Purification and Characterization of Broad Bean Lipoxygenase Isoenzymes

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Two lipoxygenase isoenzymes, BBL-1 and BBL-2, were purified from broad beans. Fractionation of globulins and albumins by ionic strength was preferred to the classical water extraction system and the ammonium sulfate fractionation as initial purification steps. From the albumin fraction, BBL-1 and BBL-2 were purified 17.6 and 35.7-fold, respectively, by conventional gel filtration and ion-exchange chromatography. The molecular weight of both BBL-1 and BBL-2 was 97 kDa with a maximal activity around pH 5.8; however, they showed a significant difference in their K_m values for linoleic acid: 2.3 and 0.25 mM for BBL-1 and BBL-2, respectively. BBL-1 produced hydroperoxides and ketodienes while BBL-2 produced exclusively hydroperoxides.

Keywords: *Broad bean; lipoxygenase; purification; isoenzymes; hydroperoxides; ketodienes*

INTRODUCTION

Legumes are an important and inexpensive source of vegetable protein that might be used as an alternative of dietary protein in human nutrition. Recently, legume protein products such as concentrates and isolates have been considered as a potential ingredient in the food industry (Endres, 1989; Sánchez-Vioque et al., 1999a). Although soybean is the vegetable source most widely used, other legumes such as peas and chickpeas have been successfully used (Clemente et al., 1999). In this sense, broad beans have also been reported as a good source of protein with adequate nutritional quality that has been the object of increasing interest over recent years (Pastuszewska et al. 1993; Rubio et al., 1991). Therefore, broad bean flour might be used to produce protein products and incorporate them into human foods for nutritional enrichment.

Because of the role that fats play in the physicochemical properties, nutritional quality, and acceptability of the protein products, the presence of lipids, even in small amounts, needs to be considered. Sanchez-Vioque et al. (1999b) reported losses of essential amino acids and the decrease of the protein digestibility due to the interaction of legumin, the major protein component of chickpea protein isolates, with oxidized linoleic acid. Matheis and Whitaker (1987) demonstrated that products formed by lipoxygenase (LOX) activity cause cross-linking of proteins. Besides, undesirable flavors produced by lipid oxidation have been identified as one of the major limiting factors as regards increasing the use of soybean flours, concentrates, and protein isolates as ingredients and extenders or in fabricated foods (Rackis et al., 1979). Many of them can result from the presence of residual LOX activity in such products.

It is well-known that lipoxygenase (linoleate: oxigen 13-oxidoreductase, EC 1.13.11.12), also known as lipoxidase and carotene oxidase, catalyzes the oxidation of polyunsaturated fatty acids containing *cis*,*cis*-1,4-pentadiene moieties and some related molecules to produce hydroperoxides (Axelrod et al., 1981). They constitute the starting point for a series of reactions forming a cascade of products, including short-chain aldehydes, ketone alcohols, acids, ciclopentanoids, epoxides, and other products, many of which are responsible for the development of off-flavors (Gardner, 1991; Hamilton et al., 1997).

Interest in LOX from legumes is arising due to their implication in the undesirable “beany”, “green”, and “grassy” flavors during processing and storage of protein products. Four soybean lipoxygenase isoenzymes (SBL) have been purified and characterized, showing differences in pH optimum, substrate, and product specificity. SBL-1 exhibits maximal activity at pH 9−10 and converts linoleic acid preferentially into the 13-hydroperoxide derivative (Shibata et al., 1987). SBL-2, characterized by a peak of activity at pH 6−7, forms the 9-hydroperoxide compound (Gardner, 1991). Recently, the presence of SBL-3a and SBL-3b with a maximal activity at pH 6 and production of a mixture of hydroperoxides as products has been reported (Kato et al., 1992). Yoon and Klein (1979) isolated four pea LOX isoenzymes (PL), showing PL I and PL II isoforms with differences in pH profiles, substrate specificity, carotene, and chlorophyl-bleaching activity and in their ability to produce carbonyl compounds during the linoleate oxidation reaction. Sanz et al. (1992) reported the presence of two isoenzymes in chickpeas, one of which formed mainly 13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid from linoleic acid, while the other yielded approximately equal proportions of the 9- and the 13-hydroperoxides and the 9- and 13-ketodienes. Beaux and Drapon (1974) isolated the LOX from horse beans and studied some of its physicochemical properties. Besides, Al-Obaidy and Siddiqui (1981) reported a partial purification of broad bean lipoxygenase (BBL) with maximal activity around pH 6.0.

