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Inhibition by lipoxygenase-3 of *n*-hexanal generation in soybeans

Hitoshi Takamura[1], Keisuke Kitamura[2] and Makoto Kito[1]

[1]*Research Institute for Food Science, Kyoto University, Uji, Kyoto 611, Japan* and [2]*National Agriculture Research Center, Tsukuba, Ibaragi 305, Japan*

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Soybean seeds contain three lipoxygenase isozymes. The functions of these lipoxygenase isozymes in *n*-hexanal generation were investigated by using mutant lines which lack two or three isozymes. In the presence of linoleic acid, the level of *n*-hexanal produced was highest in the lipoxygenase-1, -3 double deficient line, followed by the lipoxygenase-2, -3 double deficient, wild type, and lipoxygenase-1, -2, -3 triple deficient lines in that order, and lowest in the lipoxygenase-1, -2 double deficient line. This suggests that lipoxygenase-3 itself cannot produce the *n*-hexanal precursor and inhibits the *n*-hexanal generation through other pathways.

n-Hexanal generation; Lipoxygenase; Soybean

1. INTRODUCTION

Soy protein is an important protein resource, because it is economical, and because of its high nutritional quality and functionality [1,2]. However, its unfavorable grassy flavor prevents its wide utilization. The major component of its flavor is *n*-hexanal, which is generated from linoleic acid through peroxidation by lipoxygenase, followed by decomposition by hydroperoxide lyase [3–5].

Soybean seeds contain three lipoxygenase isozymes, lipoxygenase-1 (L-1), L-2 and L-3 [6]. It has been reported from this laboratory that the L-2 isozyme is responsible for the generation of *n*-hexanal in soybean homogenates by using mutants lacking lipoxygenase isozymes [4]. However, the functions of the other two isozymes remained obscure. Recently, Hildebrand et al. reported that the L-3 enzyme added exogenously to a soybean homogenate reduced *n*-hexanal production [7]. However, this was not confirmed using a soybean mutant, since no mutant line lacking both L-1 and L-2 has been obtained due to the tight linkage of these isozymes.

In this study, we aimed to elucidate the function of each lipoxygenase isozyme, especially L-3, in *n*-hexanal generation, using mutants lacking both L-1 and L-2 that we developed for the first time.

Abbreviation: L-1 (2, 3), lipoxygenase-1 (2, 3).

Correspondence address: M. Kito, Research Institute for Food Science, Kyoto University, Uji, Kyoto 611, Japan. Fax: (81) (774) 333004.

2. MATERIALS AND METHODS

2.1. *Soybean cultivars*

All seeds were produced in a greenhouse at the National Agriculture Research Center during the winter of 1990-1991. The normal (wild type), L-2, -3 double deficient, and L-1-, -3 double deficient seeds were from cv. Suzuyutaka, Kanto No. 101, and Kanto No. 102, respectively. The L-1, -2, -3 triple deficient seeds were obtained from a selfed M_5 soybean plant lacking all three lipoxygenase isozymes [8]. The L-1, -2 double deficient (F_3) seeds were from an F_2 line lacking L-1 and L-2 derived from a selfed F_1 plant obtained through a cross between cv. Suzuyutaka and the M_4 line lacking all three lipoxygenase isozymes (M. Hajika, K. Igita, and K. Kitamura, manuscript in preparation). Distribution of lipoxygenase activities in those seeds are shown in Table I.

2.2. *Reagents*

Linoleic acid (99%) was purchased from Sigma (St. Louis, MO, USA). Acetonitrile and tetrahydrofuran were of HPLC grade. Other chemicals were of guaranteed reagent grade.

2.3. *Resolution of lipoxygenase isozymes by SDS-polyacrylamide gel electrophoresis*

The lipoxygenase isozymes in soybeans were separated by single dimension SDS-polyacrylamide gel electrophoresis as described by Kitamura et al. [9], except for the following modifications: solutions A and B were changed to solutions A (36.3 g Tris, 48 ml 1 N HCl, and 0.4 g SDS in a total volume of 100 ml) and B (48 ml 1 N HCl, 0.4 g SDS, adjusted to pH 7.0 with Tris in a total volume of 100 ml), respectively.

