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The Effect of the Selective Removal of Hemagglutinins on the Nutritive Value of Soybeans

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The hemagglutinating activity of a crude extract of unheated soybean flour was removed by passage through a column of Sepharose-bound concanavalin A. The trypsin inhibiting activity of the extract was not affected by this treatment. The hemagglutinin-free extract when fed to young rats produced a rate of growth and had a protein efficiency ratio which was not significantly different from that obtained with the original extract from which the hemagglutinin had not been removed. Heat treatment of the crude extract effected a marked improvement in growth response similar to that produced by heating the raw soybean flour itself. It was concluded that the soybean hemagglutinin plays a relatively minor role in the deleterious effects of unheated soybean flour.

The poor nutritive value of raw soybeans has been generally attributed to the deleterious effect of trypsin inhibitors and other heat-labile components (Liener, 1972; Rackis, 1972). Kakade et al. (1973) have recently reported that approximately 40% of the growth-depressing effect of unheated soybeans fed to rats could be accounted for by the trypsin inhibitors. The remainder of the growth depression was attributed to the poor digestibility of the native, undenatured protein and possibly to other growth inhibitors. In the latter instance, serious consideration should be given to a class of substances, known as phytohemagglutinins or lectins, which are widely distributed in the plant kingdom, particularly among the legumes (Sharon and Lis, 1972). Although their role as growth inhibitors in beans belonging to the genus *Phaseolus* would appear to be well established (Jaffé, 1969; Liener, 1974), their nutritional significance in the soybean is still uncertain. The soybean hemagglutinin (SBH) was first isolated by Liener (1951) and later more fully characterized by Sharon and his group (Lis et al., 1964; Lotan et al., 1974). Liener (1953) had shown that when purified SBH was added to a diet containing heated soybean meal, at a level approximating its occurrence in the raw meal, the growth of rats was significantly retarded. Growth inhibition, however, was not observed when the food intake of the control diet containing heated soybean meal was restricted to the food intake of the same diet containing SBH. Birk and Gertler (1961) reported poor correlation between the hemagglutinating activity and growth-depressing activity of various fractions of soybeans, and hence concluded that

SBH must play a minor role in the detrimental effect of raw soybean meal.

In order to resolve this issue, a more rigorous approach to the problem was undertaken, one which involved the selective removal of SBH from a crude soybean extract. This approach, which is similar to the one which was employed for assessing the contribution of the trypsin inhibitors to deleterious effects of raw soybeans (Kakade et al., 1973), takes advantage of the fact that hemagglutinins strongly bind specific sugars and glycoproteins containing these sugars (Sharon and Lis, 1972). In the case of SBH, Sepharose-bound concanavalin A, which is itself a phytohemagglutinin, can be used to bind SBH (Bessler and Goldstein, 1973) since the latter is a glycoprotein containing mannose for which concanavalin A is specific (Goldstein et al., 1965). Thus, by comparing the growth response of rats to diets containing SBH-free soybean protein extract with the untreated extract it becomes possible to evaluate directly the role of SBH as a factor contributing to the poor nutritive value of unheated soybean protein.

MATERIALS AND METHODS

Preparation of Soybean Extracts. Fifty grams of defatted soybean flour (Central Soya, Chicago, Ill.) was suspended in 1 l. of 0.05 *M* phosphate buffer (pH 7.6) containing a 10^{-4} *M* concentration of each of the following salts: $CaCl_2$, $MgCl_2$, and $MnCl_2$. The suspension was stirred for 1 hr at room temperature and then centrifuged at 5000 rpm for 10 min. The supernatant which contained 85 to 90% of the protein of the flour was divided into two equal portions. One portion was immediately frozen for later use as the control, to be referred to as "extract (+SBH)," and the other half of the extract was rendered free of SBH by the procedure described below.

