Hexanal Production and TBA Number Are Reduced in Soybean [*Glycine max* (L.) Merr.] Seeds Lacking Lipoxygenase Isozymes 2 and 3

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Lipoxygenase (LOX) isozymes (linoleate:oxygen oxidoreductase, EC 1.13.11.12) catalyze the hydroperoxidation of unsaturated fatty acids with a *cis,cis*-1,4-pentadiene moiety. This reaction is the first step leading to the production of several compounds which appear to be directly related with the off-flavor characteristic of soybean protein products. In our breeding program toward cultivars yielding better tasting protein products, we determined the hexanal production and 2-thiobarbituric acid (TBA) number for the variety Cristalina (CR) and for lines derived from Cristalina lacking LOX 1 (CR1), LOX 2 (CR2), LOX 3 (CR3), LOX 1,3 (CR1,3), and LOX 2,3 (CR2,3). Removal of LOX 1 did not affect hexanal production or TBA number. However, removal of LOX 3 caused a significant decrease on both parameters. LOX 2 proved to be the most important isozyme for hexanal production. The double-recessive line lacking LOX 2 and LOX 3 yielded the lowest TBA numbers and hexanal levels. This was true for seeds with moisture contents ranging from 10.9 to 21.3%.

INTRODUCTION

One of the major obstacles for the use of soybean as a protein source for human consumption in Western countries is the presence of off-flavors which are typical of soybean products. Several authors have shown evidence that lipoxygenase (LOX) isozymes present in soybean seeds are the main factors responsible for such undesirable features (Axelrod, 1974; Rackis et al., 1979; Arai et al., 1970). The initial hydroperoxidation of unsaturated fatty acids which is catalyzed by LOX may lead to the formation of short carbon chain acids, ketones, and aldehydes (Leoni et al., 1985). These compounds, mainly ketones and aldehydes, are responsible for the typical taste and flavor of soybean products (Rackis et al., 1979). Soybean seeds are a rich source of LOX and their substrates, linoleic and linolenic acids. Physical damage during harvesting, transport, and storage allow direct contact between enzymes and substrates. High humidities and temperatures create ideal conditions for this interaction to occur (Rezende et al., 1988). Since the external factors that favor the enzymatic hydroperoxidation of unsaturated fatty acids cannot be easily controlled, the genetic elimination of LOX from the seeds has been proposed by several authors (Kitamura, 1984; Davies et al., 1987). Mutants lacking each of the individual LOX isozymes have been reported in the literature (Hildebrand and Hymowitz, 1981; Kitamura, 1984; Kitamura et al., 1983); more recently, a mutant lacking all three types of LOX in the seeds has been obtained through mutagenesis induced by γ-ray irradiation (Hajika et al., 1991). The fact that the absence of these individual isozymes does not seem to cause any detectable physiological and/or agronomical consequence to the soybean plant has motivated our group to develop a commercial cultivar devoid of LOX in the seeds. We have crossed mutant lines lacking each of the LOX isozymes to a commercial cultivar (Cristalina) widely grown in the central part of Brazil, and through a series of backcrosses of specific F2 segregants we have been able to obtain lines lacking each one of the isozymes as well as double-recessives LOX 1 and 3 minus and LOX 2 and 3 minus. Here we compare the hexanal production and thiobarbituric acid number between these lines and Cristalina. Hexanal was determined as a function of seed moisture content, and TBA number was measured in flours and extracts at various homogenization periods.

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MATERIALS AND METHODS

Genetic Material. All soybean genotypes used were from the breeding program conducted at the Federal University of Viçosa, Viçosa, MG, Brazil. Seeds were harvested during the agricultural year 1987/1988 at the Federal University of Viçosa Experimental Station. Cristalina (CR) is a commercial variety which is mainly grown in the central part of Brazil. Lines CR1, CR2, CR3, CR1,3, and CR2,3 are derived from Cristalina lacking LOX 1, 2, 3, 1 and 3, and 2 and 3, respectively. They were obtained by crossing Cristalina with mutants lacking each of the LOX isozymes. CR1, CR2, and CR3 were in the third backcross generation, after being submitted to two field selections. CR1,3 and CR2,3 derived from crosses between CR1 and CR3 and between CR2 and CR3, respectively. Seeds from 50–80 plants from each line were bulked in separate lots and used for all experiments.

Reagents. Butylated hydroxytoluene, 2-thiobarbituric acid, antifoam A, and 1,1,3,3-tetramethoxypropane were purchased from Sigma Chemical Co.

Increase of Moisture Content of Soybean Seeds. Two lots of seeds from each soybean line each containing approximately 30 g (10.9% average moisture content) were kept in a germinator at high relative humidity, 30 °C, for 6 and 16 h. The average moisture contents of the seeds increased to 16.1 and 21.3%, respectively. After this treatment, the seeds were ground and the flour was used for triplicate hexanal determinations. Water gain was calculated by weighing the seeds before and after the treatment.

