as level of specific activity are given in Table 1. Concerning the variable «level of specific activity», the ANOVA of data was significant for the factors: time, cultivar and for the interaction, with a variation coefficient of 6,345%.

Comparing the three cultivars in each germination time, it was observed that frequently the Davis cultivar differed statiscaly from Santa Rosa and FT-2 cultivars. At zero and six hours of germination time it showed similar results to those obtained for Santa Rosa. The FT-2 cultivar, in its turn, differed statistically from Santa Rosa in all germination times, except at 72 hours, where significant statistical difference was not observed in the level of specific activity between the two cultivars (Table 1).

TABLE 1
MEANS OF THE INTERACTION GERMINATION TIME X CULTIVAR FOR THE VARIABLE «SPECIFIC ACTIVITY» AND DECREASE PERCENTAGE (DEC %) OF ACTIVITY OF LIPOXYGENASE-1 PER MILIGRAM OF FULL-FAT SOYBEAN FLOURS-CULTIVARS SANTA ROSA, FT-2 AND DAVIS.

Time (hs)	Cultivar					
	Santa Rosa	dec(%)	FT2	dec(%)	Davis	dec(%)
0	1.166[1] abc B	0,00[2]	1,397 e A	0.00	1,035 a B	0,00
6	1,029 bc B	11,75	1,312 e A	6.084	1,028 a B	0,67
12	1,035 bc B	11,23	1,335 e A	4,44	0,841 ab C	18,74
18	1,182 ab B	-1,36	2,844 a A	-103,58	0,801 bc C	22,60
24	1,343 a B	-15,52	2,717 ab A	-94,49	0,756 bc C	26,96
30	1,280 a B	-9,78	1,949 d A	-39,51	0,689 bcd C	33,43
36	1,322 a B	-13,38	2,621 b A	-87,62	0,684 bcd C	33,91
42	1,055 bc B	9,52	2,174 c A	-55,60	0,659 bcd C	36,33
48	1,023 bc B	12,26	2,515 e A	-8,44	0,683 bcd C	34,01
54	0,962 cd B	17,49	1,450 e A	-3,70	0,611 cde C	41,06
60	0,778 de B	33,28	1,047 f A	25,05	0,531 de C	44,69
72	0,708 e A	39,28	0,805 g A	42,38	0,438 e B	57,68

1 Means followed by the same lower case letter in each column and same upper case letter in each row, are not significantly different at P=0.05 using Tukey's multiple range test.
2 In relation to time zero.

TABLE 2
MEANS OF THE INTERACTION GERMINATION TIME X CULTIVAR FOR THE VARIABLE «PROTEIN» AND INCREASE PERCENTAGE (INC %) OF PROTEIN IN FULL-FAT SOYBEAN FLOURS OBTAINED FROM THE CULTIVARS SANTA ROSA, FT-2 AND DAVIS DURING THE GERMINATION PROCESS.

Time (hs)	Cultivar					
	Santa Rosa	inc(%)	FT2	inc(%)	Davis	inc(%)
0	135,06[1] g B	0,00[2]	38,50 f A	0,00	38,29 g A	0,00
6	40,19 e A	11,05	39,49 e B	2,56	39,68 f B	0,98
12	39,25 f C	8,92	41,52 c A	7,83	39,93 ef B	1,63
18	39,13 f C	8,56	41,19 d A	6,97	40,89 c B	4,08
24	40,56 d B	12,54	41,57 c A	7,95	40,44 d B	2,93
30	40,92 dc B	13,50	41,60 c A	8,04	40,23 de C	2,40
36	40,61 cd B	12,67	42,30 b A	11,67	40,40 d B	2,83
42	40,63 cd C	12,72	31,80 c A	8,55	40,98 c B	4,29
48	41,05 b C	13,89	43,19 a A	12,16	42,61 ab B	8,44
54	40,57 d C	12,56	43,22 a A	12,24	42,38 ab B	7,88
60	40,44 dc C	12,19	40,37 a A	4,84	42,30 b B	7,66
72	41,51 a C	15,17	43,38 a A	12,65	42,71 a B	8,69

1 Means followed by the same lower case letter in each column and same upper case letter in each row, are not significantly different at P=0.05 using Tukey's multiple range test.
2 In relation to time zero.