Although the presence of two BBL isoenzymes has been suggested (Eskin and Henderson, 1974), isoenzyme purification has not been carried out. The object of this

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paper was the characterization of BBL isoenzymes in order to prevent, remove, or mask off-flavors in protein products that could affect their acceptability and nutritional quality. A deeper knowledge of BBL isoenzymes could greatly enhance their potential utilization as ingredients without producing adverse flavor effects, maintaining nutritional quality.

MATERIALS AND METHODS

Material and Reagents. Certified broad beans (*Vicia faba* cv. Flor de otoño) were obtained. Linoleic acid and poly(ethylene glycol) 20000 were purchased from Sigma Chemical Co. (St.Louis, MO) and Merck (Darmstadt, Germany), respectively. Acrylamide, *N*,*N*-methylenebisacrylamide and Coomassie Brilliant Blue G-250 were from Serva (Heidelberg, Germany). Sephadex G-100 and standards proteins were from Pharmacia (Pharmacia/ LKB, Uppsala, Sweden). DEAE-cellulose DE-52 was from Whatman Biosystems Ltd. Octadecyl (C_{18}) disposable extraction column (3 mL) and *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were obtained from J. T. Baker Chemical Co. and Supelco (Bellefonte, PA), respectively. All other reagents were of analytical grade.

LOX Isoenzyme Purification. Broad beans were ground to a fine flour (0.4 mm mesh), and this was defatted with hexane at room temperature. Extraction and fractionation of broad bean proteins were performed as described by Singh et al. (1988). Defatted broad bean flour (15 g) was extracted with 150 mL of 50 mM sodium phosphate buffer, pH 6.8, for 2 h and centrifuged at 10000*g* for 30 min. The supernatant was dialyzed against 25 mM sodium citrate buffer (pH 4.6) for 36 h and centrifuged at 25000*g* for 20 min. The supernatant and pellet obtained were the albumin and globulin fractions, respectively. To concentrate the LOX activity, albumin fraction was dialyzed against 40% polyethylene glycol 20000 for 16 h and centrifuged at 25000*g* for 20 min. To purify BBL, dialyzed albumins were loaded onto a Sephadex G-100 gel filtration column (100 × 2 cm) at a flow rate of 20 mL/h using the extraction buffer. The fractions containing LOX activity were pooled and applied to a DEAE-cellulose DE-52 column (3.2 × 40 cm). BBL isoenzymes were eluted with a gradient linear of 50 mM sodium phosphate (100 mL) to 200 mM (100 mL), pH 6.8, at a flow rate of 30 mL/h. Protein elution was detected at 280 nm, and fractions were assayed for LOX activity. Two separated peaks with LOX activity were found and designated BBL-1 and BBL-2. All purification steps were carried out at 4 °C.

Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was performed according to the method of Laemmli (1970). The gel system, containing 0.2% (w/v) SDS, consisted of a 12% polyacrylamide resolving gel (pH 8.8) and a 3% stacking gel (pH 6.8). The lengths of the resolving and stacking gels were 10 and 2 cm, respectively, with a gel thickness of 0.75 mm. Electrophoresis was performed at a constant current of 25 mA. Protein bands were stained by immersion of the gels in a 0.05% (w/v) Coomassie Brilliant Blue G-250 solution, in 45% methanol, and in a 9% acetic acid solution. Molecular masses of both isoenzymes were determined using molecular weight standards from Sigma.

LOX Activity Assay. A stock solution of 10 mM sodium linoleate in Tween 20 was prepared as described by Axelrod et al. (1981). LOX activities were determined spectrophotometrically. The standard assay mixture consisted of 3 mL of sodium phosphate buffer 50 mM at appropriate pH, 25 μL of substrate solution (10 mM linoleic acid), and the appropriate volume of the enzyme solution. The reaction was initiated by addition of the enzyme solution, and formation of both hydroperoxides and ketodienes was followed by change in absorbance at 234 ($\epsilon = 2.5 \times 10^4$ M^{-1} cm^{-1}) and 274 nm ($\epsilon = 2.2 \times 10^4$ M^{-1} cm^{-1}), respectively, at 25 °C (Axelrod et al., 1981). One unit of LOX activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of the hydroperoxide/min.