2.4. *Preparation and incubation of a soybean homogenate*

A soybean seed (1 grain, about 0.10–0.25 g) was soaked in water at 4°C overnight. After removal of the seed coat, the soaked seed was homogenized in 3–5 ml of cold water with a glass homogenizer (Potter–Elvejhem type) under cooling in an ice bath. The supernatant obtained on centrifugation (1500 × *g*, 10 min, 0°C) was used in the subsequent experiment. An aliquot (0.8 ml) of the homogenate was incubated with 20 μl of substrate buffer (0.05 M sodium phosphate containing 0.2% Tween 20, pH 7.0, with or without 0.2% linoleic acid) at 25°C for 30 min [4].

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2.5. *Determination of n-hexanal and the protein content*

n-Hexanal was determined as its 2,4-dinitrophenylhydrazone derivative by HPLC according to Matoba et al. [4]. The 2,4-dinitrophenylhydrazone derivative of *n*-heptanal was used as an internal standard. The protein content of the soybean homogenate was determined by the procedure of Lowry et al. [10] with bovine serum albumin as a standard.

3. RESULTS AND DISCUSSION

Fig. 1 shows typical results of the SDS-polyacrylamide electrophoresis of the lipoxygenase isozymes in homogenates of five soybean varieties. We obtained mutant lines with no lipoxygenase isozyme (lane 2), and with only L-1 (lane 3), L-2 (lane 4), or L-3 (lane 5).

The *n*-hexanal generation in soybean homogenates prepared from these mutant lines is shown in Table II. In the absence of free linoleic acid, *n*-hexanal was only generated in the wild type and the L1-, -3 double deficient line. The level of *n*-hexanal generation was higher in the L-1, -3 double deficient line than in the wild type. On the other hand, *n*-hexanal was generated in all the soybean lines after the addition of free linoleic acid as substrate. The level of *n*-hexanal generation was the highest in the L-1, -3 double deficient line, followed by the L-2, -3 double deficient, wild type, and L-1, -2, -3 triple deficient lines in that order, and the lowest in the L-1, -2 double deficient line. Although little *n*-hexanal generation was expected in the L-1, -2, -3 triple deficient line which lacks all three lipoxygenase isozymes, the level of *n*-hexanal generation was 69% of that in the wild type in the presence of free linoleic acid. This suggests the existence of an unidentified type of lipoxygenase-like enzyme or another pathway for *n*-hexanal generation.

Table I

Lipoxygenase activities of soybean mutant lines

Soybean	Lipoxygenase activity (Δ 234 nm/min/mg protein)	
	pH 6.5	pH 9.5
Wild type	11.90±0.33[a]	19.40±0.68
L-1, -2, -3 deficient	0.10±0.05	0.33±0.10
L-2, -3 deficient	1.75±0.13	18.93±2.28
L-1, -3 deficient	8.38±0.10	0.38±0.03
L-1, -2 deficient	0.53±0.10	0.10±0.03

Mature seeds were stored for measurement of lipoxygenase activity after storage for 5–7 days in a desiccator. Crude seed extracts were prepared by homogenizing 50 mg tissue in 2.5 ml 50 mM Tris-HCl buffer, pH 8.0, containing 20 mM $CaCl_2$ with a mortar and pestle at 4°C. The homogenate was centrifuged at 10 000 × *g* for 5 min. The supernatant was used within 1 h. Lipoxygenase activity was determined by measurement of formation of conjugated dienes at 234 nm. The assay was performed in the presence of 2.5 mM linoleic acid with 100 mM phosphate buffer (pH 6.5) for L-2 and L-3 activities or 100 mM borate buffer (pH 9.5) for L-1 activity.

[a]Values are means ±SD (n=3).