Preparation of Crude Extracts. Crude extracts from whole soybean seeds were prepared according to the method of Davies et al. (1987). Briefly, 50 g of soybean seeds (ca. 10% moisture content) were soaked in 150 mL of distilled water for 12 h at 4 °C. The tegument was removed, and the seeds were washed with water. They were then homogenized in 150 mL of cold distilled water for 2 min at high speed in a commercial blender and filtered through two layers of cheesecloth into a beaker. Aliquots of the filtrate were used for triplicate determinations of hexanal and TBA number.

Determination of Hexanal. Hexanal was determined by headspace gas chromatography based on the method of Rezende

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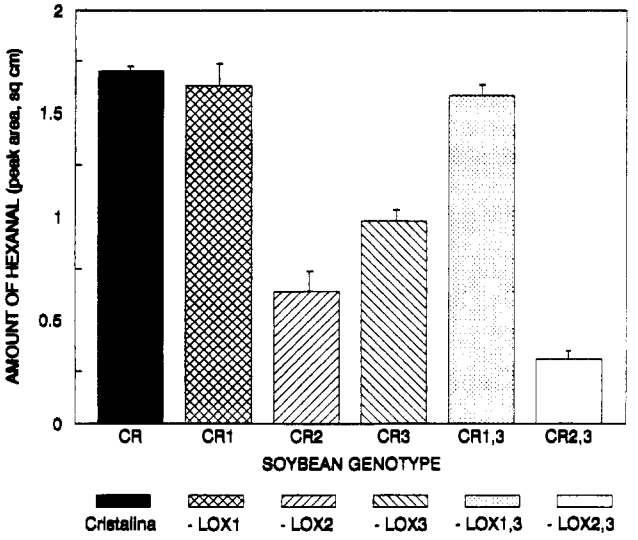

Figure 1. Hexanal determination in soybean extracts from seeds of the backcross lipoxygenase mutant lines and the wild-type recurrent parent Cristalina (CR). Each point corresponds to an average of three determinations from one lot of seeds.

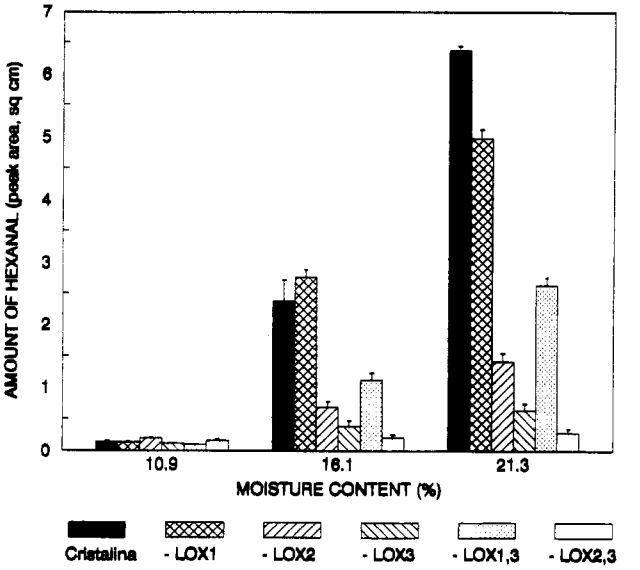

Figure 2. Hexanal determination in soybean flours from seeds at different moisture levels. Genotypes used are defined in Figure 1. Each point corresponds to an average of three different determinations from one lot of seeds.

et al. (1988). Three grams of soybean flour or 10 mL of extract was put in 33-mL vials and left at room temperature for 1 h. Then, 18 mL of 0.5 M NaOH was added to the flour or 9 mL of 1 M NaOH to the extract. After 5 min of agitation in a vortex agitator, the pH was reduced to 0.8 by adding 3 mL of 9 M HCl. The vials were tightly sealed with a septum secured by an aluminum cap, agitated again for 5 min, and incubated at 150 °C for 22 min. One-milliliter headspace vapor samples were then analyzed by gas chromatography in a 3 m × 3 mm column filled with 10% (v/v) Triton X-305 in Teflon. The oven temperature was 110 °C; the injector and detector temperatures were 180 and 250 °C, respectively. Hexanal was quantified by determination of the area of the corresponding peak and expressed in square centimeters.