Analysis of Reaction Products. Linoleic acid (20 μmol) was incubated with 2 units of BBL activity in 20 mL of oxygen-saturated 50 mM sodium phosphate buffer at appropriate pH (5.8 for BBL-1 and 5.6 for BBL-2). Reactions were carried out at 20 °C with a constant flow of oxygen for 10 min and were stopped by adjusting to pH 1.5 with 2 N HCl. The products were extracted from the incubation mixture on a reversed-phase C_{18} microcolumn and eluted with methanol. The concentrated products were esterified with diazomethane (Cohen, 1984), and isomers were analyzed by HPLC with a LiChrosorb Si-60 5 μm (4 mm × 250 mm) column (Merck), eluted with *n*-hexane/diethyl ether (92:8 v/v) with a flow rate 1.25 mL/min and detected at 234 and 274 nm (Sanz et al., 1993). To identify the reaction products, a large-scale isolation was carried out using a LiChrosorb Si-60 7 μm (10 mm × 250 mm) column (Merck) with a flow rate 2 mL/min. Each separated product was identified by IR and GC/MS. GC/MS analysis (MS-30/70, VG Analytical, Manchester, England) was performed on a 30-m SP-2380 BP capillary column (Supelco), ionization potential 70 eV. Hydroperoxides were reduced with $NaBH_4$ and analyzed after hydrogenation with H_2/PtO_2 as trimethylsilyl derivatives (BSTFA was used as reagent). The capillary column was operated isothermally at 190 °C. Ketodienes were analyzed, without derivatization, isothermally at 200 °C during 5 min and then programmed at 2 °C/min to 250 °C.

Protein Measurements. Protein concentrations were determined by the method of Bradford (1976). Bovine serum albumin (BSA) was used as standard.

Table 1. Comparison of Efectiveness of Different Initial Steps in the Purification[a] of Lipoxygenase Isoenzymes of Broad Bean

step	protein (mg)	total activity (units[b])	specific activity (units mg^{-1})	yield (%)	purification
crude extract	1856	570	0.3	100	1
$(NH_4)_2SO_4$	524	330	0.6	57.8	2.0
Osborne's method[c]	715	450	0.6	78.9	2.0
Singh's method[d]	409	465	1.1	81.5	3.8

[a] Purification was from 15 g of defatted flour. [b] One enzyme unit corresponds to the formation of 1 mol of hydroperoxide per minute. [c] Osborne and Campbell (1897). [d] Singh et al. (1988).

Table 2. Purification[a] of Lipoxygenase Isoenzymes of Broad Bean

step	protein (mg)	total activity (units[b])	specific activity (units mg^{-1})	yield (%)	purifiacation
crude extract	1856	570	0.3	100	1
albumin fraction	409	465	1.1	81.5	3.8
Sephadex G-100	50	160	3.2	28.1	10.7
DEAE DE-52					
BBL-1	1.8	9.6	5.3	1.6	17.6
BBL-2	1.1	11.8	10.7	2.1	35.7

[a] Purification was from 15 g of defatted flour. [b] One enzyme unit corresponds to the formation of 1 mol of hydroperoxide per minute.

RESULTS AND DISCUSSION

LOX Isoenzyme Purification. Preliminary studies were carried out to optimize procedures and conditions for extraction and isolation of BBL isoenzymes. There was a great reduction in BBL activity (42%) of broad beans when the ammonium sulfate fractionation was used as initial purification step (Table 1). In other legumes, such as french beans (Abbas et al., 1989) and horse beans (Eskin and Herderson, 1974), a 50% loss of LOX activity after ammonium sulfate fractionated has been reported. To reduce these important losses in BBL activity, the search for alternative methods was investigated. Obtaining an albumin-enriched fraction according to Singh's method (1988) constituted an important initial step in continuing the purification

