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Purification and Thermal Inactivation of Two Lipoxygenase Isoenzymes from Potato Tubers

Young-Myeong Kim, Chang-Won Lee* and Kwan-Hwa Park

Department of Food Science and Technology, Seoul National University, Suwon.
** Department of Microbiology, Gyeongsang National University, Jinju.*

Abstract

Two lipoxygenases (F-I and F-II) were purified from potato tubers by ammonium sulfate fractionation and ion-exchange column chromatographies. The purified isoenzymes were apparently homogeneous on polyacrylamide gel electrophoresis. Both enzymes showed a similar optimum pH of 5.5-6.0. From thermal inactivation experiments with the purified enzymes in the range of 50 to 65°C, D- values of 13.3 min and 4.3 min at 65°C, and z-values of 11.8°C and 10.3°C were obtained respectively for F-I and F-II. By applying absolute reaction rate equation, thermodynamic parameters were also determined for the activation part of the inactivation process.

Introduction

Lipoxygenase (linoleate: oxygen oxidoreductase EC 1. 13. 11. 12) is a non-heme iron dioxygenase catalyzing the hydroperoxidation of polyunsaturated fatty acids with a methylene-interrupted *cis, cis*-diene system, like linoleic acid, to produce the conjugated *cis, trans*-diene hydroperoxide. This enzyme is known to occur in various plants such as soybean[1-4], pea[5-7], potato tuber[8-10], wheat[11-13], rice bran[14,15], bean[16], flax seed[17], and other vegetables[18-21]. Also, the enzymes having similar activity were reported for microorganism[22] and animal[23].

In foodstuffs, lipoxygenase acts upon lipids and causes destruction of essential fatty acids such as linoleic linolenic, and arachidonic acid. As consequence, volatile compounds such as hexanal and pentanal are produced[17,24]. These compounds are known to be a major cause of rancidity and off-flavor occurring in the foodstuffs, when processed and stored for a long period. The secondary products arising from hydroperoxides decomposition also damage proteins and amino acids through formation of covalent bonds. The enzyme has also carotene-bleaching activity[17,25]. In the light of these potential damage on food qualities that can be caused by the enzyme, it is of utmost importance to know its inactivation characteristics by heat, the form of energy most routinely employed in food processing.

In this study, we report the purification of two lipoxygenases from potato tubers and their thermal inactivation characteristics.

Materials and Methods

Linoleic acid and Tween 20 were purchased from Kanto, Japan; Sephadex G-25 from Sigma, USA; CM- and DEAE-cellulose from Serva, FRG. All other chemicals were reagent grade and were used without further purification.

Lipoxygenase activity assay

The reaction mixture contained 2.4*ml* of 0.1*M* phosphate buffer, pH 5.7, 0.1*ml* of enzyme solution. The reaction was started by adding 25 μl of substrate stock solution (see below), and the absorbance increase at 234 nm was recorded as a function of reaction time with a double-beam UV-visible spectrophotometer (Shimadzu UV-200). Enzyme activity was calculated from the linear portions of recorded kinetic curves. One unit of lipoxygenase activity was defined as the amount of enzyme causing an increase of 0.1 absorbance unit per minute at 25°C under the specified conditions. The substrate stock solution was prepared by adding 2.0 *ml* of 1 *N* NaOH to a mixture containing 0.50 g each of linoleic acid and Tween 20. Then, the volume was brought to 50 *ml* with distilled water. This stock solution was stored at 4°C up to 7 days.

Protein determination

Protein content was usually determined by the method

of Lowry *et al.* [26] using crystalline bovine serum albumin as the standard. Protein concentrations of column eluates, however, were routinely monitored by the absorbance at 280 *nm*.

Preparation of potato tuber homogenate

Potato tubers (*Solanum tuberosum* variety Dejima) were obtained from the Horticulture Experimental Station at Suwon. They were stored frozen at -18°C until use. Usually, five hundred grams of peeled potato tubers cut into small pieces were homogenized in a Waring blender, in 0.5 liter of cold 0.1 *M* phosphate buffer, pH 6.8 containing 2 *mM* each of ascorbic acid and sodium metabisulphite. The resulting homogenate was filtered through nylon cloth and then centrifuged for 15 min at 13,000 × *g*. The supernatant is the crude potato tuber extract used in purification experiments.

