itor. This was achieved by heating the blanched extracted flakes for 10 min at 100 C. The raw bean flakes required a process of 30 min at 120 C, similar to the conditions used industrially.

The flakes thus processed were evaluated by a taste panel. This involved admixing the flours in varying proportions with precooked, drum-dried rice. Triangle difference tests were used to determine the highest level of incorporation at which the mixtures could not be distinguished from the rice alone. The data in Table I show that 5% raw, defatted soybean flour in the rice adversely affected the taste but as much as 15% of the blanched defatted soybean flour could be incoporated without affecting flavor of the rice ($p = 0.05$).

Economics

There are two cost considerations. One is that the soybeans must be hydrated to about 18% for heat treatment. Since this moisture is too high for oil extraction, the soybeans must be dried to about 12%. The other is that the yield of crude oil from enzyme inactive soybeans was reduced by 0.5 out of about 20%.

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Two Soybean Genotypes Lacking Lipoxygenase-1

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ABSTRACT

The U.S. Department of Agriculture soybean germplasm collection (6,499 accessions) was screened for genotypes with greatly reduced or missing lipoxygenase-1 (L-1) [linoleate: O_2 oxidoreductase, EC 1.13.11.12] and lipoxygenase-2 and L-3 (L-2 and L-3) activity. The L-1 assay used linoleic acid dispersed in Tween-20 at pH 9.0 as the substrate (acid assay) and the L-2 and L-3 assay used linoleic acid methyl ester dispersed in ethanol at pH 7.0 as the substrate (ester assay). The spectrophotometric assay based on conjugated diene formation at 234 mm was used in the qualitative screening procedure. Two plant introductions (PI), 133226 from Indonesia and PI 408251 from Korea lacked L-1 activity. Oxygen uptake, electrophoresis and isoelectric focusing confirm the lack of detectable L-1 activity in the seed of these two genotypes. Radial diffusion against soybean seed lipoxygenase antiserum showed that the two genotypes are missing a precipitin band that normal soybean genotypes and purified lipoxygenase from soybean seed exhibit. Neither the L-1 variants nor any other accessions tested had greatly reduced activity with the ester assay.

INTRODUCTION

Lipoxygenase [linoleate: O_2 oxidoreductase, EC 1.13.11.12] has been implicated as the principal cause of the undesirable flavors of soybean products (1,2) especially soymilk (3). Additionally, the lipid hydroperoxides resulting from lipoxygenase action can lead to loss of nutritive value by the destruction of certain vitamins and protein (4). Also, lipid hydroperoxides and their breakdown products may have toxic effects (4).

In commercial soybean crushing operations, there is usually from 15 to 20 min between flaking of the seeds and extraction of the oil. Thus lipoxygenase has ample time to initiate the oxidation of the linoleic and linolenic acids and their esters in the oil (5). Heat treatment of soybeans prior to oil extraction increases the stability of the oil, presumably due to the inactivation of lipid oxidizing enzymes such as lipoxygenase, but has the undesirable consequence of reducing the solubility of the protein (5-7).

Soybean seeds contain at least three lipoxygenase isozymes, all having molecular weights (MW) of about 100,000. With linoleic acid as the substrate, lipoxygenase-1 (L-1) has a pH optimum at 9.5, lipoxygenase-2 (L-2) has a pH optimum at 6.5 and lipoxygenase-3 (L-3) has a broad pH optimum from 4.5 to 9.0. The isoelectric points of the three isoenzymes also are different; L-1 is the most acidic (8,9). L-1 is the most reactive with free linoleic acid, whereas L-2 and L-3 are most reactive with methyl linoleate or trilinolein (10). On an equal protein basis, L-1 is 2.5 times as active as L-2 at its pH optimum, and L-2 is 2.5 times as active as L-3 or L-3b (11). L-1 is at least 36 times more stable than L-2 at 69 C (12).