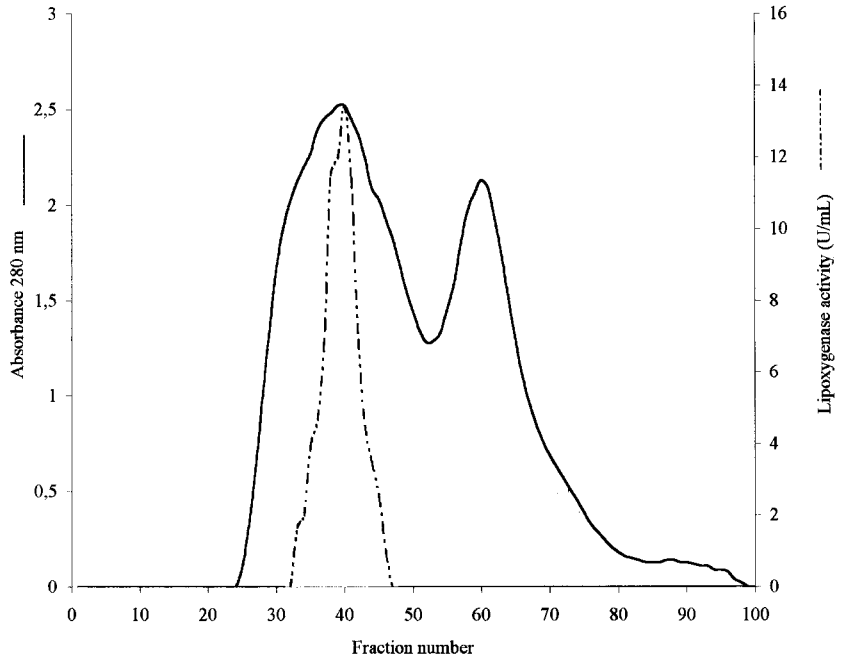

Figure 1. Gel filtration of broad bean albumins on a Sephadex G-100 column. Fractions of 2.5 mL were collected and assayed for proteins and lipoxygenase activity as described under Materials and Methods.

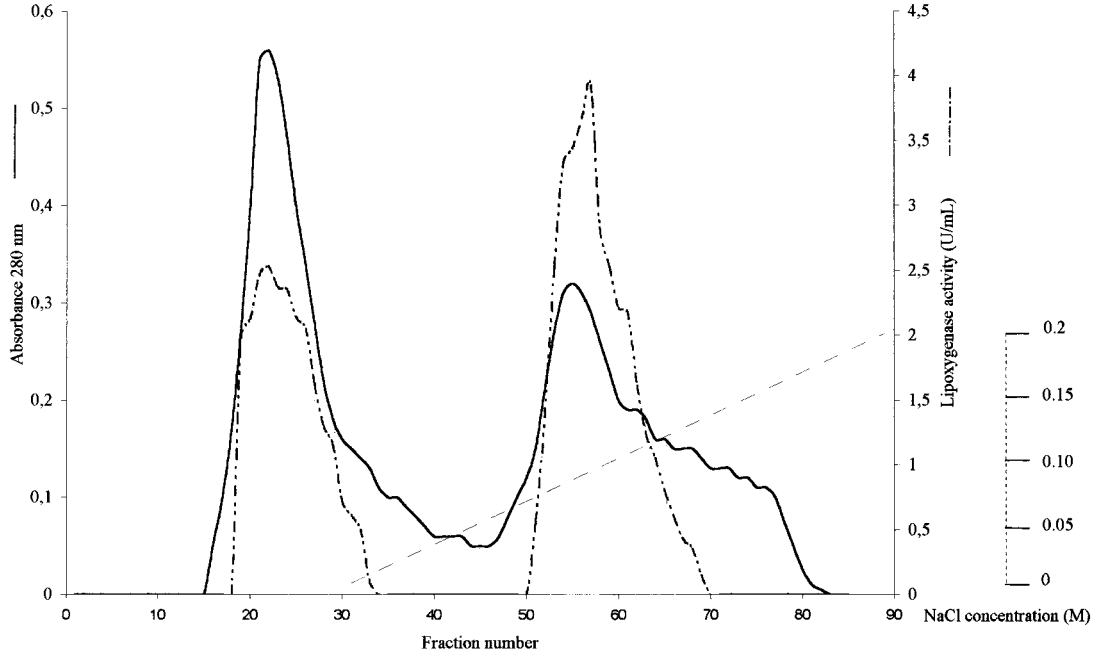

Figure 2. Separation of broad bean lipoxygenase isoenzymes on DEAE ion-exchange column. Fractions of 3 mL were collected and assayed for proteins and lipoxygenase activity as described under Materials and Methods.