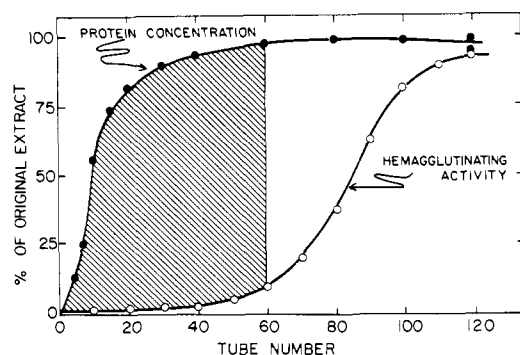Department of Biochemistry, College of Biological Sciences, University of Minnesota, St. Paul, Minnesota 55101.

Figure 1. Removal of hemagglutinin from soybean extract by affinity chromatography on insolubilized concanavalin A. Soybean extract, prepared as described in the text, was continuously pumped through a column (4.5 × 8.0 cm) of Sepharose-bound concanavalin A at a flow rate of 220 ml/hr. Each fraction contained 20 ml. The shaded area between the two curves denotes those tubes which were pooled to give "extract (−SBH)" used in feeding experiments.

Table I. Composition of Diets Used to Evaluate the Nutritional Effects of the Soybean Hemagglutinin (SBH)

Ingredient, %	Designation of diet					
	1	2	3	4	5	6
Extract (−SBH)[a]	14.6					
Extract (+SBH)[b]		14.4				
Extract (+SBH), heated[b]			14.4			
Raw soy flour[c]				18.7		
Heated soy flour[c]					18.7	
Casein[d]						10.8
Corn oil	6.0	6.0	6.0	6.0	6.0	6.0
Mineral mix[e]	4.0	4.0	4.0	4.0	4.0	4.0
Vitamin mix[f]	1.0	1.0	1.0	1.0	1.0	1.0
Corn starch	74.4	74.6	70.3	70.3	70.3	78.2

[a] Contains 68.3% protein. [b] Contains 69.5% protein. [c] Contains 53.4% protein. [d] Contains 93% protein. [e] Prepared according to Hubbell et al. (1937); purchased from General Biochemicals, Chagrin Falls, Ohio. [f] Purchased from General Biochemicals.

Concanavalin A, prepared from jack bean meal (Sigma) by the method of Agrawal and Goldstein (1967), was coupled to cyanogen bromide activated Sepharose 4B (Pharmacia) as described by Porath et al. (1973). The crude soybean extract was pumped through a column (4.5 × 8 cm) of Sepharose–concanavalin A which had been previously washed with 1 M NaCl and then 0.05 M phosphate buffer (pH 7.6), both of the latter solutions containing 10^{-4} M $CaCl_2$, $MgCl_2$, and $MnCl_2$ which are required for maximal binding capacity of concanavalin A (Agrawal and Goldstein, 1968). The dimensions of this column permitted a high flow rate of 220 ml/hr. The column had to be operated at room temperature in order to prevent precipitation of protein which occurs in the cold. The protein in the effluent was monitored by measuring absorbance at 280 nm, and hemagglutinating activity in the eluted fractions was determined as previously described (Liener, 1955). A typical run is shown in Figure 1. Only those fractions which had less than 10% of the activity of the original extract were pooled and frozen and will be hereafter referred to as "extract (−SBH)." The column was regenerated by first washing out all of the residual extract remaining on the column with the pH 7.6 phosphate buffer, followed by washing with the same buffer containing 1% α-methylmannopyranoside (Pfanstiehl) which serves to elute SBH plus any other glycoproteins which may have been bound to the concanavalin A. It was estimated that approximately 2% of the protein in the original extract was bound to the column and eluted with α-methylmannopyranoside (unpublished data). About 50% of this fraction was SBH and the other half a mixture of three unidentified glycoproteins. Amino acid analysis of extracts (+SBH) and (−SBH) indicated that the removal of this small amount of protein from the original extract did not significantly affect its amino acid composition. After washing the column with 1 M NaCl and phosphate buffer as described above, the column was ready for reuse; columns treated in this fashion were used repeatedly over a period of 9 months without any noticeable loss in activity. A total of 24 runs were necessary in order to produce sufficient material (180 g of lyophilized product) for the feeding experiments described below.