Hammond et al. (13) and Chapman et al. (14) investigated the genetic and environmental influences on a number of chemical components of soybean seed that

affect soybean oil quality. They found that the level of unsaturated fatty acids was strongly influenced by the environment, but lipoxygenase activity was predominantly under genetic control. They therefore suggested that low lipoxygenase activity be used as a criterion for selection in soybean breeding programs.

Hymowitz has taken a different approach for improving the nutritional quality of soybean. He theorized that, unless a chemical component of soybean seeds is absolutely necessary for the survival of the domesticate, genotypes should exist in nature which do not have the chemical component. Hymowitz and coworkers have found soybean genotypes in the U.S. Department of Agriculture soybean collection that lack a seed lectin (15), the Kunitz trypsin inhibitor (16) and seed β-amylase (17).

The objective of this study was to screen the U.S. Department of Agriculture soybean germplasm collection for genotypes with greatly reduced or missing L-1, L-2, or L-3 activity in mature seeds.

MATERIALS AND METHODS

Germplasm

The 5904 soybean [*Glycine max* (L.) Merr.] accessions and 595 wild soybean (*G. soja* Sieb. & Zucc.) accessions evaluated for L-1, L-2 and L-3 activity were obtained from Drs. R.L. Bernard, Urbana, IL, and E.E. Hartwig, Stoneville, MS, curators of the northern and southern U.S. Department of Agriculture soybean germplasm collections, respectively (Table I).

Lipoxygenase Extraction

Lipoxygenase was extracted from seed of each accession by grinding one or more whole seeds (totalling more than 100 mg) in 3 ml buffer (0.06 M Tris, 0.015 M $CaCl_2$, 13% sucrose, pH 8.2) at 4 C for 5 sec using a Brinkmann Polytron (Model PT 20) at maximal setting. The extracts were centrifuged at 3,000 × g at 4 C for 15 min. The supernatant was saved and used immediately for all analyses except for the qualitative screening procedure in which extracts that were frozen were analyzed. Extracts from seeds of the cv. Williams were used as standards in all assays and Williams seed extracts which had been boiled for 1 hr were used as controls.

TABLE I

Accessions of the U.S. Department of Agriculture Soybean Germplasm Collection Evaluated for Lipoxygenase-1, Lipoxygenase-2 and Lipoxygenase-3

Collection	Maturity group[c]	Number evaluated
FC strains[a]		22
PI strains[b]	00	72
	0	172
	I	273
	II	572
	III	706
	IV	1780
	V	942
	VI	322
	VII	226
	VIII	183
	IX	86
	X	113
U.S. and Canadian cultivars[d]		394
Glycine gracilis[e]		41
Glycine soja (wild soybeans)[f]		595

[a]Numbers assigned by the former Forage and Range Research Branch, USDA.

[b]Plant introduction numbers assigned by Germplasm Research Lab., USDA.

[c]Soybean germplasm has been classified into 19 maturity groups based on day-length response. Groups 00 to IV are maintained at Urbana, IL, and groups V to X are maintained at Stoneville, MS.

[d]A collection of cultivars released by public institutions.

[e]A collection of accessions, intermediate between the soybean (*G. max*) and its wild ancestor, which taxonomically belongs to *G. max*, but for this report separated from *G. max*.

[f]The wild ancestor of the soybean.

L-1 Substrate

An aqueous linoleate stock solution containing Tween-20 as the dispersant was used as described by Yoon and Klein (18) except that it contained 0.02% citric acid to chelate possible prooxidant contaminants, such as copper and iron, and 5×10^{-3} M NaCN to inhibit heme oxidases (19). The stock solution was diluted with 4 vol of 0.2 M borate buffer, pH 9.0, for all assays giving a final concentration of 2.57 mM linoleic acid. The substrate always was prepared immediately prior to use from linoleic acid stored frozen under nitrogen.