The Tukey Multiple Range Test (P=0.05) showed that for the FT-2 cultivar there was higher differentiation among the average multiple comparison groups. On the contrary, for the Santa Rosa and Davis cultivars this did not occur (Table 1).

The Davis cultivar showed, from the 12 hours time of, a gradual decrease of the level of specific activity, while the Santa Rosa and FT-2 cultivars showed more variation during the germination times studied (Table 1). Considering the cultivars within germination times, the percentages of decrease were 57.68, 42.38 and 39.28% for the Davis, Santa Rosa and FT-2 cultivars, respectively. These values were very similar to those found by Suburbine et al. (8) (aproximately 55%) using seeds of cultivar Jupiter. Since in this study the size of the germinated seeds were not measured during germination, it is difficult to compare these results with those reported by Hildebrand et al (24), which were based on the size (mm) of the seeds and not on the germination time.

The regression analysis of the experimental data, obtained from the variable «level specific activity/mg of flour» during germination, indicated that the quadratic polinomial model fits best the Davis cultivar (R^2=0.9197) and the polinominal model of the fourth degree fits best the Santa Rosa and FT-2 cultivars (R^2=0.9041 and 0.7486, respectively).

Effect of the germination on the amount of protein of the full-fat soybean flours: The average of total protein values in the full-fat germinated soybean flours from Santa Rossa, FT-2 and Davis cultivars are shown in Table 2.

The ANOVA of these results was significant to the factors: time, cultivar and to the interaction time X cultivar, with a variation coefficient of 0.29%.

Comparing the amount of protein of the three cultivars at each germination time, it was observed that the FT-2 cultivar primarely showed higher levels of protein. Besides, the values of total protein of this cultivar statiscally differed from those obtained for the Davis and Santa Rosa cultivars in almost all the germination times. Only at 0 and 15 hours no statistical differences were found when compared to the Davis cultivar.

The Santa Rosa cultivar showed lower values of the total protein in seven different germination times, differing statiscally from the FT-2 and Davis cultivars in almost all the germination times (Table 2).

After studing the protein amount according to the germination time in each cultivar it was observed that in the FT-2 cultivar there was a higher differentiation among the average multiple comparison groups, while for the Santa Rosa and Davis cultivars this did not hapen. Despite showing the lowest percentage of total protein, the cultivar Santa Rosa showed the highest increase of this variable (15.17%) followed by FT-2 (12.65%) and Davis (8.69%) (Table 2). Generally it was observed that in the three cultivars studied protein increased similarly.

The increase in the protein contents (expressed on dry basis), for the three cultivars in relation to the control, after 72 hours of germination (Santa Rosa= 15.17%, FT-2=12.65% and Davis =8.69%) were higher than those found by Jiménez

et al, (25) (4.4% for the Siatsa cultivar germinated for 72 hours), by Bates et al (26) (5.8% for the Bragg cultivar germinated for 96 hours), by Mostafa & Rhama, (7) (decrease of 0.4% for the Calland cultivar germinated for 72 hours). These increase are relative according to Mostafa & Rhama, (7), since they occur due to the decrease of other compounds during the germination.

Among the regression models, the polinomial of the fifth degree had the best fit for the variable «total protein» of the cultivar Davis, with a determination coefficient of 0.8537. For the cultivars FT-2 and Santa Rosa the quadratic model had the best fit, with determination coefficients of 0.8981 and 0.8470, respectively.

ACKNOWLEDGMENTS

The authors would like to acknowledge the financial support provided by CPG/UEL, CONCITEC/PR e CNPq.