Extract (+SBH) and extract (−SBH) were exhaustively dialyzed against distilled water and lyophilized. A portion of extract (+SBH) which had been lyophilized was dispersed in 5 parts of distilled water and autoclaved for 20 min at 15 psi. The resulting coagulum was homogenized in a Waring Blendor and again lyophilized. A portion of the unheated soybean flour was subjected to the same heat treatment by autoclaving the flour which had been

spread out as a thin layer (0.5 in.) in a shallow glass tray. All preparations were analyzed for nitrogen (Ballentine, 1957), trypsin inhibitor activity using benzoyl-DL-arginine-p-nitroanilide as substrate (Kakade et al., 1969), and hemagglutinating activity (Liener, 1955).

Rat Feeding Experiments. Six diets, formulated to provide in all cases a level of 10% protein, were prepared as shown in Table I. Data pertaining to the trypsin inhibitor and hemagglutinating activities provided by these diets are included in Table II. Diet 1 containing extract (−SBH) had less than 10% of the hemagglutinating activity of diet 2 which contained extract (+SBH) and diet 4 which contained raw soy flour; there was no appreciable difference, however, in the trypsin inhibitor activity contained in these three diets. Heating eliminated practically all of the trypsin inhibitor activity in diets 3 and 5, containing extract (+SBH) and soyflour, respectively. About 98 and 93% of the hemagglutinating activity of extract (+SBH) and soyflour, respectively, were destroyed by heat treatment.

Male 21-day-old weanling rats (Holtzman) were divided into six groups of six animals each, equalized as closely as possible with respect to initial weight, and housed in individual wirebottom cages. The protein efficiency ratio (PER, grams gained per gram of protein consumed) was calculated from the weight gains and food consumption recorded over a period of 17 days. At the termination of the experiment each rat was sacrificed by exposure to chloroform, and the pancreas was excised and weighed.

RESULTS

The results of the feeding experiments are summarized in Table II. The weight gains and PER values of the animals fed extract (+SBH), diet 2, were not significantly different from those fed the unheated soyflour, diet 4. As expected, heat treatment in both cases (diets 3 and 5, respectively) produced a marked increase in both weight gains and PER. Of special interest here is the fact that diet 1, containing the extract from which over 90% of the SBH had been removed, produced slightly better growth and had a somewhat higher PER than diet 2 containing the original extract containing SBH; these small differences, however, did not prove to be statistically significant at a probability level of 5%. Another important point to note here is the fact that, although the food consumption of the rats receiving the extract (−SBH) was some-

Table II. Summary of Data Obtained with Rats Fed Soybean Extract from Which SBH Had Been Removed, Compared with the Original Extract, with and without Heat Treatment, and Soy Flour, with and without Heat Treatment

Diet no.	Protein component	Trypsin inhibitor act.[a] TUI/g of protein × 10⁻³	Hemag- glutina- ing act.[b] HU/g of protein × 10⁻³	Wt gain,[c] g	Food consumption, g	PER[c]	Wt of pancreas, g/100 g body wt
1	Extract (−SBH)	99	29	13.4 ± 2.6ᵃ	119.9 ± 14.9ᵃ	1.13 ± 0.24ᵃ	0.65 ± 0.06ᵃ
2	Extract (+SBH)	85	324	9.8 ± 3.0ᵃ	103.7 ± 12.7ᵃ	0.91 ± 0.39ᵃ	0.66 ± 0.11ᵃ
3	Extract (+SBH), heated	2	6	34.5 ± 8.9ᵇ	152.5 ± 13.6ᵇ	2.25 ± 0.51ᵇ	0.45 ± 0.11ᵇ
4	Soy flour, raw	98	430	13.0 ± 3.5ᵃ	127.4 ± 10.9ᵃ	1.01 ± 0.21ᵃ	0.62 ± 0.07ᵃ
5	Soy flour, heated	2	32	35.9 ± 5.8ᵇ	157.0 ± 15.7ᵇ	2.30 ± 0.35ᵇ	0.41 ± 0.09ᵇ
6	Casein			33.3 ± 6.9ᵇ	116.0 ± 16.5ᵃ	2.91 ± 7.3ᵇ	0.46 ± 0.08ᵇ