L-2 and L-3 Substrate

For the stock solution, 0.2 ml methyl linoleate was dissolved in 50 ml 95% ethanol containing 0.02% citric acid and 5×10^{-3} M NaCN. This was diluted with 4 vol of 0.1 M phosphate buffer, pH 7.0 giving a final concentration of 2.41 mM methyl linoleate. The substrate was prepared immediately prior to use.

Qualitative Screening Procedure

In screening for variants for L-1 activity, 10 μl of the seed extracts were mixed with 2 ml aerated L-1 substrate and the change in absorbance at 234 nM (conjugated diene formation) at 22 C relative to the control was monitored with a Beckman UV recording spectrophotometer (20). The same procedure was used in screening for L-2 and L-3 variants except that the L-2 and L-3 substrate was used.

Quantitative Assays

The same procedures were used as in the qualitative screening procedure except that extracts from 6 seeds of each accession were analyzed using the substrates as above and with a 1:10 and 1:100 dilution of the L-1 substrate in pH 9.0 buffer. Ten μl of the undiluted seed extracts was used for the ester assay and 10 μl of a 1:100 dilution of the seed extracts was used for the acid assay. One unit of activity was defined as an absorbance increase of 0.01 $(min)^{-1}$ at 234 nm. The protein content of the extracts was determined by the Lowry method (21).

Oxygen uptake at 28 C using the ester and acid substrates was monitored using 4.0-ml glass temperature-controlled reaction cells equipped with an oxygen electrode (Yellow Springs Instrument Co.) which were placed in a light path of a Bausch and Lomb Spectronic 70 coupled to an Ominscribe recorder (20).

Electrophoresis

Electrophoresis of the seed extracts on 10% polyacrylamide was performed according to the method of Davis but at pH 8.3 (22). The current was 1 mA/gel for 5 min and 3.5 mA/gel for 50 min. The buffer was at 4 C initially and electrophoresis was performed at room temperature. A volume of seed extract containing about 6 mg of protein was applied to each gel. Lipoxygenase activity in the gels was detected according to the starch-iodine method of Guss et al. (19) and using a specific lipoxygenase staining technique involving o-dianisidine (23,24) at pH 9.0

Isoelectric Focusing

Isoelectric focusing of the soybean seed extracts was performed as described by Catsimpoolas (25) but with the following modifications: the anode electrode solution was 0.01 M phosphoric acid and the cathode electrode solution was 0.02 M sodium hydroxide. Both solutions initially were at 4 C. The gels contained 10% sucrose and 3.07 mM ammonium persulfate instead of a riboflavin catalyst. Also, the gels contained 2% ampholytes (LKB) of four parts pH 5 to 7 to one part pH 3 to 10 ampholyte solution. The seed extracts applied to the gels were extracted as described above (ca. 2 mg protein/gel) except that extracts also contained 2% ampholytes (as for the gels). The gels were stained for lipoxygenase in pH 9.0 buffer using the o-dianisidine stain (23,24).

Immunochemical Study

Soybean lipoxygenase specific antibodies were obtained by immunizing each of two young adult male New Zealand white rabbits (*Oryctologus cuniculus* L.) with 5 mg soybean seed lipoxygenase (Type IV, Sigma Chemical Co.) plus 1 ml Freund's complete adjuvant emulsion (Grand Island Biological Co.) for the primary injection. A booster injection consisting of an emulsion of 5 mg lipoxygenase plus 1 ml Freund's incomplete adjuvant was given to each rabbit 6 weeks after the primary injection. The emulsions were injected into the rabbits subcutaneously in the top of the neck. All bleedings were made from the marginal ear vein of the rabbits. Each radial diffusion plate was prepared by adding 1 ml of the lipoxygenase antiserum to 10 ml 1% agar at 60 C in 0.1 M phosphate buffer, pH 8.0. Twenty μl of seed extracts and lipoxygenase (1 mg/ml) was placed in 4-mm diameter wells in the agar.

