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Antioxidative and Antihemolytic Activities of Soybean Isoflavones

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Two properties of soybean isoflavones were examined in in vitro systems: (1) their antioxidative potency by measuring the extent of inhibition of lipoxygenase action and (2) their antihemolytic activity by measuring their ability to prevent peroxidative hemolysis of erythrocytes. The extent of the antioxidative capacity of isoflavones is positively correlated to the number of hydroxyl groups in the isoflavone nucleus. Glycosidation of isoflavones depressed their antioxidative activity considerably. A different susceptibility of erythrocytes of sheep, rats, and rabbits to the antihemolytic activity of isoflavones was found. Very high antihemolytic activity of some isoflavones such as daidzein was exerted toward sheep erythrocytes, very little and only by genistein was exerted toward rat erythrocytes, and no antihemolytic effect was noted at all on rabbit erythrocytes. A hemolysis enhancing activity of small amounts of isoflavones on rabbit erythrocytes, but not on those of sheep and rats, has been observed. The antioxidative and antihemolytic activities of isoflavones toward sheep erythrocytes were differently affected by structural differences of isoflavones.

In a previous paper (Naim et al., 1974) procedures for isolation and separation of isoflavones from soybeans and for their quantitative determination have been described, and studies on their fungistatic action reported. The wide use of soybeans in foods and feeds calls for the study of some of the biological properties of soybean isoflavones in order to find out whether detrimental and/or beneficial effects on humans and animals are exerted by the presence of isoflavones in soybean foods and feeds. Observations on antioxidative and antihemolytic activities exerted by 6,7,4′-trihydroxyisoflavone, a compound isolated from fermented soybeans, were reported by György et al. (1964) and Ikehata et al. (1968). According to some data reported in the literature, isoflavones are absorbed from the digestive tract of animals and can be partly recovered in the plasma of sheep (Lindner, 1967) and guinea pigs (Shutt and Braden, 1968), after ingestion of isoflavone-containing leguminous forages. Therefore, a systematic study of the antioxidative and antihemolytic properties of soybean isoflavones is desired and the present work deals with these activities of free isoflavones and isoflavone glycosides. Occurrence or nonoccurrence of a relationship between the extent of these activities, when examined, may permit us to draw some conclusions with regard to the mode of the antihemolytic activity of isoflavones. In the present work hemolytic assays were performed with red blood cells of rams, rats, and rabbits in order to study a possible influence of species differences in the structure of components of the erythrocyte membrane which are expressed in the effects of isoflavones.

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EXPERIMENTAL SECTION

Materials. Individual isoflavones and isoflavone glycosides were prepared from soybeans as described by Naim et al. (1973). Quercetin and lipoxygenase type 1 were purchased from Sigma Chemical Co. Dimethoxydaidzein was prepared by treatment of daidzein with CH_2N_2. The potent antioxidant quercetin (Crawford et al., 1961) was used as a reference compound for comparing antioxidative and antihemolytic activities of isoflavones.

Inhibition of Lipoxygenase by Isoflavones. The following two procedures were used for determining the extent of inhibition of lipoxygenase activity by isoflavones. (a) The inhibitory effect of isoflavones on the coupled oxidation of β-carotene and polyunsaturated fatty acids by soybean lipoxygenase was measured by spectrophotometric determination of the disappearance of carotene according to Ben Aziz et al. (1971). Tween 80 was replaced by Tween 20 for solubilizing carotene and linoleic acid in the suitable buffers. (b) Uptake of oxygen by linoleate in the presence of lipoxygenase was measured according to the procedure developed by Grossman et al. (1969). Procedure b permits the performance of the lipoxygenase test in the presence of higher concentrations of isoflavones since the measurement of oxygen absorption, unlike the spectrophotometric determination of carotene bleaching, is not interfered by turbidity resulting from the insufficient solubility of isoflavones, even in the presence of Tween.