process of BBL. This procedure was carried out under milder conditions and was strongly preferred to the ammonium sulfate fractionation due to the minimization of losses in total BBL activity (19%) and an improvement in the purification degree. The classical water extraction system used by Osborne and Campbell (1897) to obtain different protein fractions was also considered. However, due to the presence of cross-contamination between globulin and albumin fractions a lesser effectiveness with respect to Singh's method was found. The obtained albumin fraction by Singh's method had a 3.8-fold-purification and a 81.5% in yield with respect to the initial sample. Different chromatography systems were considered in preliminary attempts to further purify the BBL isoenzymes. Although fast protein liquid chomatography (FPLC) has been used to purify LOX in soybeans (Kato et al., 1992; Sanz et al., 1993), significant losses in BBL activity (>90%) were found (data not shown). Thus, a conventional size-exclusion chromatography using Sephadex G-100 was employed in this study, obtaining a purification degree of 10.7 and a yield higher than 28% (Table 2). One single LOX active peak with an apparent molecular weight of 100 ± 5 kDa (Figure 1) was observed, assuming a uniform spherical shape for the enzyme molecule. The collected fractions were applied to a DEAE-cellulose DE-52 column, and two LOX active peaks were resolved and designated as BBL-1 and BBL-2 (Figure 2). BBL-1 was eluted with 50 mM sodium phosphate buffer pH 6.8 and BBL-2 using a gradient linear of 50 to 200 mM sodium

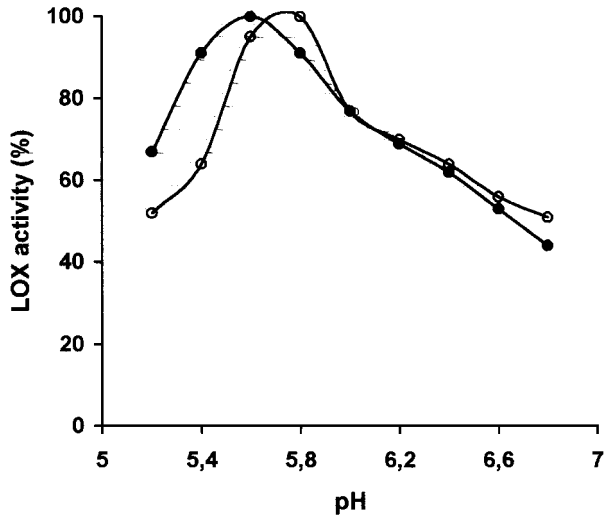

Figure 3. Effect of pH on the activity of BBL-1 (●) and BBL-2 (○). Assay conditions are described under Materials and Methods.

phosphate buffer pH 6.8. In such conditions, a better purification factor was obtained for BBL-2 (35.7) in comparison to BBL-1 (17.6).

The average values for the molecular weights of both BBL-1 and BBl-2 as determined by SDS−PAGE was 97 kDa. This weight appeared to be similar to that reported in small faba beans (Eskin and Henderson, 1974) and higher than those reported for pea (Reynolds and Klein, 1984) and lupin (Olias and Valle, 1988) with 64 and 92 kDa, respectively.

pH Profile and K_m Determination. BBL showed a narrow range of pH activity, the maximal being pH 5.6 and 5.8 for BBL-1 and BBL-2, respectively (Figure 3). The BBL isoenzymes significantly reduced their activities below pH 5.0 or above pH 7.0 with reduced values higher than 60%. For the determination of apparent K_m and V_{max} of highly purified BBL isoenzymes at optimun pH, linoleic acid concentrations were varied from 0.05 to 0.50 mM and the data plotted as a Lineweaver−Burk graph (Figure 4). Both isoenzymes showed a significant difference in their K_m values as determined by a Lineweaver−Burk plot. Apparent K_m values for BBL-1 and BBL-2 obtained at optimum pH were 2.3 and 0.25 mM, respectively, when activities were measured in air-saturated solutions using linoleic acid as substrate. The K_m values were lower than the reported data for horse bean (0.57 mM) (Eskin and Henderson, 1974) and type-2 pea lipoxygenases, which ranged from 2.2 to 3.6 mM (Reynolds and Klein, 1982).

