The Oxidation-Reduction Enzymes of Wheat. III. Isoenzymes of Lipoxidase in Wheat Fractions and Soybean[1]

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ABSTRACT

Crude aqueous extracts from wheat milling fractions, soybeans, and mung beans, in addition to a commercially purified soybean lipoxidase, were subjected to electrophoresis on polyacrylamide gels. A specific staining procedure involving incubation of gels containing 0.5% soluble starch with linoleate substrate, followed by treatment of the gel with acidic potassium iodide, revealed lipoxidase as brown to blue bands. Mung beans and purified commercial soybean lipoxidase each showed one band. Break shorts, reduction shorts, red dog, and flour from Selkirk, Lee, Bison, and Triumph varieties of wheat showed two major bands; the break shorts of Selkirk and Lee also showed two minor bands with lower R_f values. Crude aqueous extracts of soybean showed four bands that stained positive for lipoxidase.

Lipoxidase is a plant enzyme occurring in some cereal grains which catalyzes the peroxidation of the cis, cis pentadiene double bond system (1). The importance of lipoxidase to the baking industry has been established for some time (2).

Experiments by Koch *et al.* (3) suggest that there is more than one lipoxidase in soybeans. Previous work from this laboratory on wheat dehydrogenases (4) demonstrated the existence of a number of isoenzymes.

The present study was undertaken to develop a staining procedure for lipoxidase on polyacrylamide gels and to study the occurrence of lipoxidase isoenzymes in wheat *(Triticum vulgare)* as well as soybean *(Glycine max)* and mung bean *(Phaseolus aureus).*

MATERIALS AND METHODS

Extraction of Lipoxidase

Wheat Mill Fractions. Reduction shorts, break shorts, and red dog from Selkirk, Lee, Bison, and Triumph varieties of wheat were extracted with 12.5% sucrose solution (1:6 w./v.) for 75 min. at approximately 4°C.; a magnetic stirrer was operated at moderate speed so that foaming was minimal. Nondefatted wheat fractions extracted with severe agitation (as with a Virtis homogenizer) exhibit considerable smearing of the lipoxidase activity on polyacrylamide gels. After extraction, the mixtures were centrifuged in a Servall SS-1 at approximately 5,000 × *g* for 15 min., filtered through Whatman No. 1 filter paper, and frozen in approximately 1½-ml. aliquots. Lipoxidase activity in the frozen extracts appeared to remain essentially constant for periods up to 6 months.

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Twenty grams of flour from each of the above varieties was extracted at 4°C. with water (1:6 w./v.) and centrifuged, and the supernatant was freeze-dried. The lyophilized supernatant solids were taken up in 10 ml. of 12.5% sucrose, and 0.1 ml. of this solution was subjected to electrophoresis. Total weight of dried extract applied to the gel ranged from 3.8 to 7 mg. The large amount was necessary because of the relatively low lipoxidase activity of the flour.

These wheat fractions were all shown to contain lipoxidase as measured by the method of Surrey (5).

Soy and Mung Beans. Whole soy and mung beans were ground to a coarse powder with mortar and pestle. This material was then treated with 10 volumes of cold acetone in a Waring Blendor for 1 min. The solvent extraction was repeated twice, after which the powders were allowed to dry under vacuum at room temperature. The defatted powders were then extracted with sucrose solution (1:10 w./v.) as described above.

Disk Gel Electrophoresis

Electrophoresis on polyacrylamide gels was performed essentially by the method of Ornstein and Davis (6), with the exception that the small-pore solution No. 2 contained 1% soluble potato starch for iodometry (J. T. Baker) and was made fresh daily. Sample sizes ranging from 30 to 100 μl. per tube were subjected to electrophoresis at 4°C. with a current of 2.5 ma. per tube.

Staining Procedure

When electrophoresis was complete, the gels were removed from the tubes and placed in 10 $\times$ 100-mm. test tubes, and substrate solution was added to completely cover the gels. The substrate consisted routinely of $2 \times 10^{-3}M$ linoleate in $0.05M$ tris-HCl buffer at pH 8.3. Substrate was prepared by dispersing 0.5 g. linoleic acid ($> 99\%$, Hormel) with a drop of Tween 20 in 25 ml. redistilled water. This suspension was ultrasonically dispersed under nitrogen in an ice bath in a Branson sonifier (model LS-75) for 5 min. at a power level setting of 8 and tuned to 9.5 amperes. The dispersed linoleic acid was made up to 50 ml. with redistilled water. This stock solution was diluted appropriately with tris-HCl buffer, pH 8.3, to yield $2 \times 10^{-3}M$ substrate. The gels reacted with the substrate for 20 min. at room temperature; the tubes were inverted frequently to ensure aeration.