Determination of TBA Number. This procedure was developed by Tarladgis et al. (1960) and modified by Rhee and Watts (1966) and Davies et al. (1987). It is based on the determination of dialdehydes released from lipid oxidation. These products can form a colored complex when reacted to TBA. Briefly, 4 g of soybean flour or 15 mL of crude extract was homogenized with a Polytron homogenizer at low speed in 50 mL of distilled water at room temperature for different periods. Enzymatic activity was then inactivated by addition of 4 M HCl up to pH 1.2. One hundred and fifty microliters of 15% (w/v) butylated hydroxytoluene (BHT) in ethanol plus 0.5 mL of antifoam A were added to the homogenate, and the volume was completed to 100 mL with water. Products released from lipid oxidation were separated by distillation with a Barret distilling apparatus (Vidrolabor, São Paulo, Brazil). Fifty milliliters of the distillate was collected, and 3-mL aliquots were mixed with the same volume of 20 mM TBA in acetic acid. Color reaction developed for 35 min in a boiling water bath. Absorbance was read at 538 nm, and the product was expressed as milligrams of malonaldehyde per 1000 g of sample. A standard curve was prepared via distillation with 1,1,3,3-tetramethoxypropane ranging from 6.6 to 33.3 μM. Malonaldehyde recovery factor was close to 1.

RESULTS

Hexanal Production in Soybean Extracts. We determined hexanal production in soybean extracts of cultivar Cristalina (CR) and of lines derived from Cristalina lacking at least one of the LOX isozymes. CR1 and CR1,3 released basically the same amounts of hexanal compared to the normal line CR (Figure 1). On the other hand, CR3, CR2, and CR2,3 produced, respectively, around 40%, 62%, and 82% less hexanal than CR (Figure 1).

Hexanal Production in Soybean Flour. Soybean flour obtained from seeds at different moisture contents were tested for hexanal production. At low moisture contents (10.9%) there was no significant difference between the amounts of hexanal released from flours prepared with seeds from cultivar CR and CR-derived lines (Figure 2). However, as the moisture content of the seeds was artificially raised to 16.1% and 21.3%, the effect of the different LOX isozymes on hexanal production could be noticed. CR and CR1 produced the highest amounts of hexanal, followed by CR1,3, CR2, CR3, and CR2,3 (Figure 2). This last genotype, which lacks LOX 2 and LOX 3, released a low steady amount of hexanal essentially independent of the moisture content.

TBA Number in Soybean Crude Extracts and Flours. The TBA number increased for most genotype extracts and flours as a function of homogenization time (Figures 3 and 4). Flours and extracts prepared with seeds from genotype CR had the highest TBA number and CR2,3 the lowest. Similarly for the hexanal production, CR2,3 presented a low steady TBA number independent of the homogenization time.

DISCUSSION

Hexanal production and TBA number have been reported to be correlated with off-flavors in soybean products (Arai, 1970; Davies et al., 1987). Therefore, these parameters were determined as a measure of the quality of soybean seeds obtained in our breeding program toward cultivars yielding better tasting protein products. Determination of hexanal in extracts of soybean seeds lacking at least one of the LOX isozymes (Figure 1) indicates that while LOX 1 does not seem to contribute much for hexanal production, LOX 2 is the most active isozyme leading to the formation of such aldehyde. This result confirms data from other authors using linoleic acid as an exogenous substrate (Matoba et al., 1985; Zhuang et al., 1991). LOX 3 appears to have a moderate but significant role in hexanal production. CR3 released an intermediate amount of hexanal, between that produced by CR1 and CR2 (Figure 1). Our results did not show the negative effect of LOX 3 proposed by Hildebrand et al. (1990) on C_6 aldehyde production. Our experimental conditions, however, differ

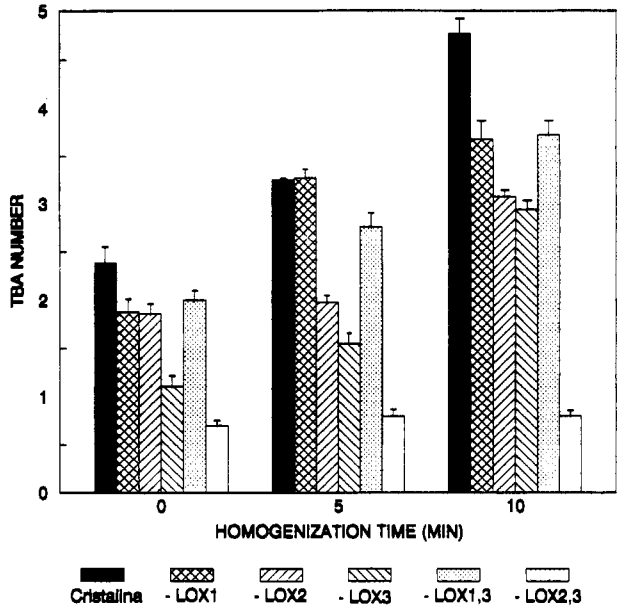

Figure 3. Determination of TBA number of extracts from soybean seeds submitted to different homogenization periods. Genotypes used are defined in Figure 1. Each point corresponds to an average of three different determinations from one lot of seeds.