Seed Age Study

Lipoxygenase was extracted from each of 10 seeds of 2 lots of cv. Harosoy. The first lot was stored 6 years at 10 C, then 3 years at room temperature. The second lot of Harosoy seed was harvested in 1979 and stored for 6 months at 10 C. The seeds were assayed for both L-1 (acid assay) and L-2 and L-3 (ester assay).

RESULTS

Both the L-1, L-2 and L-3 activity of cv. Harosoy seeds stored for 6 years at 10 C, then 3 years at room temperature were not significantly different from Harosoy seeds stored 6 months at 10 C.

Two accessions out of the 6499 screened were found to lack detectable L-1 activity. One of the accessions with undetectable L-1 activity was PI 133226 introduced into the U.S. in 1939 from Indonesia and the other was PI 408251 introduced into the U.S. in 1976 from South Korea. All other accessions evaluated had high L-1 activity. All of the accessions evaluated had high L-2 and L-3 activity.

The conjugated diene formation of the two L-1 variants was not different from the boiled control for the quantitative acid assay. The results were the same when the assay was conducted against the L-1 substrate diluted 1:10 and 1:100 with the 0.2 M borate buffer, pH 9.0. The two L-1 variants showed similar ester activity as the cv. Williams and a randomly selected plant introduction, PI 423800 (Table II).

Oxygen uptake is at least 200 times greater in normal soybean genotypes than in either of the two L-1 variants with the acid assay. There was little difference between the L-1 variants and the normal soybean genotypes for oxygen uptake with the ester assay (Table II).

TABLE II

Conjugated Diene Formation and Oxygen Uptake of Standard Soybean Genotypes and Lipoxygenase-1 Variants with the Linoleic Acid, pH 9.0 (acid assay) and Linoleic Acid Methyl Ester, pH 7.0 (ester assay) Substrates

Genotypes	Conjugated diene formation[a]		Oxygen uptake[b]	
	Acid assay	Ester assay	Acid assay	Ester assay
Williams	246[c]	3.0	6.9×10^{-4}	571.7
PI 133226	0	1.5	15.4	173.1
PI 408251	0	1.4	114.3	423.0
PI 423800[d]	132	2.7	2.4×10^{-4}	489.5

[a]One activity unit is defined as a change in absorbance of 0.01 at 234 nm min^{-1} (mg protein)$^{-1}$ after adjustment for the boiled control.

[b]Equals nmol O_2 min^{-1} (mg protein)$^{-1}$ adjusted for boiled control.

[c]All values are the means of 6 determinations.

[d]PI 423800 is a randomly selected soybean genotype.

Electrophoresis of seed extracts of cv. Williams and PI 423800 and staining for lipoxygenase activity at pH 9.0 produced lipoxygenase-1 bands. However no bands were produced by the variants PI 133226 or PI 408251 with either the starch-iodine staining technique (19) or the o-dianisidine staining technique (23,24).

As with electrophoresis, only soybean genotypes with normal L-1 activity showed lipoxygenase bands with the o-dianisidine stain (23,24).

Radial diffusion in agar containing soybean lipoxygenase antiserum showed 3 bands with the commercial source of soybean lipoxygenase, cv. Williams and PI 423800. The middle precipitin band was missing for the two L-1 variants, PI 133226 and PI 408251 (Fig. 1).