After substrate incubation, the gels were removed from the tubes, rinsed thoroughly with distilled water, and placed in clean test tubes, 10 $\times$ 100-mm. The tubes were then filled with a solution of acidic potassium iodide containing 5 ml. saturated aqueous KI per 100 ml. 15% acetic acid and immediately stoppered with a cork. The brown-to-blue activity bands begin to appear within 2 to 5 min. and are optimally developed within 15 to 20 min.

For optimal sensitivity and to prevent background staining due to autoxidation of the potassium iodide, the acetic acid solution is degassed by vacuum and purged with nitrogen just prior to the addition of the KI. Further, these two components are mixed under nitrogen just prior to use.

With commercially purified soybean lipoxidase, bands have been observed with amounts as low as 500 units as defined by Sigma.[2]

RESULTS AND DISCUSSION

Staining Specificity

Since many systems will produce a colored complex in the presence of acidic potassium iodide and soluble starch, measures were taken to ensure the specificity of the reaction. The bands appeared after incubation of the gels in linoleic acid or its salt, but no reaction was obtained with chromatographically pure oleic acid or with 99% trans-trans linolelaidic acid (Sigma).

Heme-catalyzed peroxidations are inhibited by cyanide (1); however, incubation of the gels in substrate containing $10^{-3}M$ cyanide had no apparent effect on the enzyme pattern; thus, nonspecific heme-catalyzed lipid peroxidation was eliminated. The stain for peroxidase, with guaiacol as a hydrogen donor, was completely blocked in the presence of $10^{-3}M$ cyanide.

Finally, addition of antioxidants such as alpha-tocopherol and hydroquinone to the substrate appears to inhibit the band intensity. Alpha-tocopherol at $10^{-3}M$ decreased band intensity approximately 50% over a control; hydroquinone at $10^{-3}M$ eliminated the response. With hydroquinone, the background darkens rapidly when the gels are placed in the acidic KI.

The above results indicate that banding produced on polyacrylamide gels under the conditions described is due to lipoxidase activity.

Effects of pH and Substrate Concentration

Lipoxidase activity has been observed in extracts from wheat and soybeans over a substrate pH range from 5.5 to 8.3. Band intensity increases with increasing pH, probably owing to increased substrate solubility and consequently enhanced diffusion of the substrate into the gel. Substrate concentration was not found to be critical with $2 \times 10^{-3}M$ linoleate optimal. Koch *et al.* (3) have presented evidence suggesting the presence of two different lipoxidase enzymes in soybeans, one active on linoleic acid alone and the other having greater activity on trilinolein than linoleic acid. In experiments in which trilinolein or methyl linolenate was used as substrate, no lipoxidase activity could be detected on polyacrylamide gels of aqueous soybean extracts, even though concurrent manometric data indicated that sufficient activity was present. Since these substrates are much less soluble in aqueous solutions than linoleic acid, these results are probably caused by the inability of the substrate to diffuse into the gel.

Comparison of Lipoxidase Patterns

Figure 1 shows typical lipoxidase patterns obtained from unfractionated aqueous extracts from wheat (Selkirk break shorts), soybeans, and mung beans, as well as that from a commercially purified soybean lipoxidase.

The two faster-migrating bands seen in the wheat sample were common to all milling fractions, including the flour of the four varieties of wheat tested thus far: two HRW (Bison and Triumph) and two HRS (Lee and Selkirk). The two slower-moving minor bands in the wheat sample have been observed

[2] Sigma Chemical Co., St. Louis, Mo. Price list, Jan. 1966.

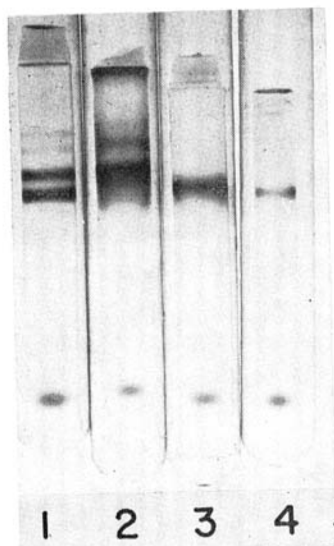

Fig. 1. Lipoxidase patterns obtained from: 1, wheat (Selkirk break shorts); 2, crude soybean extract; 3, commercially purified soybean preparation; 4, crude extract of mung bean.

in break shorts of Selkirk and Lee but not in that of Triumph and Bison. The pattern for the crude soybean extract exhibits considerably more smearing than that from wheat, although distinct banding is evident. R_f values relative to the dye band of the two minor bands in the crude wheat extracts (0.25 and 0.20) are almost identical with those of the two minor bands in the crude soy extract (0.24 and 0.21). All four sources of the enzyme shown contain a major band at R_f 0.33; wheat has a major band at R_f 0.40, and crude soybean exhibits moderate activity at the origin.

The appearance of more than one band for lipoxidase, at least for soybean, is not entirely unexpected, since Koch *et al.* (3) reported the existence of two different enzymes in soybeans. The multiplicity of bands may suggest the existence of isoenzymes of lipoxidase or may reflect polymerization of the enzyme.

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