ANTINUTRIENT OCCURRENCE AND SOME PHYSICOCHEMICAL PROPERTIES OF THE PROTEIN FRACTIONS OF FIVE BRAZILIAN SOYBEAN VARIETIES

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SUMMARY

Protein solubility under different pH conditions, amino acid composition of proteins in the flours, and proteins isolated at a pH of 4.5 (P.I.), as well as trypsin inhibitor and hemagglutinin activities were studied in five Brazilian soybean varieties. The levels of protein and non-protein amino compounds were similar for all varieties. More protein was extracted in slightly alkaline (pH 8.5) water solution than with plain water. Lowering the pH of the extract to 4.5 caused the precipitation of 83% to 86% of the protein. Of the nitrogen remaining in the whey 31% to 39% could be precipitated with 5% trichloroacetic acid. Protein isolated inhibited amino acid patterns similar to those of defatted flours. Trypsin inhibitor activities were higher in the plain water extracts than in the extracts at pH 8.5. Most of the trypsin inhibitory activity was associated with the whey fraction, soluble at a pH of 4.5, while the hemagglutinin activities decreased considerably in the pH 4.5 supernatant, indicating precipitation of the hemagglutinins in this pH.

INTRODUCTION

Soybean is recognized as the most important protein source

Manuscrito modificado recibido: 19–5–80.

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of vegetable origin. The high nutritive value of soybean protein has been observed for quite a long time and has been well documented (1-3). Osborne and Mendel (1) were apparently the first investigators to observe, however, that the raw soybean was toxic to the rat, and that heat in the presence of moisture increased considerably the nutritive value of this legume seed.

Bowman (4), and Ham and Sandstedt (5) were the first researchers to discover a substance in the crude soybean extract which *in vitro* inhibited the trypsin activity on casein. Kunitz (6-8) described the isolation and crystallization of a soybean trypsin inhibitor which was precipitable by TCA, non-dialyzable, and having a molecular weight of 24,000.

Chernick, Lepkovsky and Chaikoff (9) demonstrated that rats and chicks fed with a diet containing crude soybean flour exhibited depressed growth and an enlarged pancreas, with a greater secretion of digestive enzymes and protein.

Using DEAE-cellulose chromatography, Rackis *et al.* (10) isolated from the pH 4.5 soybean whey, two trypsin inhibitors which they named $SBTIA_1$ and $SBTIA_2$. In a similar manner, Rackis and Anderson (11) isolated directly from a crude soybean extract four different trypsin inhibitors which they called $SBTIA_1$, $SBTIA_2$, $SBTIB_1$ and $SBTIB_2$, all of them with high inhibitory activities, their molecular weights varying between 8,000 and 24,000.

Another type of antinutritional factor in soybean is the hemagglutinin, a name given by Liener (12). Liener and Pallansch (13) were the first workers to purify a toxic protein from soybean which strongly inhibited growth when given orally to the rat and killed the animal if used intraperitoneally. This protein was essentially free of antitryptic activity but showed a marked ability to agglutinate red blood cells from various species *in vitro*. The presence of multiple molecular forms was suggested by several workers and demonstrated by Lis *et al.* (14), who separated four different hemagglutinins (HG_I, HG_{II}, HG_{III}, and HG_{IV}) on DEAE-cellulose chromatography. All four hemagglutinins appeared to be glycoproteins containing 4.5% mannose and 1% glucosamine, having a molecular weight of about 110,000.

Using isoelectric focusing, Catsimpoolas and Meyer (15) showed the presence of four types of hemagglutinins (A, B, C, and D) which were immunochemically identical. The most abundant was the B form which seemed to be identical to HG_I identified by Lis *et al.* (14), and also to the form purified previously by Liener (13).

The objective of the present study was to compare the extent of proteins extractability and the activities of trypsin inhibitors and hemagglutinins in five Brazilian soybean varieties.

MATERIALS AND METHODS

The soybean varieties used in this study were obtained from the Leguminous Plant Section of the Agronomic Institute of Campinas, State of São Paulo, Brazil.