[a] Expressed as trypsin units inhibited (TUI) as defined by Kakade et al. (1969). [b] Expressed as hemagglutinating units (HU) as defined by Liener (1955). [c] Mean of six rats per group ± standard error of the mean; values within each column having different roman superscripts are statistically different ($P < 0.05$) using the F test for unpaired data (Davies, 1949).

what greater than that of the rats fed extract (+SBH), this difference again did not prove to be statistically significant. The data in Table II also reveal that the elimination of SBH from the crude soybean extract did not alter the pancreatic enlargement which is characteristically produced in animals fed raw soybeans, an effect which can be attributed to the trypsin inhibitor and the enzyme resistant nature of the native protein itself (Kakade et al., 1973).

DISCUSSION

From these data one would be forced to conclude that SBH is a relatively minor factor contributing to the poor nutritive properties of raw soybeans. This conclusion would appear to be at variance with our earlier report (Liener, 1953) that SBH exerted a significant growth-inhibiting effect when added to a diet containing heated soybean meal. It should be emphasized, however, that growth inhibition in that instance was observed only when the animals were fed ad libitum, and growth depression was accompanied by a corresponding decrease in food intake. If, on the other hand, the food intake of rats receiving the basal diet (containing heated soybean meal) without SBH was restricted to the level of food consumed by the animals fed the diet to which SBH had been added, no difference in growth performance between the two groups was apparent. It will be noted from the food consumption data given in Table II that there was no significant difference in the food intake between the two groups of animals fed either the original extract or the extract from which the SBH had been selectively removed. Thus, the failure to demonstrate a difference in growth between these two groups of animals serves to reinforce our earlier conclusion that, under conditions of equalized food intake, SBH does not exert a growth depressing effect on the growth of rats.

The key question to be answered, therefore, should be: which set of experimental conditions provides a truer picture of the nutritional effects of SBH, feeding animals: (a) a diet containing heated soybean protein to which pure SBH has been added, or (b) a diet containing unheated soybean protein from which SBH has been selectively removed? In situation (a) the effect of SBH is being assessed under conditions in which all of the protein, including such biologically active factors as the trypsin inhibitor, has been denatured or inactivated. In the case of situation (b) the effect of SBH is being tested under conditions in which all of the other proteins are in their native and biologically active state. In the latter case, therefore, complex interactions between various protein fractions could occur which might modify the biological activity of any one fraction. For example, it is conceivable that the trypsin inhibitor might somehow suppress the adverse physiological effect which the SBH might otherwise have on the animal. That this sort of thing may in fact occur is suggested by the experiments of Sambeth et al. (1967) which involve an evaluation of the biological activity of various fractions of soybean protein; they concluded that many of the biological effects manifested by the soybean fractions are the result of interactions among the various components contained therein. If our data can be interpreted in the light of these considerations, we are inclined to favor the view that SBH probably does not make a significant contribution to the overall deleterious nutritive properties of unheated soybeans.

It should be emphasized that the results reported here are applicable only to the soybean and may have little relevance to other legumes which also contain hemagglutinins. Experiments, similar to those reported here for soybeans, are now in progress with other legumes which enjoy popular consumption in many parts of the world and represent a potentially rich source of protein which should be more fully exploited (Milner, 1973).