DISCUSSION

All of the evidence suggests that two soybean accessions

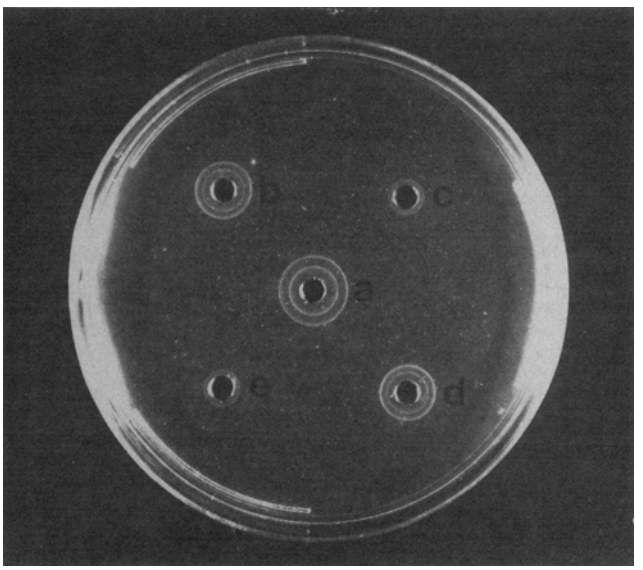

FIG. 1. Radial diffusion in 1 ml soybean lipoxygenase antiserum in 10 ml 1% agar in 0.1 M phosphate buffer, pH 8.0. (a) 20 μl Sigma Chem. Co. Type IV soybean lipoxygenase (1 mg/ml); (b) 20 μl Williams seed extract (50 mg/ml); (c) 20 μl PI 408251 seed extract 50 mg/ml); (d) 20 μl PI 423800 seed extract (50 mg/ml); and (e) 20 μl PI 133226 seed extract (50 mg/ml).

have been found that lack lipoxygenase-1, the major form of lipoxygenase present in soybean seeds in terms of in vitro activity (11). The two accessions have normal L-2 and L-3 activity indicating, as expected, that L-1 is coded for by a DNA sequence at a different locus than L-2 and L-3. All three forms of lipoxygenase in soybean seeds probably are non-allelic because of the differences in, e.g., substrate specificy, pH optimum, calcium activation and reaction products (8,9,11,26,27). Thus, the different lipoxygenase forms in soybean seeds probably are true isozymes rather than allozymes (28).

The acid and ester assays (for conjugated diene formation and oxygen uptake) provide a rapid means of screening for variants of the different lipoxygenase isozymes. The high pH, polar substrate, and the use of Tween-20 in the substrate preparation of the acid assay is optimal for L-1 activity and should show little activity of L-2 and L-3 (10,12). The discovery of genotypes with no detectable acid activity but near normal ester activity indicates that the acid assay indeed was specific for L-1. The low pH and nonpolar substrate in the ester assay is more optimal for L-2 and L-3 and the use of ethanol instead of Tween-20 as the dispersing agent gives greater activity for L-2 (10,12). The soybean assessions screened in the study displayed some variability with the ester assay (as measured by conjugated diene formation). However, the two L-1 variants showed reduced but readily detectable activity with the ester assay, suggesting that the ester assay also picks up L-1 activity. However, modifier genes may also be involved in these differences in L-2 and L-3 activity.

No rapid assay could be developed which clearly differentiated L-2 from L-3. Inclusion of calcium in the reaction media may have given some separation (8,29) but the effects of calcium on the lipoxygenase isozymes in crude extracts is unknown. L-2 appears to have greater specific activity than L-3 (11), but no quantitative information is available on the relative amounts of the different lipoxygenase isozymes in soybean seeds. The ester assay was designed to show a clear genotypic difference of those accessions which lacked the major ester isozymes (L-2 and L-3) or either of them assuming they are of equal activity. All 6,499 accessions evaluated had essentially equal ester activity.

A number of physiological roles have been proposed for lipoxygenase, including the initiation of lipid oxidation in germinating seedlings (30), involvement in pathogen defense (31), and ethylene biosynthesis (32), but the evidence is not conclusive for any of these roles. Also, there has been no differentiation of the lipoxygenase isozymes in any of these physiologial studies. The discovery of the two soybean genotypes lacking L-1 activity will be of considerable utility in the study of the physiologial roles of lipoxygenase. The results reported here also demonstrate the importance of collecting and maintaining extensive germplasm collections of our major crops.

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