Nitrogen content in the flours and in different extracts was determined by the semimicro-Kjeldahl method and the protein content by multiplying %Nx6.25, or by applying the method of Lowry *et al.* (16). Non-protein nitrogen was determined in the supernatants after precipitation of the proteins with TCA at a final concentration of 5%. For the preparation of defatted flour the material was extracted with petroleum ether (BP 30-60°C), thus reducing the residual oil to less than 2% on a dry basis.

The protein extraction was performed by suspending the flour in distilled water using a solid/liquid ratio of 1:20 (w/v). The pH was adjusted to 8.5 with 1*N* NaOH solution, following agitation for two hours and, afterwards, through centrifugation (8,000 xg) during 10 minutes. Most of the extracted proteins were precipitated from the alkaline solution by adjusting the pH to 4.5 with 1*N* HCl solution. The isoelectric proteins were separated by centrifugation.

Determinations of amino acids were performed on the hydrolysates using the Beckman Model 120C Amino Acid Analyzer and the procedure recommended by the manufacturer.

For the determination of trypsin inhibitor activity the method used was essentially the casein digestion method described by Kunitz (8) and modified by Kakade, Simons and Liener (17).

The hemagglutinin activity was determined by the method of Liener (18) subjected to some modifications. In a 150 ml erlenmeyer, containing 5g of defatted flour, 50 ml of a saline solution (0.85% NaCl) were added and placed in a shaker at 200 rpm for 2 hours. The pH of the suspension was about 6.4. A supernatant was obtained by centrifuging the suspension at 8,000 xg during 20 minutes. The volume was adjusted to 50 ml with saline solution. Part of the saline extract was treated with HCl to bring the pH to 4.5, in order to precipitate most of the proteins.

For the alkaline extraction the flour was suspended in water

and the pH raised to 8.5 with 1*N* NaOH solution adjusting periodically the pH during the 2-hour extraction period. The rest of the procedure was identical to that used for extraction at pH 6.4. For preparing the blood suspension, fresh rabbit blood was obtained by heart puncture, and centrifuged at 1,500 rpm in a clinical centrifuge to separate the plasma. The red cells were washed three times with saline solution. A 4% suspension was prepared in saline solution and the pH adjusted to 7.0 with phosphate buffer. Nine volumes of the suspension were mixed with one volume of a 1% trypsin solution and incubated in a water bath at 37°C for one hour. The trypsinized cell suspension was then centrifuged at low speed, washed three times with saline solution and suspended to a 4% concentration. With this stock suspension a standard solution (1:40 dilution) was prepared to give 0.5 absorbance at 620 nm, using saline as a blank.

The hemagglutinin activity test consisted of serial dilutions of the extract with the saline solution, to find the minimum concentration of protein still capable of giving a positive hemagglutinating reaction. In the reaction mixture, equal volumes of the standard cell suspension and of the diluted extract were mixed and allowed to stand for one hour at room temperature. After this time, the agglutination could be detected visually.

RESULTS AND DISCUSSION

The crude protein content of the five varieties studied ranged from 43 to 49.4% in the defatted flours on a dry-weight basis.

Results of the extraction and fractionation of the nitrogenous substances from defatted flours are shown in Table 1. The fractionation was based on the isoelectric point of the majority of the soybean proteins (pH 4.5), and in the precipitation with TCA solution at a 5% final concentration. As the data reveal, a total of 83% to 86% of the extracted protein precipitated at pH 4.5, leaving a supernatant (whey) which contained 14% to 17% of the extracted nitrogenous substances. Non-protein nitrogen (NPN) which appeared in the supernatant after treating the extract with TCA, accounted for 9% to 11% of the extracted nitrogen (% Nx6.25). Of the remaining nitrogen in the whey, about 31% to 39% could be precipitated with 5% trichloroacetic acid.