Purification procedure

The following steps were carried out at 2 to 4°C and the buffer used throughout was 50 *mM* potassium phosphate buffer, pH 6.8 and will be referred to simply as the 'buffer' unless specified otherwise.

The crude extract was fractionated with ammonium sulfate and the proteins precipitated between 30 to 60% saturation were dissolved in a small volume of the buffer. After desalting by passing through a Sephadex G-25 column (4 × 50 *cm*) which had been equilibrated with the buffer, the enzyme solution was applied to a CM-cellulose column (3.5 × 30 *cm*). Unadsorbed proteins containing lipoxygenases were washed down with the buffer at a flow rate of 35 *ml* h^{-1} and fractions of 10 *ml* were collected. Active fractions were pooled and concentrated with gum arabic. The concentrated enzyme solution was applied to a DEAE-cellulose column (3.5 × 30 *cm*). After washing with 200 *ml* buffer, the column was eluted (35 *ml* h^{-1}) corresponding to the two activity peaks of Fig 2 were pooled concentrated and dialyzed against the buffer. A second DEAE-cellulose column chromatography was carried out to further purify each of the two active peaks from the first DEAE chromatography. A column of 3.0 × 18 *cm* was used, and linear NaCl gradients of 0.0 *M*-0.2 *M* and 0.0 *M*-0.3 *M* were used respectively to purify F-I and F-II. After dialysis and concentration, the resulting enzyme preparations were used for further experiment.

Polyacrylamide gel electrophoresis

Disc gel electrophoresis was carried out on 7% and 15% gels at pH 8.3 by the method of Davis[27] or at pH 4.3 according to Reisfeld *et al.*[28]. Samples were applied routinely at 50 to 200 *mg* proteins per tube and bromophenol blue was used as the tracking dye. Protein staining was done with Amidoschwarz 10B at 1%.

Thermal inactivation of enzymes

Experiments of thermal inactivation of lipoxygenase were carried out essentially as described by Park[29], in 0.1 *M* phosphate buffer, pH 6.8. From the data of residual enzyme activities after various time intervals of heat treatment in the temperature range of 50 to 65°C, kinetic and thermodynamic constants were estimated according to Aylward & Haisman[30] and Labuza[31].

Results and Discussion

Two lipoxygenase activities (F-I and F-II) were purified from potato tubers by ammonium sulfate fractionation, CM-cellulose and DEAE-cellulose ion exchange column chromatography. The single activity peak appeared on the CM-cellulose chromatography (Fig. 1) was resolved into two lipoxygenase isoenzymes by the first DEAE-cellulose chromatography (Fig. 2). These isoenzymes will be referred to as F-I and F-II, in the eluting order from DEAE-cellulose column chromatography at pH 6.8. Each activity peak was further purified by the second DEAE chromatography (Figs 3 and 4). F-I was eluted at NaCl concentration of 0.11 *M* and F-II at 0.17M, and the final purification fold was 26 and 13, respec-

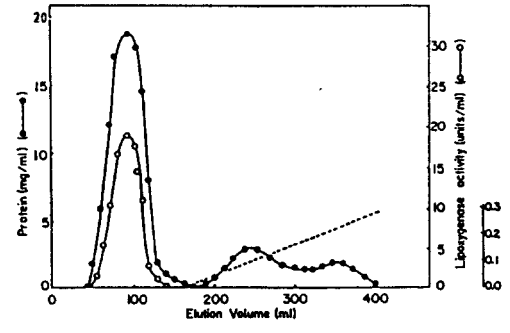

Fig. 1. CM-cellulose column chromatography of potato tuber lipoxygenase. 630 *mg* of protein precipitated by ammonium sulfate between 30% and 60% was applied to a CM-cellulose column (3.5 × 30 *cm*) preequilibrated with 50 *mM* phosphate buffer, pH 6.8, and was eluted with the same buffer