Product Specificity. To characterize the product specificity of purified BBL isoenzymes, linoleic acid was used as substrate. The kinetics of formation of hydroperoxides at 234 nm absorbance (Axelrod et al., 1981) and ketodienes at 274 nm absorbance (Vioque and Holman, 1962) were monitorized. To establish that the 274 nm absorption in our study was due to ketodiene

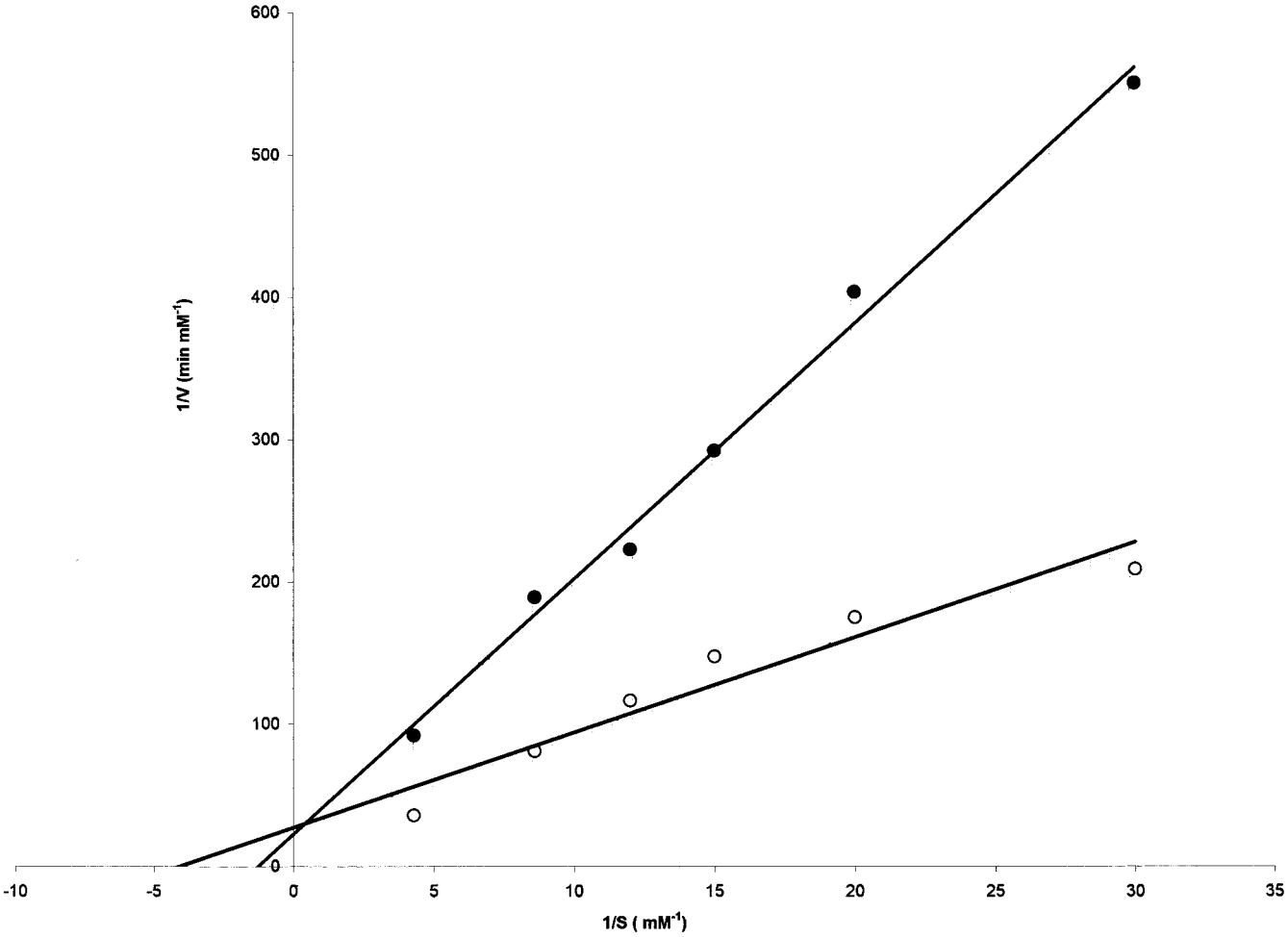

Figure 4. Double-reciprocal plot of the linoleic acid concentrations vs the initial rate of conjugated diene formation catalyzed by BBL-1 (●) and BBL-2 (○) at optimun pH. Assay conditions are described under Materials and Methods.