Table 2 shows the amino acid composition of the defatted flours (D.F.) and of the protein isolates (P.I.) for the five varieties studied. No significant differences in their amino acid profiles

TABLE 1

EXTRACTION AND FRACTIONATION OF THE NITROGENOUS SUBSTANCES FROM DEFATTED FLOUR OF FIVE SOYBEAN VARIETIES (%N x 6.25)

Fraction	Soybean varieties				
	Santa Rosa	Industrial	IAC-1	IAC-72-1385	UFV-1
Protein in the flour (% dry basis)	47.7	48.7	42.9	46.1	49.4
Protein in the water extract (% of total in the flour)	75.8	71.0	75.3	84.5	71.0
Protein in the pH 8.5 extract (% of total in the flour)	85.9	83.7	80.9	90.7	82.8
Insoluble protein at pH 4.5 (% of total extracted at pH 8.5)	84.3	86.1	83.0	86.2	85.7
Nitrogenous substances soluble at pH 4.5 (% of total extracted at pH 8.5)	15.7	13.9	17.0	13.8	14.3
Protein soluble at pH 4.5 precipitable with TCA (% of total extracted at pH 8.5)	6.1	4.4	6.1	4.8	4.8
Non-protein nitrogenous substances (% of total extracted at pH 8.5)	9.6	9.5	10.9	9.0	9.5

TABLE 2

AMINO ACID COMPOSITION OF THE DEFATTED FLOUR AND PROTEIN ISOLATE OF FIVE SOYBEAN VARIETIES (g amino acid/16 g N)*

	Santa Rosa		Industrial		I.A.C.–1		I.A.C.–72–1385		UFV–1	
	D.F.	P.I.	D.F.	P.I.	D.F.	P.I.	D.F.	P.I.	D.F.	P.I.
Lys	7.0	6.8	6.8	6.2	6.3	6.8	6.8	7.0	6.7	6.6
His	2.2	2.4	2.4	2.2	2.0	2.2	2.3	2.3	2.3	2.3
NH_3	2.2	2.2	2.2	2.2	2.0	2.2	2.8	2.3	2.2	2.2
Arg	6.2	6.7	6.7	6.7	5.8	6.7	6.3	7.2	6.2	6.8
Asp	15.0	14.6	16.3	14.2	14.5	14.5	15.6	15.6	17.3	14.8
Thr	4.4	4.4	4.2	4.0	4.0	3.9	4.2	4.1	4.2	3.8
Ser	6.7	6.0	6.4	5.9	6.0	5.9	6.6	6.3	6.7	5.9
Glu	30.7	31.0	30.0	31.0	27.6	30.7	31.3	33.3	30.7	31.0
Pro	3.9	3.8	3.7	3.6	3.5	3.6	3.7	4.0	4.0	3.7
Gly	5.0	4.6	5.0	4.5	4.7	4.6	5.0	4.8	5.2	4.6
Ala	5.0	4.6	5.0	4.4	5.1	4.5	4.9	4.7	4.9	4.4
1/2-Cys	1.0	1.2	0.8	1.3	0.8	0.8	0.9	0.8	1.0	1.0
Val	3.6	4.8	3.6	4.8	3.4	4.6	3.7	5.0	3.5	4.7
Met	1.1	0.9	1.2	1.0	1.0	1.1	1.0	1.1	1.3	1.0
Ileu	3.5	4.8	3.4	4.6	3.0	4.7	3.9	5.0	3.2	4.6
Leu	8.7	9.2	8.8	8.9	7.7	9.0	8.4	8.7	8.4	8.9
Tyr	3.7	3.7	3.7	3.6	3.3	3.6	3.7	3.9	3.7	3.5
Phe	4.9	5.4	4.8	5.1	4.7	5.3	5.2	5.8	4.8	5.2

* Results are means of duplicate determinations.
D.F. = Defatted flour.
P.I. = Protein isolate.

were apparent either among varieties or between the defatted flour and the protein isolate within each variety. As expected for the soybean proteins, all varieties were low in sulphur-containing amino acids. As is known, in most legume foods, including soybean, protein quality is also affected differently by the methionine availability, and not only by their content of this amino acid as determined by chemical methods.