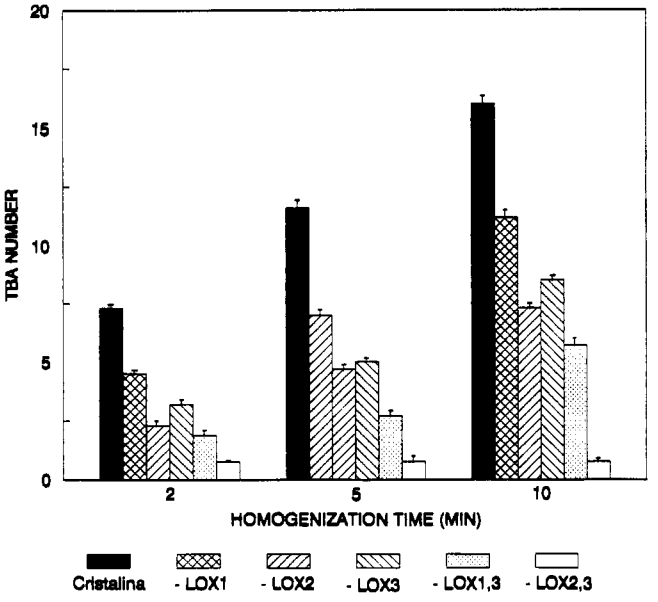

Figure 4. Determination of TBA number of flours from soybean seeds submitted to different homogenization periods. Genotypes used are defined in Figure 1. Each point corresponds to an average of three different determinations from one lot of seeds.

significantly from those used by that group, particularly in relation to the preparation of the samples for headspace gas chromatography (see Materials and Methods). Furthermore, unlike these authors, we did not use an exogenous substrate (linoleic acid) for lipoxygenase.

Moisture content of the seeds is an important factor affecting quality of soybean protein (Rezende et al., 1988). We determined hexanal evolution in seeds of the various CR lines with different moisture contents to learn about the combined effect of LOX deficiency and moisture content (Figure 2). Determination of hexanal in soybean flours with low moisture content (10.9%) confirmed the importance of this factor on hexanal production and, indirectly, on protein quality. All genotypes released a very low amount of hexanal at such moisture level (Figure 2). Higher moisture contents caused a general increase of hexanal evolution and confirmed the importance of LOX 2 and LOX 3 for the production of this aldehyde (Figure 2). Hexanal release from CR2,3 flour was essentially independent of moisture content in the range 10.9–21.3% under the conditions of our assay (Figure 2).

TBA numbers obtained from flours and extracts homogenized for different times also confirmed the important role of LOX 2 and LOX 3 on lipid hydroperoxidation (Figures 3 and 4). Seeds lacking these isozymes yielded flours and extracts with low TBA numbers which did not vary significantly with increasing homogenization time (Figures 3 and 4). Lipid oxidation was also measured by TBA analysis in cultivar Century and its near-isogenic lines lacking at least one of the LOX isozymes (Davies et al., 1987). In that case, the authors found a good correlation among TBA number, sensory evaluation, and absence of LOX 2 isozyme. Their results indicated that genetic removal of LOX 2 might reduce off-flavors in soy products. In our case, the line lacking LOX 2 and LOX 3 (CR2,3) yielded the lowest values for hexanal production and TBA number (flour and extract). Unfortunately, Davies et al. (1987) did not determine the TBA number for Century minus LOX 2 and LOX 3 or hexanal production for any of the lines they studied. Additionally, in this work we have analyzed the effect of the seed moisture content on hexanal production. Our results point to CR2,3 (minus LOX 2 and LOX 3) as the most promising line for the food industry. Preliminary sensory evaluation in our laboratory points to the same direction (data not shown).

The experimental approach we have chosen has many intrinsic variables. First, we assumed that the activities of hydroperoxide lyase and of other enzymes that might be involved with hydroperoxide catabolism were essentially the same in all genotypes tested. Second, we were aware that by preparing the crude extracts in water (pH ~ 6.5) we favored the action of type II LOX (LOX 2 and LOX 3). These isozymes have a pH optimum around 7.0, while LOX 1 reaches maximum activity at pH 9.0 (Axelrod et al., 1981). Finally, one should consider that since soybean seeds contain extremely low amounts of free fatty acids (Frankel et al., 1987), which are preferred by LOX 1, the majority of the hexanal measured was due to the action of LOX 2 and LOX 3, which are more effective in the hydroperoxidation of esterified fatty acids (Zhuang et al., 1991).

The two variables tested, i.e., moisture content and homogenization time, are extremely important for the food industry. It is as critical to harvest seeds with a low moisture content as it is to determine the amount of time the soy extract should be kept soaked in water. It is clear that the elimination of LOX 2 and LOX 3 allows more flexibility during harvesting and processing of the seeds. The next step is to determine if the protein products obtained from these modified lines will indeed present better organoleptic properties.

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