Table 3. Product Specificity of Broad Bean Lipoxygenases Using Limoleic Acid as Substrate (Percentage within Each Class of Product)

product	BBL-1	BBL-2
13-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid	29.3	83.1
13-hydroperoxy-(9*E*,11*E*)-octadecadienoic acid	8.6	7.2
9-hydroperoxy-(10*E*,12*Z*)-octadecadienoic acid	43.7	6.2
9-hydroperoxy-(10E,12*E*)-octadecadienoic acid	18.4	3.5
13-keto-(9*Z*,11*E*)-octadecadienoic acid	36.7	–
13-keto-(9*E*,11*E*)-octadecadienoic acid	19.8	–
9-keto-(10*E*,12*Z*)-octadecadienoic acid	31.4	–
9-keto-(10*E*,12*E*)-octadecadienoic acid	12.1	–

formation, reduction with $NaBH_4$ was carried out. Upon reduction, the 274 nm peak disappeared completely and the intensity at 232 nm increased; this is attributed to the formation of the hydroxy conjugated dienes from the ketodienes (Vioque and Holman, 1962).

To eliminate the interference from isomerization, the oxidation, extraction, and methylation were carried out swiftly at low concentration and temperature and the determination of the isomeric ratio was performed inmediately by HPLC. To establish the chemical structure, the products were collected separately from HPLC and analyzed by IR and GC/MS according to the Materials and Methods. The IR spectra of hydroperoxides had absorption bands at 951 and 986 cm^{-1} for the *Z*,*E*-isomers and a single high intensity at 990 cm^{-1} for the *E*,*E*-isomers. The IR spectra of ketodienes showed absorbed slightly at 964 and 997 cm^{-1} for *Z*,*E*-isomer hydrogenation yielded hydroxystearates that were converted into trimethylsilyl ethers (TMSi) and analyzed by GC/MS. The TMSi derivatives from the presumed 13-isomers gave rise to the expected ions due to α,α-fragmentation of the TMSi group (*m*/*z* 173 and 135), as did the derivatives of the 9-isomers (*m*/*z* 229 and 259). In the case of ketodienes from BBL-1, enzymatic oxidations were analyzed without derivatization and rendered characteristic fragments, from McLafferty rearrangement, of *m*/*z* 252 for 13-isomers and *m*/*z* 166 for the 9-isomers.

Table 3 shows the percentage of the oxidation products from linoleic acid catalyzed by BBL-1 and BBL-2. BBL-1 produced hydroperoxides and ketodienes while BBL-2 produced exclusively hydroperoxides and none of the 274 nm absorbing product. In its ability to form solely the hydroperoxyoctadecanoic acid from linoleic acid, BBL-2 appears to be similar to soybean L_2 (Pistorius, 1974). Similar results were obtained in chickpeas by Sanz et al. (1992). They resolved two active forms of LOX, one of them catalyzed the formation of conjugated diene hydroperoxides from linoleic acid while the other formed hydroperoxides and ketodienes as major products. The apparence of ketodienes has been considered as a diagnostic of insufficient oxygenation. However, ketodienes from linoleic acid aerobically formed by LOX isoenzymes from kidney beans, peas, and chickpeas have been reported (Sanz et al., 1992, 1993). BBL-2 showed a high regiospecificity since it formed predominantly 13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (83.1%); however, BBL-1 seems to be a lesser regiospecific and geometric-specific LOX with similar proportions of the 9- and the 13-hydroperoxy isomers with high levels of *E*,*E*-isomers. These differences in the ratio of 13- to 9-hydroperoxy isomers produced by BBL-1 and BBL-2 could result in differences of off-flavors due that hydroperoxides produce different breakdown products (Sessa, 1979). Hexanal, the main contributor for the grassy-beany and green flavor, is formed from 13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid plus subsequent cleavage of the product by hydroperoxide lyase (Hatanaka et al., 1987). Because BBL-2 produces mainly 13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid, we suggest that could be a major source of 3(*Z*)-hexanal, in broad bean protein products. On the other hand, BBL-1 could reduce hexanal formation by converting the lipoxygenase product, 13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid, into forms unavailable for conversion to hexanal (Hildebrand et al., 1990).

Data suggest that deleterious secondary products responsible of undesirable off-flavors and compounds that can attack proteins might be formed by BBL isozymes. As a result of that, a decrease in the functional and nutritional properties as well as in the acceptability of protein products from broad beans may occur. Special attention should be paid to prevent the presence of lipoxygenase activity during the processing and storage of protein products from broad beans and different inactivation methods of BBL (e.g., heat treatment, acid pH, or antioxidants) should be explored in order to avoid the appearance of off-flavors.

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