The typsin-inhibitor activities of the water (pH 6.4) extracts, the alkaline (pH 8.5) extracts and their corresponding whey protein fractions are detailed in Tables 3 and 4. The results are expressed in terms of units of trypsin inhibited per ml of the diluted extracts (1:50 dilution), and also as units per mg of the protein extracted. The main findings in this study were the following: a) the protein solubility and extractability were higher in the alkaline (pH 8.5) solutions, but the inhibitor activity, both per ml of extract and per mg of protein, was higher in the water extracts; b) for both the water and the mild alkaline extracts, most of the inhibitory activity remained in the supernatant after isoelectric precipitation of the proteins at a pH of 4.5.

TABLE 3

FRACTIONATION OF TRYPSIN INHIBITOR ACTIVITY DURING ISOLATION OF PROTEIN FROM WATER EXTRACTS OF DEFATTED FLOURS OF FIVE SOYBEAN VARIETIES

Varieties	Water extract* (pH 6.4)		Supernatant* (pH 4.5)	
	TUI/ml Extract	TUI/mg Protein	TUI/ml Supernatant	TUI/mg Protein
Santa Rosa	147.2	357.8	114.6	1,512.3
Industrial	155.8	249.0	109.8	1,146.3
IAC-1	108.9	322.9	84.2	1,155.3
IAC-72-1385	112.1	219.1	69.8	1,078.7
UFV-1	114.8	303.7	72.0	611.8

* Adjusted to the same volume.

TUI = Trypsin units inhibited.

TABLE 4

FRACTIONATION OF TRYPSIN INHIBITOR ACTIVITY DURING ISOLATION OF PROTEIN FROM ALKALINE EXTRACTS (pH 8.5) OF DEFATTED FLOURS OF FIVE SOYBEAN VARIETIES

Varieties	Alkaline extract* (pH 8.5)		Supernatant* (pH 4.5)	
	TUI/ml Extract	TUI/mg Protein	TUI/ml Supernatant	TUI/mg Protein
Santa Rosa	127.8	307.5	81.3	1,298.0
Industrial	93.0	157.6	58.3	732.5
IAC-1	92.7	202.8	33.5	662.5
IAC-72-1385	93.8	169.0	29.0	443.0
UFV-1	106.1	152.9	87.7	1,268.4

* Adjusted to the same volume.

TUI = Trypsin units inhibited.

The results of the hemagglutinating activities of the saline extracts and of the alkaline (pH 8.5) extracts are shown in Tables 5 and 6, respectively. The hemagglutinating capacity varied from 1.59 μg protein/ml for the Santa Rosa variety, to 0.4 μg protein/ml for the IAC-1 in the saline (pH 6.4) extracts. In the alkaline (pH 8.5) extracts the hemagglutinating capacity presented less variation, fluctuating from 0.63 μg protein/ml for the UFV-1 variety, to 0.91 μg protein/ml for the Santa Rosa.

In both types of extracts, the activities decreased substantially in the pH 4.5 supernatant. This decrease was from 6 to 45-fold in the saline solution, and from 7 to 19-fold in the alkaline extracts supernatant, indicating precipitation of the hemagglutinins at a pH of 4.5, both from the saline and from the alkaline extracts.

The nutritional significance of hemagglutinins still constitutes a somewhat debatable issue.

Turner and Liener (19) recently removed the hemagglutinins from a soybean extract by affinity chromotography, and after conducting some PER measurements, concluded that such factors are a relatively minor contribution to the poor nutritive value of

TABLE 5

HEMAGGLUTININ ACTIVITIES IN DIFFERENT PROTEIN FRACTIONS FROM SALINE EXTRACTS OF FIVE SOYBEAN VARIETIES*

Varieties	Saline extract (pH 6.4)	Supernatant (pH 4.5)
Santa Rosa	1.590	9.136
Industrial	0.700	8.391
IAC-1	0.400	18.272
IAC-72-1385	0.865	11.419
UFV-1	0.770	14.654

* Hemagglutinating activity is expressed as mcg of protein/ml, capable of causing hemagglutination.

