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A NOVEL METHOD FOR ISOLATION OF LIPOXYGENASE-1 ISOENZYME FROM SOYBEAN MEAL

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A novel method has been elaborated for the isolation of lipoxygenase-1 (Lox-1) isoenzyme from soybean meal. The essential step of the procedure is the selective heat treatment of soybean meal extract at 70 °C in a medium of 0.05 ionic strength and pH 5.2 for 5 min. The heat treated extract practically free from lipoxygenase-2 (Lox-2) and lipoxygenase-3 (Lox-3) isoenzymes was purified by DEAE-cellulose ion exchange chromatography. The resulted lipoxygenase-1 isoenzyme was homogeneous as judged by PAGE. A tenfold purification and a relatively high yield (39 %) was achieved.

Keywords: soybean; Glycine max; lipoxygenase isoenzymes; selective heat treatment

Introduction

Soybean seeds contain an iron-containing dioxygenase namely lipoxygenase (linoleate : oxygen oxidoreductase, EC 1.13.11.12) catalyzing the hydroperoxidation of polyunsaturated fatty acids and esters containing a *cis,cis*-1,4-pentadiene system [1]. The hydroperoxides formed are cleaved by hydroperoxide lyase, resulting in C_6 aldehydes in plants. There are at least three lipoxygenase isoenzymes in soybean seeds, named lipoxygenase-1 (Lox-1), lipoxygenase-2 (Lox-2) and lipoxygenase-3 (Lox-3), which exhibit differences in substrate and product specificity, optimum pH for catalytic activity, isoelectric point and thermal stability [2-4]. The levels of the lipoxygenase isoenzymes are influenced to different degrees by both cultivar and climatic effects. The weather conditions can exert a greater effect than the cultivar effect [5].

With linoleic acid as substrate, linoleic acid 13-hydroperoxide and 9-hydroperoxide can be formed in the reaction catalyzed by lipoxygenase. The ratio of isomers derived from the reaction depends on the isoenzyme(s) present and the reaction conditions [6].

Soybean lipoxygenase can be used to produce flavour compounds, but in some cases the presence of Lox-2 and Lox-3 isoenzymes is undesirable because of side reactions. A large number of methods have been suggested for separation of the isoenzymes, e.g. ion exchange chromatography [7-9], chromatofocusing [10-11], electrophoresis [12] and isoelectric focusing [7, 10].

We have found [13] that the selective heat treatment of the soybean meal extract makes the elimination of Lox-2 and Lox-3 isoenzymes possible. Based on this finding, a novel method for the isolation of Lox-1 isoenzyme has been elaborated.

Materials and methods

Reagents

Linoleic acid, Tween 20, DEAE-cellulose and soybean lipoxygenase (Type I-B) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acrylamide, N,N,N',N'-tetramethylethylenediamine and Coomassie Brilliant Blue R-250 were supplied by Reanal Factory of Laboratory Chemicals (Budapest, Hungary) and N,N'-methylene-bis(acrylamide) was obtained from Fluka A.G. (Buchs, Switzerland). The other chemicals were of analytical grade. The sodium linoleate substrate was prepared according to Axelrod et al. [14].

Apparatus

A Biochrom 4060 spectrophotometer was used for enzyme activity measurements and a 2117 Multiphor II Electrophoresis System for electrophoresis (Pharmacia LKB, Uppsala, Sweden).

Lipoxygenase Assay

The method of Axelrod et al. [14] as modified by Márczy et al. [5] was applied, except that 0.2 M sodium borate buffer was used for Lox-1. One unit of activity was defined as the amount of enzyme producing 1 μmol of linoleic acid hydroperoxide per min at room temperature at pH 9.0 (Lox-1) or 6.8 (Lox-2 and Lox-3).

Protein Content

Protein contents were determined according to Lowry et al. [15], with bovine serum albumin as standard or by absorbance at 280 nm.