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Development of Novel Free Radicals during the Amino–Carbonyl Reaction of Sugars with Amino Acids

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Novel free radicals were generally found to be developed in an early stage of the amino–carbonyl reaction with sugars and amino acids by using ESR spectrometry. The radicals showed characteristic hyperfine structures and apparently differed from those existing in melanoidin formed with browning. The results of many combined reactions of various sugars and their related carbonyl compounds with various amino acids and amines indicate that the spectral patterns were mainly influenced by the structure of amino compounds as primary and secondary amino groups but not by the structures of sugars and most of their analogous carbonyl compounds. On the development of free radicals, effects of oxygen, pH, temperature, molar ratio, and some additives were investigated. These results suggest that the radicals did not locate in a highly conjugated structure such as melanoidin but they may exist at a particular position in some products formed at an early stage of the sugar–amino acid reactions and still containing the residues of either reactant.

The amino–carbonyl reaction of sugars with amino acids has been studied extensively as a principal reaction which causes important changes in food qualities such as browning along with melanoidin formation and development of various roast flavors. Concerning the reaction mechanism a proton transfer chain reaction has been proposed as an early stage of the reaction (Isbell and Frush, 1958), but little is known about the formation of free radicals except for the presence of stable free radicals in melanoidin (Mitsuda et al., 1965).

Recently, the authors have found the development of free radicals in some amino–carbonyl reaction systems (Namiki et al., 1973, 1974), and this report deals with the details of development of novel free radicals in an early stage of the browning reaction of sugars with amino acids.

EXPERIMENTAL AND RESULTS SECTION

Experimental procedures employed generally were as follows. In a Pyrex test tube was placed a solution containing equimolar amounts of sugar and amino acid prepared with distilled water and heated in a boiling water bath. Development of free radicals was checked at regular intervals of the heating time by use of a JES-ME-1X ESR spectrometer with a quartz tube for liquid samples, and development of browning was determined with the absorbancy at 420 nm. The reagents used were Guaranteed Grade and distilled water was prepared with Pyrex apparatus.

Reactions of α- and β-Alanine with Various Sugars. A mixture of D-arabinose and β-alanine was employed representatively, since a pentose–β-alanine system has been

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known as a remarkable one in browning with sugars and amino acids at neutral pH (Kato, 1956). When a mixture of D-arabinose (1.0 g) and β-alanine (0.6 g) in distilled water (2.0 ml) was heated in a boiling water bath, development of the ESR signal could be observed as soon as the reaction was started. As shown in Figure 1, relative intensity of the signal increased rapidly during several minutes in an initial stage of the reaction where the spectrum showed a characteristic hyperfine structure as shown in Figure 1a ($g = 2.0034$, 23 lines of a splitting constant of 3 G). The intensity of the signal then started to fall with further heating and decreased gradually to a constant level along with changes in the ESR spectral pattern from a to b at 10 min and finally to a broad singlet (c) after 90 min; the development of type a radical appeared likely to precede the browning reaction.

Figure 2 shows the reaction of D-arabinose with α-alanine, where the development of either free radical and browning were observed to proceed in a similar manner to the above case, although here either one proceeded with more moderation, and it was clearly demonstrated that the development of a characteristic ESR spectrum occurred prior to browning and the hyperfine structure ($g = 2.0036$, 17 lines of a splitting constant of 3 G) differed apparently from that presented above. Thus, the change in the relative intensity of the ESR signal shown in Figure 1 can be seen as a sum of the changes in those of spectra a and c as indicated by the broken lines A and C, respectively.

Further ESR studies were done on similar reactions of various sugars and some carbonyl compounds with α- or β-alanine, and the results are summarized in Table I with some features of their characteristic ESR spectra. As to the line number of the hyperfine structure, it is to be noted that almost all the sugars and their related carbonyl compounds gave essentially the same ESR spectra with a