TABLE 6

HEMAGGLUTININ ACTIVITIES IN DIFFERENT PROTEIN FRACTIONS OF EXTRACTS AT A pH OF 8.5 FROM FIVE SOYBEAN VARIETIES*

Varieties	Alkaline extract (pH 8.5)	Supernatant (pH 4.5)
Santa Rosa	0.910	8.316
Industrial	0.780	7.695
IAC-1	0.560	11.145
IAC-72-1385	0.650	13.419
UFV-1	0.630	4.320

* Hemagglutinating activity is expressed as mcg of protein/ml, capable of causing hemagglutination.

raw soybeans. From earlier work, however, Liener (12) had demonstrated that the overall growth rate of rats could be depressed if hemagglutinins were added to a diet and then feed the animals *ad libitum*. A highly significant difference in toxicity was observed in various lines of beans by Jaffé and Brucher (20). This can be distinguished by means of a simple test, wherein different blood preparations are used. It has been consistently observed that extracts of bean seeds which are active in agglutinating trypsin-treated blood cells of cows are of a toxic nature. This toxicity can be demonstrated by injecting mice intraperitoneally with a crude seed extract or by feeding tests.

Irrespective of the degree of importance of hemagglutins in the nutrition of the rat, we were interested in assessing the levels of such antinutrients in Brazilian soybean in connection with our concern in minimizing the cooking time of specific bean mixtures for human consumption. As already showed by Antunes and Sgarbieri (21), cooking the Santa Rosa soybean in water at atmospheric pressure requires at least 30 minutes heating at 100°C to eliminate completely the hemagglutinin activity.

The nutritional significance of the trypsin inhibitors is not better established either. Kakade *et al.* (22) carried out a biochemical and nutritional study on different varieties of soybeans. They found no correlation between chymotrypsin and trypsin inhibitor activity, growth and protein efficiency ratio (PER); on the other hand, a good negative correlation ($r = -0.77$) was determined between the weight of the pancreas and PER. These findings confused even further the issue of the nutritional importance of the protease inhibitors in soybean, since it was generally assumed that trypsin inhibitors were mainly responsible for the pancreas hypertrophy and the poor growth of animals fed raw soybeans (23, 24).

A lack of correlation between trypsin inhibitor activity and PER was also found by Antunes and Sgarbieri (21), who suggested the possible existence of one or more antinutritional factors in soybean, perhaps even more toxic than trypsin and chymotrypsin inhibitors and the hemagglutinins. Such "factor" seems to be extremely heat-labile, and may cause pancreatic hypertrophy and growth inhibition.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Dr. Manoel

Albino Coelho de Miranda of the Leguminous Plant Section of the Agronomic Institute of Campinas, State of São Paulo, Brazil, for his Cooperation in providing seeds for this study; to the State of São Paulo Research Foundation (FAPESP) for the scholarship granted to the senior author during the execution of the work reported herein, and to Dr. Jaime Amaya-Farfán for his valuable suggestions concerning the elaboration of the present article.

RESUMEN

EXISTENCIA DE FACTORES ANTINUTRICIONALES Y CARACTERISTICAS FISICOQUIMICAS DE FRACCIONES PROTEINICAS DE CINCO VARIEDADES BRASILEÑAS DE SOYA

Se estudiaron en cinco variedades brasileñas de soya las siguientes propiedades: solubilidad de las proteínas a diferentes pH, composición de aminoácidos de las proteínas de las harinas y aislados en sus puntos isoeléctricos, y las actividades de inhibición tríptica y de hemaglutinación. El nivel de proteínas y de compuestos aminados no-proteínicos fue similar en todas las variedades. Con un pH de 8.5 se extrajo algo más de proteínas que con agua. Al ajustar el pH de los extractos a 4.5 se precipitó entre 83 y 86% de las proteínas. Del nitrogeno que quedó en la solución, se pudo precipitar el 31 y 39% usando ácido tricloroacético al 5%. Los aislados proteínicos mostraron una composición de aminoácidos parecida a la de la harina. El extracto acuoso acusó mayor actividad antitríptica que el obtenido al pH de 8.5. La mayor parte de la actividad antitríptica quedó en la solución, al ajustar el pH a 4.5; en cuanto a la actividad hemaglutinante, ésta disminuyó mucho en el sobrenadante obtenido al pH de 4.5, lo que indica precipitación de las hemaglutininas a este pH.

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