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed in a continuous buffer system of 0.1 M sodium phosphate buffer (pH 7.5). The total monomer concentration [acrylamide + N,N'-methylene-bis(acrylamide)] in the gel was 5 %, the concentration of N,N'-methylene-bis(acrylamide) being 2.7 % of the total monomer concentration. Samples of 15 μg protein in 0.01 M sodium phosphate buffer (pH 7.5) were applied onto a gel slab (12 cm x 4 cm). Electrophoresis was carried out for 2 h at 200 mA at 5 °C. The gel was stained for protein with 0.25 % of Coomassie Brilliant Blue R-250 dissolved in methanol:water:acetic acid (23:23:4).

Experimental

Preparation of Crude Extracts

Soybean meal was prepared from a Chandor cultivar harvested in 1993, kindly provided by the Cereal Research Institute (Szeged, Hungary). Ten grams of soybean meal was defatted with 20 cm^3 portions of petroleum ether at 0 °C. Defatted meal was extracted with 5 volumes (v/w) of 0.05 M acetic acid by mechanical stirring for 1 h at 4 °C. The suspension was then filtered through cheesecloth and centrifuged at 10,000 g for 20 min. The resulting supernatant was used in the further experiments.

Selective heat Treatment

The crude extract was heated to 70 °C in a water bath under continuous stirring and was kept at this temperature for 5 min [13]. Immediately after heat treatment the suspension was cooled to room temperature in an ice bath and then was centrifuged at 10,000 g for 20 min at room temperature.

Ion Exchange Chromatography

Anion exchange chromatography of heat treated soybean meal extract was performed according to Axelrod et al. [14] and Weber et al. [16] with modifications on DEAE-cellulose. 14.5 cm^3 heat treated soybean meal extract was dialyzed against 3 x 500 cm^3 0.01 M sodium phosphate buffer (pH 7.0). The enzymatically inactive precipitate was discarded and the supernatant was loaded on a DEAE-cellulose column (1.85 x 21 cm) equilibrated with 0.01 M sodium phosphate buffer (pH 7.0). Elution (22.5 cm^3/h) was performed with a linear gradient formed from equal volumes of 0.01 M and 0.22 M sodium phosphate buffer (pH 7.0). Fractions of 6 cm^3 were collected and the protein contents were determined by absorbance at 280 nm. Activities were assayed at pH 9.0 (Lox-1) and 6.8 (Lox-2 + Lox-3). The enzymatically active fractions of Lox-1 were pooled and concentrated by ultrafiltration.

Results and discussion

Data from a typical Lox-1 isoenzyme isolation and purification experiment are summarized in *Table 1*. The data show that the selective heat treatment of soybean meal extract at 70 °C is a crucial step in the isolation of Lox-1. Though the purification of Lox-1 was less than twofold in respect of specific activity as an effect of heat treatment, the amounts of Lox-2 and Lox-3 isoenzymes were markedly reduced by this simple method. The anion exchange chromatography makes the removal of further protein impurities possible (*Fig.1*). Tenfold purification and a relatively high yield (39 %) was achieved by the two purification steps. The purified Lox-1 isoenzyme was electrophoretically homogeneus as judged by PAGE (*Fig.2*).

Table 1 Isolation of lipoxygenase-1 isoenzyme from soybean meal[a]

Purification step	Volume (cm^3)	Total Lox-1 activity (units)	Lox-1 yield (%)	Total protein (mg)	Lox-1 specific activity (units/mg protein)	Activity ratio*	Purification
extraction	17.0	582.6	100.0	317.6	1.83	5.4	1.0
heat treatment	14.5	392.5	67.4	130.8	3.00	20.1	1.6
DEAE-cellulose chromatography	30.0	227.3	39.0	11.1	20.48	20.8	11.2

[a]In the experiment, 5 g soybean meal was processed
*Lox-1 activity / (Lox-2+Lox-3 activity)