Determination of Antihemolytic Activity of Isoflavones. The preventive action of isoflavones and of quercetin against the hemolytic effect of H_2O_2 solutions on washed red blood cells of sheep, rats, and rabbits was examined by the in vitro test of Segal et al. (1966) as adopted for our purposes, using citrated blood. The blood

Table I. Inhibition of Lipoxygenase by Isoflavones from Soybeans

Inhibitor assayed	Concn, M	% inhibition, as measured by: Oxidation of linoleate[a]	% inhibition, as measured by: Destruction of carotene[b]
Quercetin (reference)	5.0×10^{-6}	30 ± 3	49 ± 4
	1.0×10^{-5}	50 ± 4	65 ± 2
	5.0×10^{-5}	83 ± 7	90 ± 2
Genistein	5.0×10^{-5}	32 ± 4	15 ± 2
	2.5×10^{-4}	57 ± 5	40 ± 3
	1.0×10^{-3}	70 ± 5	
Genistin	1.0×10^{-4}	0	0
	2.5×10^{-4}	10 ± 2	13 ± 1
	1.0×10^{-3}	30 ± 2	
Daidzein	1.0×10^{-4}	12 ± 2	11 ± 1
	2.5×10^{-4}	16 ± 2	30 ± 5
	1.0×10^{-3}	26 ± 4	
Daidzin	5.0×10^{-4}	0	13 ± 1
	1.0×10^{-3}	15 ± 3	
Glycitein	1.0×10^{-4}	15 ± 3	16 ± 2
	2.5×10^{-4}	32 ± 4	33 ± 2
Glycitein 7-*O*-β-glucoside	2.5×10^{-4}	12 ± 2	15 ± 3
	1.0×10^{-3}	25 ± 5	
Dimethoxydaidzein	1.0×10^{-3}	12 ± 3	
	4.0×10^{-3}	35 ± 5	

[a] The rate of oxygen absorption in the control samples was 1.99 μl/min. [b] The rate of carotene destruction in the control samples was 1.78 μg/min.

was withdrawn from the jugular vein of Awassi rams, from the left ventricle of the heart of rats (*Albino ratus norvegicus*, 1.5–3 months), or from the ear vein of rabbits (local breed). The erythrocytes were separated by centrifugation and washed with saline or isotonic sodium phosphate buffer (pH 7.4), until the supernatant was colorless. The erythrocytes were then diluted with saline or phosphate buffer to give a 4% suspension. Varying amounts of quercetin or isoflavones homogenized with saline or buffer were added to 2 ml of the suspension of erythrocytes and the volume was made up to 3.5 ml with saline or buffer. This mixture was preincubated for 5 min and then 0.5-ml H_2O_2 solutions of appropriate concentration in saline or buffer were added. The concentration of H_2O_2 in the reaction mixture was adjusted so as to bring about 90% hemolysis of sheep blood cells after 120-min incubation. Because of varying conditions the exact amount of H_2O_2 had to be determined in each instance by a preliminary experiment. In the case of sheep blood, the concentration of H_2O_2 was between 0.1 and 0.2%, while in the case of rats and rabbits erythrocytes a concentration between 0.2 and 0.3% was used. About 80–90% hemolysis of rat and rabbit erythrocytes was obtained after 4–6 h. Incubation was concluded after these time intervals by centrifugation during 5 min at 1000*g* and the extent of hemolysis was determined by measurement of the absorbance (at 540 nm) corresponding to hemoglobin liberation.

RESULTS

Extent of Inhibition of Lipoxygenase Activity by Isoflavones. Good agreement was achieved between the results concerning the antioxidative efficiency of isoflavones measured by both procedures used for the determination of inhibition of lipoxygenase activity (Table I). Both assays were carried out in the presence of 10^{-3} M ethylenediaminetetraacetic acid (EDTA); therefore, antioxidative efficiency of isoflavones cannot be attributed to their metal-chelating action.

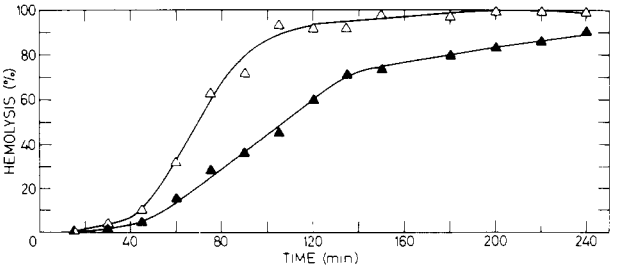

Figure 1. Effect of daidzein on rate of H_2O_2-induced hemolysis of sheep erythrocytes in saline: (△) hemolysis in control experiment; (▲) hemolysis in the presence of 7.0×10^{-6} M daidzein.