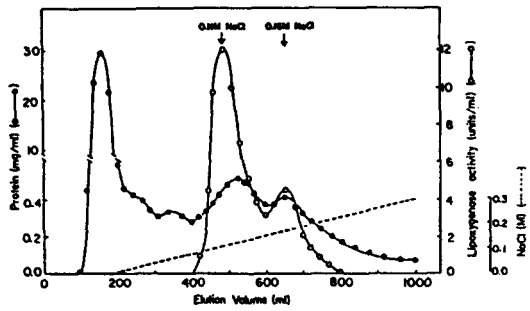

Fig. 2. First DEAE-cellulose column chromatography of lipoxygenase. The sample was 350 *mg* protein obtained from CM-cellulose chromatography. Elution was performed by NaCl gradient in 50 *mM* phosphate buffer, pH 6.8. See text for experimental details

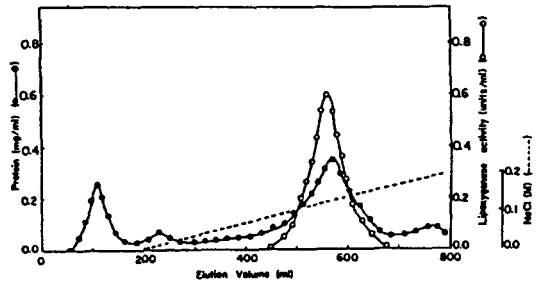

Fig. 3. Second DEAE-cellulose column chromatography of lipoxygenase F-I. 160 *mg* protein of F-I obtained from the first DEAE-cellulose chromatography was applied to a DEAE column (3 × 18 *cm*) and was eluted with a linear NaCl gradient

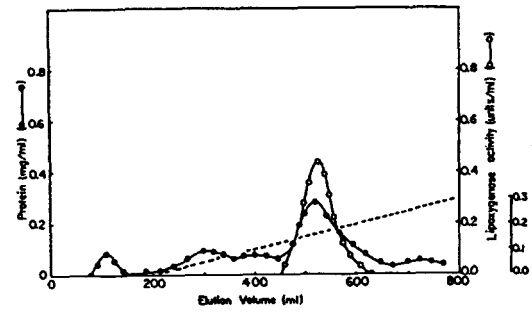

Fig. 4. Second DEAE-cellulose column chromatography of lipoxygenase F-II. 100 *mg* protein of F-II obtained from the first DEAE-cellulose chromatography was applied to the same column as in Fig. 3 Elution was performed with a linear NaCl gradient

tively. The quantitative results of the above purification procedures are summarized in Table 1. The final preparations of the two isoenzymes consisited of apparently single proteins as revealed by the polyacrylamide gel electrophoresis at pH 8.3 under non-denaturing conditions (Fig. 5). the relative mobilities on electrophoresis were 0.72 and 0.76 respectively for F-I and F-II. Also, two lipoxygenases were partially purified from the same plant tissue by Pinsky *et al.*[32] through ecteolla cellulose chromatography and gel filtration. In contrast, Sekiya *et al.*[9] identified a single lipoxygenase in potato tubers after DEAE-

Table 1. Summary of purification of lipoxygenases from potato tuber

Procedure	Protein (*mg*)	Total activity (units)	Yield (%)	Specific Activity (*units/mg*)	Purification fold
Crude extract	17482	29747	100	1.7	1.0
Ammonium sulfate fractionation, 30-60% saturation	5371	20823	70	3.9	2.3
CM-cellulose chromatography	2174	10624	36	4.9	2.9
1st DEAE-cellulose chromatography					
F-I	305	4767	16.0	15.6	9.2
F-II	162	1549	5.2	9.6	5.6
2nd DEAE-cellulose chromatography					
F-I	41.7	1821	6.1	43.7	25.7
F-II	29.6	648	2.2	22.7	13.4