REFERENCES

1. Eskin N.A.M., Grossman S., Plisky A. Biochemistry of lipoxygenase in relation to food quality. Critical Reviews in Food Sci Nutr. Boca Raton, v.9, p. 1-40, 1977.
2. Rackis J.J., Sessa D.J., Honig D.H. Flavour problems of vegetables proteins. J.A.O.C.S., Champaign, v.56, p. 262-271, 1979.
3. Gomes J.C., Visentainier J.V., Moreira M.A., et al. Atividade de lipoxigenase e teor de n-hexanal em farinhas de soja. Ar. Biol. Tecnol. Curitiba, v.30, p.481-500, 1987.
4. Aexelrod B, Cheesbroough T.M., Laakso S. Lipoxygenase from soybeans. Methods Enzimol. San Diego, v.71, p.441-451. 1981.
5. Eldrigde A.C., Warner K., Wolf W.J. Alcohol treatment of soybean and soybean protein products. Cereal Chem. St. Paul, v. 54, n.6, p.1229-1237.
6. Esaka M., Suziki K., Kubota K. Inactivation of lipoxygenase and trypsin in soybean on microwave irradiation. Agric. Biol Chem. Tokyo, v.50 p.2395-2396, 1986.
7. Mostafa M.M. & E.H. Rhama. Chemical and nutritional changes in soybean during germination. Food Chemistry, Barking, v.23, p.257-275. 1987.
8. Suburbie F., D. Mendizabal, C. Mendizabal. Germination of soybean and its modifying effects on the quality of full-fat soy flour. J.A.O.C.S., Champaign, v.48 p.192-194. 1981.
9. Ribeiro M.L.L. Efeito do processo de germinação sobre os constituintes nutricionais e antinutricionais de 2 cultivares de soja. Londrina: UEL, 98p. Dissertação (tese de mestrado) Universidade Estadual de Londrina. 1992.
10. Fischer K.H., Grosch W. Co-oxidation von Linosäure zu flüchtigen verbindungen von Lipoxygenase-Isozymen aus Sojabohnen. Z. Lebensmi-Unters. Forsch, Heidelberg, v. 165p. 137-139, 1977.
11. Matoba T., Hidaka H., Kitamura K. et al. Contribution of hydroperoxide lypase activity to n-hexanal formation in soybean. J Agric Food Chem. Washington v. 33 p.356-358, 1985 (A).
12. Barros E.G. Moreira M.a., Fontes e.P.B. Ativadade de lipoxigenase L_1 e L_1 em cultivares comerciais de soja. Arq. Biol. Tecnol. Curitiba, v.27 p.381-386. 1984.
13. Bordingnon J.R. Determinação de lipoxigenase-a: efeito da germinação sobre o teor de proteína e atividade dessa enzima em soja. Londrina: UEL, 92p. Dissertação (tese de mestrado) Universidade Estadual de Londrina. 1993.
14. Lowry O.N., Rosebrough N.J., Farr A.L. et al. Protein measurement with the folin phenol reagent. J. Biol. Chem. Baltimore, v. 193, p265-275. 1951.
15. Instituto Adolfo Lutz. Normas analíticas. São Paulo: Instituto Adolfo Lutz, 1985.
16. Lilliefors J.W. On the Kolmogorov-Smirnov Test for Normality with mean and Variance Unknown. J. Amer Statis. Ass (s.I.), v.62, p.399-402. 1967.
17. Anderson V.L., McLean R.A. Design of experiments: a realist approach. New York: Marcel Dekker, p.16-24. 1974.
18. Cochran W.G., Cox G.M. Experimental Design 29 th e. New York: J. Willey 809p. 1981.
19. Draper N.R. Smith H. Applied Regression Analysis. 2nd. New York: J. Willey, 709p. 1981.
20. Giri J., Parvathan, Santhini K. Effect of germination on the leves of pectins, phytins and minerals in three selected legumes. Ind. J. Nutr. Diet. Coimbatore, v. 18, p.87-91, 1981.
21. Abdullan A., Baldwin R., Minor H. Germination effects and antinutrients of mungbeans and two strains of small-seed soybeans. J Food Prot. Ames, v.47, n.6, p.441-444. 1984.
22. East J.W., Nakayama T.O., Parkan S.B. Changes in stachyose, raffinose, sucrose and monosaccharides during germination of soybeans. Crop Sci. Madison v.12, n.1, p.7-9, 1972.
23. Suparmom, Markakis P. Tempeh prepared from germinated soybeans. J Food Sci, Chicago v.52, p1736-1737. 1987.
24. Hildebrand D.F., Versluys R.T., Collins G.B. Changes in lipoxygenase isozyme levels during soybean embryo development. Plant Sci. v.75, p.1-8. 1991.
25. Jiménez M.F.M., Elías L.G., Bressani R. Estudios bioquímicos y nutricionales de la semilla germinada de soya. Arch. Latinoam. Nutr. v.35, p.480-490. 1985.
26. Bates R.P., Knapp F.W., Araujo P.E. Protein quality of green-mature, dry mature and sprouted soybeans. J Food Sci. Chicago, v.42, p.271-272. 1977.

Recibido: 29-09-1994
Aceptado: 21-04-1995