It appears from the results presented in Table I that the extent of the antioxidative capacity of the compounds examined is positively correlated to the number of the hydroxyl groups on the flavone or isoflavone nucleus. The highest activity was exhibited by quercetin with five free hydroxyl groups. Genistein, with three hydroxyl groups, was ten times less active than quercetin. Inhibition (30%) of soybean lipoxygenase has been attained by 5×10^{-6} M quercetin or by 5×10^{-5} M genistein. The antioxidative activities of the corresponding glycoside genistin and of both other soybean isoflavones, daidzein and glycetein, which contain two OH groups in different positions, were much lower and almost equal to each other. Glycosidation of these isoflavones, which results in daidzin and glycetein 7-*O*-β-glucoside, or substitution of the hydroxyl groups present in daidzein by methoxyl groups further depressed their antioxidative activity.

As mentioned above, genistein, the major isoflavone in soybeans (Naim et al., 1974), inhibits considerably the activity of lipoxygenase. When genistein was present in the in vitro system in a concentration similar to that found in soybeans (10^{-3} M), 70% inhibition of lipoxygenase has been found. It may be concluded that genistein, as an intermediate in the biosynthesis of genistin, exerts in soybean seeds in situ a protective action against lipoxygenase.

Antihemolytic Activity of Isoflavones toward H_2O_2-Induced Hemolysis of Sheep Erythrocytes. A characteristic curve representing the rate of the inhibition of hemolysis of sheep blood cells by daidzein is given in Figure 1. Considerable inhibition was found after 120-min incubation, i.e. the time necessary to hemolyse 90% of the blood cells in the control assay, and it was therefore chosen as the appropriate time interval for this test.

It appears from Table II that the various isoflavones differ considerably in the extent of their antihemolytic action. The antihemolytic potency of quercetin surpasses that of the individual isoflavones, even that of daidzein, which is the most active isoflavone in this respect. The extent of the activity of daidzein almost reached that of quercetin and surpassed considerably genistein and glycetein. It may be noted that the antihemolytic activity of the glycoside genistin was about 5 times higher than that of the corresponding aglycon genistein, when measured in saline. In all other instances substitution of hydroxyl groups present in isoflavones, e.g., substitution of one or two hydroxyl groups of daidzein by methoxyl or by glycosidation, depressed the antihemolytic effect considerably.

The extent of the antihemolytic activity of some isoflavones such as genistein and glycetein varies considerably with the medium of assay. Both aglycones exert a stronger antihemolytic effect in phosphate solution than in saline, whereas the respective potencies of daidzein, of quercetin, and of isoflavone glycosides were not influenced by this

Table II. Comparative Antihemolytic Effects of Soybean Isoflavones and Quercetin[a]

Antihemolytic agent	Assayed in saline: Molar concn needed to achieve 50% inhibition of hemolysis[b]	Assayed in saline: Rel antihemolytic potency, as % of that of quercetin	Assayed in buffer, pH 7.4: Molar concn needed to achieve 50% inhibition of hemolysis[b]	Assayed in buffer, pH 7.4: Rel antihemolytic potency, as % of that of quercetin
	On Ram Erythrocytes			
Quercetin	6.2×10^{-6}	100	4.9×10^{-6}	100
Genistein	5.4×10^{-5}	11	8.0×10^{-6}	61
Genistin	1.2×10^{-5}	52	1.0×10^{-5}	49
Daidzein	7.2×10^{-6}	86	5.2×10^{-6}	94
Daidzin	4.9×10^{-5}	13	3.5×10^{-5}	14
Glycitein	5.1×10^{-5}	12	7.5×10^{-6}	65
Glycitein 7-*O*-β-glucoside	6.2×10^{-5}	10	5.5×10^{-5}	9
Formononetin	1.5×10^{-4}	4		
Dimethoxydaidzein	2.8×10^{-4}	2		
	On Rat Erythrocytes[c]			
Quercetin	3.0×10^{-6}	100	3.4×10^{-6}	100
Genistein	3.2×10^{-4}	0.9	9.0×10^{-5}	4