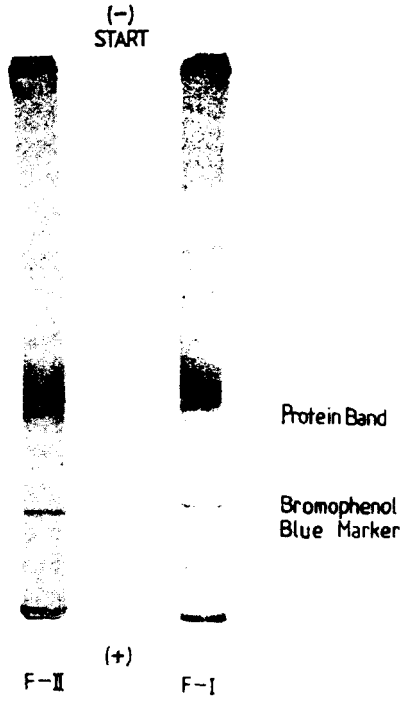

Fig. 5. Polyacrylamide gel electrophoresis of potato tuber lipoxygenases, (A) F-I and (B) F-II. The samples used were enzyme preparations obtained after the second DEAE-cellulose chromatographies. Electrophoresis was carried out on gels containing 7% acrylamide, and at pH 8.3

cellulose and DEAE-Sephadex ion-exchange chromatography. This discrepancy among investigators in the number of lipoxygenase isoenzymes of potato tubers may reflect the varietal differences in the isozymic pattern of the enzyme. Lipoxygenase in general has been reported to exist in multiple isozymes[33-36].

The low yields (Table 1) observed in this study appear to be related to the low purification fold. Because we obtained apparently homogeneous enzymes after the second DEAE-cellulose chromatography, the two isoenzymes should represent about 4% and 8% of the total proteins of potato tubers, respectively for F-I and F-II, as calculated from the values of our purification fold. Though we have no idea of the precise role of plant lipoxygenases in vivo, it seems improbable that as much as 12% of total proteins is necessary for a single, lipid-oxidizing activity for any plant. Thus, the low purification fold of our study seems to be due to the inactivation of the enzymes during purification. In fact, most reported results of lipoxygenase purification show low purification folds and low yields[35,37,38]. In this regard, Smith & Lands[39] noted a self-catalyzed destructionof soybean lipoxygenase and suggested that such enzyme inactivation might occur during isolation and handling. According to these lines of reasoning, our final enzyme preparations may contain certain portions of inactivated enzymic species and this may explain the rather diffuse bands on electrophoresis of F-I and F-II (Fig. 5).

Our both isoenzymes had nearly the same pH-activity profile with the optimum pH of 5.5 to 6.0 (Fig. 6). Similar results had been obtained for lipoxygenase from potato tuber and other sources[8,9,32,40].

The thermal inactivation of F-I and F-II was performed with the enzyme preparations obtained by second DEAE-cellulose chromatography, in the temperature range of 50 to 65°C. The data obtained were fitted to the general kinetic equation involving a single molecular species: $-dE/dt = k'E^n$, where E is the enzyme concentration or activity; k', the specific reaction rate constant; and n is the order of reaction. The order of reaction obtained in this

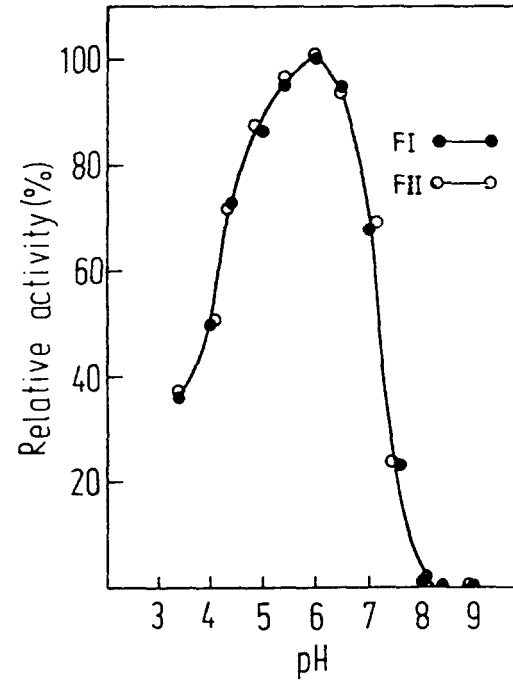

Fig. 6. pH-activity profile of potato tuber lipoxygenases, F-I and F-II. the two isoenzymes showed nearly the same profile, which is therefore represented by a single line. The following buffers were used: Na-acetate (pH 3.5-5.5); potassium phosphate (pH 5.7-8.0); and Tris-HCl (pH 8.5-9.5)