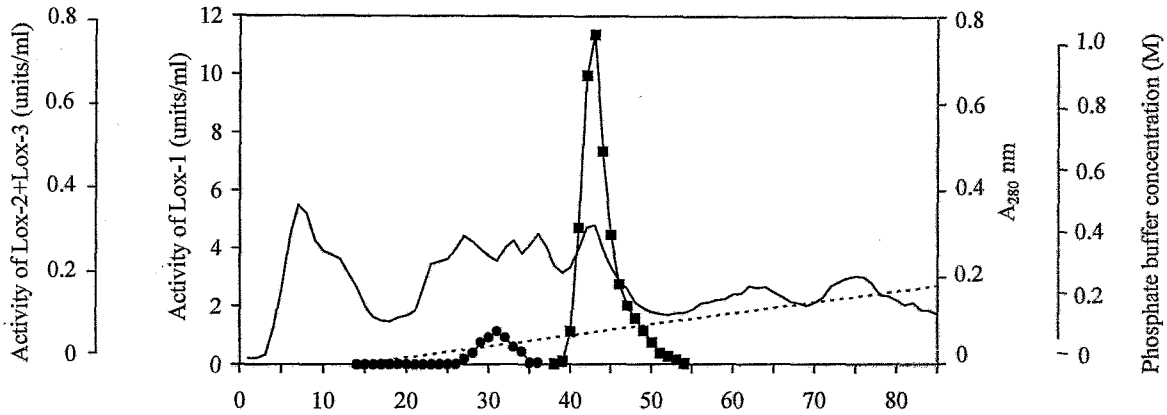

Fig.1 DEAE-cellulose column chromatography of a heat-treated soybean meal extract. Column (1.85 x 21 cm) was equilibrated with 0.01 M sodium phosphate buffer (pH 7.0). Sample (131 mg protein) was applied in the same buffer and was eluated (22.5 cm^3/h) with a linear gradient formed from equal volumes of 0.01 M and 0.22 M sodium phosphate buffer (pH 7.0).
Symbols: —●—, Lox-2 + Lox-3 activity at pH 6.8; —■—, Lox-1 activity at pH 9.0;
——, absorbance at 280 nm; ····· , concentration of sodium phosphate (M)

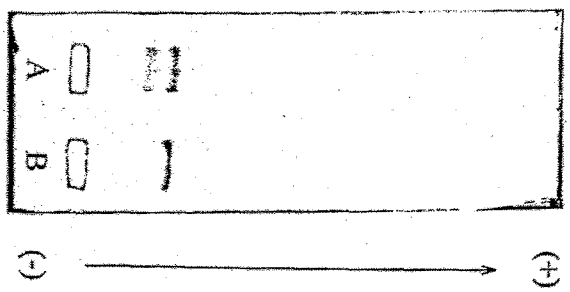

Fig. 2 Polyacrylamide gel electrophoretic pattern of purified Lox-1 isoenzyme. Electrophoresis was carried out in 5 % polyacrylamide gel slab (12 x 4 cm) for 2 h with 200 mA at 5 °C. Samples 15 μg protein each. **A**: Sigma type I-B lipoxygenase; **B**: lipoxygenase-1isoenzyme purified with the new method

Conclusion

We have found earlier [17, 18] that the selective heat treatment of aqueous extracts of animal organs as kidney and pancreas could be advantageous in enzyme isolations. It was supposed that this simple method would be useful in the isolation of plant enzymes too. The difference in the heat stability of soybean lipoxygenase isoenzymes [19] and the practical demand on Lox-1 isoenzyme make the application of heat treatment obvious. The heat treated soybean meal extract is practically free from Lox-2 and Lox-3 isoenzymes. It can be used immediately for the production of linoleic acid 13-hydroperoxide, or it can be stored frozen without deterioration for a long time. In comparison with the known methods [7-12] the proposed novel procedure is more advantageous because it does not require special equipments and chemicals. Therefore it is not expensive and is suitable for industrial purposes promoting the biocatalytic production of flavour compounds.

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