[a] The data are averages of six replicates each. [b] The deviations from the averages of the molar concentrations of the antihemolytic agents are ±20% for ram erythrocytes and ±30% for rat erythrocytes. [c] No antihemolytic activity was achieved by genistin, daidzin, and daidzein in concentrations up to 1.0×10^{-3} M when assayed on rat erythrocytes.

change of the medium. The enhancing effect of phosphate may be attributed possibly to differences in pH (7.4 in phosphate buffer and 7.0 in saline), its metal-chelating effect, or its ability to form a nonpenetrating solution which balances the osmotic pressure of intracellular hemoglobin (Mezick et al., 1970) and alters the permeability of the membrane.

Antihemolytic Activity of Isoflavones toward H_2O_2-Induced Hemolysis of Rat Erythrocytes. H_2O_2-induced hemolysis of rat blood cells was much less susceptible to inhibition by isoflavones than was found for sheep blood cells. Quercetin, however, manifested antihemolytic activity of equal extent toward rat and ram blood cells. Genistein, in concentrations of 10^{-5} to 10^{-4} M, was the only isoflavone which exerted very slight antihemolytic activity toward rat blood cells. The other isoflavones had no effect even at concentrations of 10^{-3} M. A possible antihemolytic action of these isoflavones, when present in higher concentrations, could not be examined because of their insufficient solubility in the assay medium.

Effect of Isoflavones on H_2O_2-Induced Hemolysis of Rabbit Erythrocytes. No inhibitory effect of isoflavones or quercetin on hemolysis of rabbit erythrocytes could be noted. Moreover, very small amounts of isoflavones (10^{-3}–10^{-5} M) enhanced the rate of hemolysis of rabbit blood cells when the concentration of H_2O_2 was also considerably low, 0.2–0.3% (Figure 2). Such an enhancing effect on hemolysis could not be produced on erythrocytes of rats or sheep, even in the presence of very small amounts of H_2O_2 and isoflavones. Genistein and daidzein exerted a stronger hemolysis-enhancing effect than the respective glycosides (Figure 2).

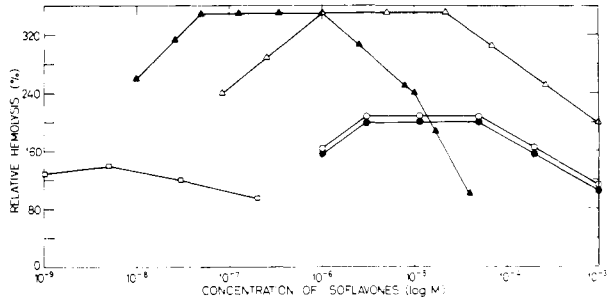

Figure 2. Enhancing effect of isoflavones on H_2O_2-induced hemolysis of rabbit erythrocytes. The concentration of H_2O_2 used produces in the control a full hemolysis after 5–6 h: (□) quercetin; (▲) genistein; (△) daidzein; (○) daidzin; (●) genistin.

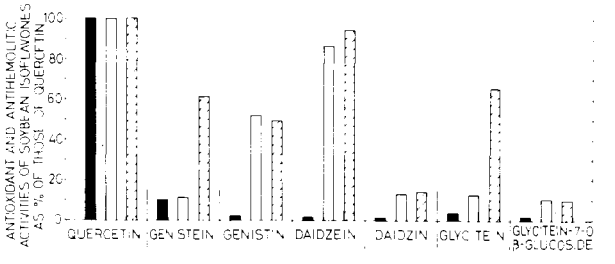

Figure 3. Comparative antioxidative and antihemolytic activities of isoflavones: (■) antioxidative activity. Antihemolytic activity assayed with: (□) sheep erythrocytes, in saline; (■) sheep erythrocytes, in buffer. The columns referring to the antioxidative activity express the ratios of molar concentration necessary to achieve 25% inhibition of lipoxygenase. The columns referring to the antihemolytic activity express the ratios of molar concentrations necessary to achieve 50% inhibition of hemolysis.