Table II

n-Hexanal generation in soybean homogenates lacking lipoxygenase isozymes in the absence and presence of linoleic acid

Soybean	*n*-Hexanal generation (nmol/mg protein)	
	−18:2[a]	+18:2
Wild type	1.34±0.06[b]	2.03±0.24
L-1, -2, -3 deficient	0.14±0.09	1.41±0.05
L-2, -3 deficient	0.15±0.01	3.56±0.74
L-1, -3 deficient	1.86±0.35	6.31±0.66
L-1, -2 deficient	0.14±0.03	0.37±0.03

[a]18:2, linoleic acid.

[b]Values are means ±SD (n=3).

The level of *n*-hexanal generation in the L-1, -2 double deficient mutant, which only has L-3, was very low (18% of that in the wild type) and much lower than that in the L-1, -2, -3 triple deficient mutant. Therefore, it is likely that L-3 itself has no ability to generate the *n*-hexanal precursor and inhibits the *n*-hexanal generation through other pathways.

The *n*-hexanal generation was higher in the L-1, -3 double deficient (only L-2 present) and the L-2, -3 double deficient (only L-1 present) mutants than in the wild type (3.1 and 1.8 times, respectively). This suggests that the L-2 isozyme predominantly takes part in the *n*-hexanal generation [4], however, the contribution of the L-1 isozyme is also significant. In addition, the enhanced

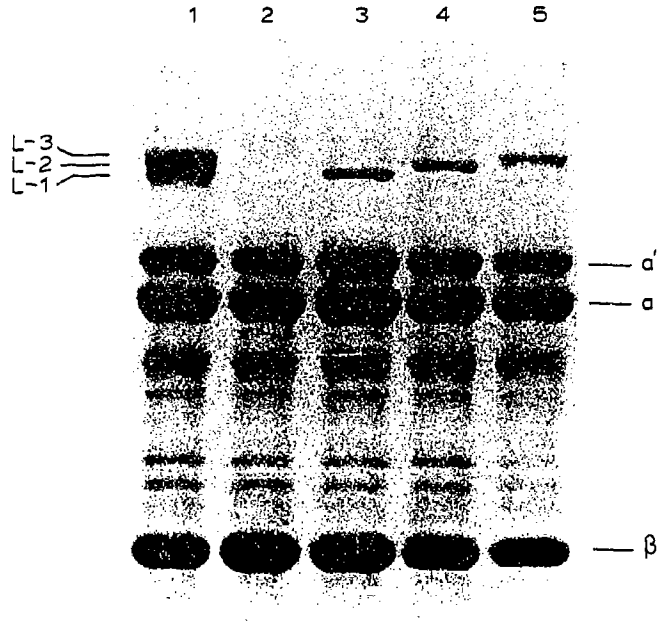

Fig. 1. Resolution of the lipoxygenase isozymes in soybean seeds by SDS-polyacrylamide gel electrophoresis. Lane 1, cv. Suzuyutaka (wild type); lane 2, a line (M_5) lacking all three lipoxygenase isozymes; lane 3, a line (Kanto No. 101) lacking L-2 and L-3; lane 4, a line (Kanto No. 102) lacking L-1 and L-3; lane 5, a line (F_2) lacking L-1 and L-2. α, α' and β, subunits of β-conglycinin.

generation of *n*-hexanal in these mutant lines which lack the L-3 isozyme may be due to the removal of an inhibitory effect of L-3 [7].

As mentioned above, the *n*-hexanal generation was lower in the L-1, -2, -3 triple deficient and L-1, -2 double deficient mutant lines, only a very small amount of *n*-hexanal being generated in the latter. Hence, it is feasible that the use of these mutant lines will lead to the production of soybean food material with a less grassy beany flavor and thus allow a wider utilization of soy protein as a food ingredient. However, the reason for the *n*-hexanal generation in the L-1, -2, -3 triple deficient mutant and the mechanism underlying the repression of *n*-hexanal generation by the L-3 isozyme remain unknown. A study aimed at resolving these problems is in progress.

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