Table 2. Summary of thermal inactivation data for potato tuber lipoxygenases[a]

Heating temperature (°C)	D- value (*min*)		Thermodynamic constant[b]					
			F-I			F-II		
	F-I	F-II	$\Delta H^{\neq}$	$\Delta S^{\neq}$	$\Delta G^{\neq}$	$\Delta H^{\neq}$	$\Delta S^{\neq}$	$\Delta G^{\neq}$
50	267	124	169	202	103	192	281	102
55	98	65		203	102		281	100
60	44.5	24.2		203	101		282	99
65	13.3	4.3		203	100		281	97

a. Mean values from two independent experiments. Experimental errors were not greater than 10% of mean values

b. In kj mol^{-1} for $\Delta H^{\neq}$and $\Delta G^{\neq}$; in J mol^{-1} K^{-1} for $\Delta S^{\neq}$

way for F-I and F-II was respectively 1.07 ± 0.03 and 1.03 ± 0.04 (two independent experiments; confidence interval, 95%). this indicates that the thermal inactivation of potato tuber lipoxygenase follows a first-order reaction kinetics as for most other enzymes[30]. Other relevant, kinetic and thermodynamic data of thermal inactivation are summarized in Table 2. From the decimal reduction time (D-values) defined as the time taken for a 90% decrease in the activity of the enzyme, it is readily seen that F-I is more thermostable than F-II. However, z-values, the temperature change in °C necessary to produce a tenfold change in D-value, were similar for both isoenzymes. There are no data available for comparison on the thermostability of potato tuber lipoxygenases. However, the inactivation of pea lipoxygenase has been extensively studied[37,41-44] and to a less extent also for soybean enzyme[34,45,46]. Under acceptably comparable conditions, D-value for the pea enzyme was calculated to be 21 min at 65°C and pH 7.0 with a z-value of 3.7°C. For soybean enzyme, D-value at 65°C was found to be 44 min[45] in an early experiment, while Park[29] found a D-value of 10 min at 66°C and a z-value of 9.8°C. In a recent experiment from this laboratory, a z-value of 9.8°C. In a recent experiment from this laboratory, a z-value of 7.1°C and a D-value of 22.0 min at 66°C were estimated by employing differential scanning calorimetry (our unpublished results). From comparison of these data with ours, lipoxygenases from legumes appear to be more thermostable than potato tuber enzymes at around 65°C. As for the thermodynamic constants, the free energy of activation of the thermal inactivation process ($\Delta G^{\neq}$) was well in the range of those for other proteins and enzymes, i.e. 92-113 *kj* mol^{-1}[31,32]. The values of $\Delta S^{\neq}$(the entropy change of activation) (table 2) of F-I and F-II fitted well to the compensation law applied to protein/enzyme/water reactions with an isokinetic temperature of 329K[31].

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감자 Lipoxygenase isozyme의 분리와 열불활성화

김영명 · 이창원* · 박관화

서울대학교 식품공학과 · 경상대학교 미생물학과

황산암모늄분획 침전, 이온교환컬럼 크로마토그래프를 이용하여 감자로부터 2개의 Lipoxygenase isozyme (F-I 및 F-II)을 분리정제하고 각각의 isozyme에 대하여 열불활성화 실험을 행하였다.

분리된 isozyme은 polyacrylamide gel 전기 영동상에서 단일밴드를 보였으며 두 isozyme의 최적 pH는 5.5~6.0으로 비슷하였다.

열불활성화 온도 범위인 50~60℃에서 F-I 및 F-II의 D_{65} 값은 각각 13.3min 및 4.3min이었으며 Z 값은 11.8℃ 및 10.3℃ 이었다. 또한 각 isozyme의 열역학적인 상수는 절대반응속도식에 따라 구하였다.