DISCUSSION

It appears from Figure 3 that the relative activity of isoflavones as inhibitors of lipoxygenase activity, with respect to quercetin as reference agent, differs considerably from their activity as antihemolytic agents toward H_2O_2-induced lysis of ram blood cells. Thus, both activities are differently effected by structural changes of isoflavones. This lack of correlation between both activities suggests that the antioxidative properties of isoflavones are not the only factor responsible for their antihemolytic activity. Likewise, no good correlation between antioxidative and antihemolytic activities of vitamin E derivatives was found by Skinner et al. (1971). These authors assumed also that different mechanisms were involved in both effects.

Concepts proposed for explaining the mechanism of the in vitro erythrocyte hemolysis test used as an index of vitamin E deficiency in animals seem to be valid for understanding the mode of the antihemolytic action of isoflavones. The H_2O_2-induced hemolysis is inhibited by phenols of different structure (Younkin et al., 1971), tocopherols, and isoflavones. According to Horn et al. (1974) tocopherols act as inhibitors of peroxidative attack by

H_2O_2 on polyunsaturated fatty acids contained in membrane phospholipids. Such lipid peroxidation is associated with formation of "holes" in the membrane permitting the efflux of hemoglobin (Barker and Brin, 1975). Another function of phenolic compounds acting against hemolysis may be a specific interaction of these inhibitors with membranous phospholipids and proteins leading to physicochemical stabilization of the membranes and reduction of their permeability (Lucy, 1972). This concept is supported by the experimental evidence provided by Leibowitz and Johnson (1971) for the incorporation of the phenolic antioxidant BHT in membranes, which results in their stabilization.

Species differences in the composition of erythrocyte membranes, particularly the higher content of polyunsaturated fatty acids in the phospholipid fraction of erythrocyte membranes in ruminant animals (Nelson, 1967), may contribute to the different susceptibility of erythrocytes of sheep, rats, and rabbits to the antihemolytic action of isoflavones.

The hemolysis-enhancing action of small amounts of isoflavones on rabbit erythrocytes is not entirely unexpected. As outlined above the antihemolytic action of isoflavones on sheep erythrocytes is at least partly due to the antioxidative property of the isoflavones. According to the scheme generally accepted (Sherwin, 1972) for the mode of action of antioxidants, peroxide radicals of lipids (RO_2) are trapped by the antioxidant (AH):

$$ROO\cdot + AH \rightleftarrows ROOH + [A\cdot]$$

This equilibrium depends on the structure of the lipid (R) and can even be shifted to the left (Witting, 1974). This may occur also in the herewith examined system, namely H_2O_2-oxidized rabbit erythrocyte membranes + isoflavones. Thereby, the hemolysis-enhancing activity of isoflavones exerted on rabbit blood cells in the presence of small amounts of H_2O_2 could be correlated to the particular structure of membrane phospholipids present in rabbit erythrocytes (Horn et al., 1974).

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Coumestrol Content of Fractions Obtained during Wet Processing of Alfalfa

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The concentration of coumestrol was determined in alfalfa, alfalfa fractions, and 16 selected food products of plant origin. More than 80% of the initial coumestrol of alfalfa remained in the fibrous residue (pressed alfalfa) that resulted from a juicing operation. Compared to fresh alfalfa, whole leaf protein concentrate (LPC) pressed alfalfa had higher coumestrol contents, and both green LPC and white LPC had lower contents. At alkaline pH levels, coumestrol was extracted more readily from fresh alfalfa and also resisted co-precipitation with white LPC during its heat coagulation. The coumestrol content of white LPC prepared under mildly alkaline conditions or by diafiltration was within the range found in other vegetable products.

Alfalfa is a promising source for the production of leaf protein concentrates (LPC) which can help meet the protein needs of the world. Our laboratory has developed two processes for the production of LPC. (1) The Pro-Xan process produces a dehydrated alfalfa meal for ruminants, LPC for nonruminants, and alfalfa solubles for possible use in single cell protein production (Kohler et al., 1968). (2) The Pro-Xan II process yields, in addition to the above-mentioned products, white LPC which is designed for human consumption (Edwards et al., 1975).

A large number of plant species, including a number of those consumed by humans, contain many compounds which exhibit biological activity in laboratory and farm animals (Bennetts et al., 1946; Bradbury and White, 1954; Ershoff, 1954). Some of the active substances in these

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