Purification and Characterization of Two Lipoxygenase Isoenzymes from Cowpea [*Vigna unguiculata* (L.) Walp.]

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Lipoxygenase specific activity in 89 cultivars of cowpea [*Vigna unguiculata* (L.) Walp.] varied from 30 to 397 units/mg of protein. Two lipoxygenase isoenzymes L-1 and L-2 were purified from a variety UPL Cp2 by extraction with water, followed by dialysis against water, 40–60% ammonium sulfate fractionation, and DEAE-Sephadex A-50 ion-exchange and hydroxylapatite chromatography. L-1 and L-2 were both highly specific for linoleic acid and exhibited a narrow optimal activity at pH 6.2. Apparent K_m values of 0.8×10^{-3} M and 0.55×10^{-4} M linoleic acid were obtained for L-1 and L-2, respectively. L-1 and L-2 had molecular weights of 68 000 and 74 000, respectively, by $NaDodSO_4$ gel electrophoresis and had R_f values of 0.25 and 0.11, respectively, by regular gel electrophoresis. L-1 and L-2 isoenzymes were inhibited to varying degrees by different metal ions although, in general, L-2 was more sensitive. L-2 was also more sensitive to heat. On the other hand, L-1 was more strongly inhibited by antioxidants than L-2. The lipoxygenase isoenzymes were stable at pH 4–9. The enzyme in situ was highly stable even in seeds soaked in acidic solution at pH 2 for 10 h. However, blanching of soaked and unsoaked seeds resulted in total loss of activity.

Lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12) catalyzes the oxidation of polyunsaturated fatty acids containing *cis,cis*-1,4-pentadiene systems to form hydroperoxides. The mechanism of hydroperoxide degradation by the lipoxygenase reaction to odorous carbonyl compounds which account for the beany and off-flavor in soybean, peanut, and other legumes is not yet well established. The hydroperoxides formed as well as their secondary products can react with amino acids or proteins to lower the nutritive values of foods.

Lipoxygenase is well distributed in the plant kingdom, especially legumes among which soybean has been reported as the richest source (Scott, 1975) and evidence has been presented for the existence of lipoxygenase isoenzymes (Christopher et al., 1970, 1972; Eskin and Henderson, 1974, 1976; Yoon and Klein, 1979). Recently, Truong et al. (1979) observed four cowpea lipoxygenase isoenzymes by electrophoresis. They found that cowpea seeds [*Vigna unguiculata* (L.) Walp.] possess higher lipoxygenase specific activity than soybean. Some physicochemical properties of the crude enzyme were also reported. In the present investigation, we report the purification and characterization of two cowpea lipoxygenase isoenzymes, the two steps necessary in order to elucidate their roles in the beany and off-flavor formation, in the nutritional quality of cowpea, and in their possible role in the resistance of cowpea to pest and disease. Such information would help the plant breeders and the food scientists in their attempts to improve the quality of cowpea and its products.

MATERIALS AND METHODS

Materials and Reagents. Seeds of cowpea were obtained from the Institute of Plant Breeding, University of the Philippines at Los Banõs. Only one variety, UPL Cp2, was used for lipoxygenase purification.

Oleic acid, linoleic acid, linolenic acid (99% pure), monolinolein, trilinolein, Tween 20, DEAE-Sephadex A-50, and bovine serum albumin were purchased from Sigma Chemical Co. Hydroxylapatite was prepared according to the method of Tiselius as described by Bernardi (1971). Reagents for electrophoresis were obtained from Bio-Rad. Other reagents were analytical grade. Deionized distilled water was used to prepare all solutions.

Purification Procedures. Cowpea seeds were ground in a Wiley mill to pass a 60-mesh screen. The samples were kept at −20 °C until used. All extraction and purification steps were performed at 4 °C.

Enzyme Extraction. Twenty grams of cowpea meal was extracted with 400 mL of distilled water for 30 min. The slurry was passed through four layers of cheesecloth and then centrifuged at 12000*g* for 20 min. The clear supernatant referred to as the water extract was dialyzed against large volumes of distilled water for at least 24 h. The precipitate, globulin, was discarded. The supernatant, termed albumin, was used for lipoxygenase isolation and purification.

Ammonium Sulfate Fractionation. The albumin fraction was treated with ammonium sulfate to make a 40% saturated solution. The precipitate which did not contain any activity was removed by centrifugation and discarded. More ammonium sulfate was added to the supernatant to reach 60% saturation. The 40–60% precipitate was spun down by centrifugation, dissolved with a small amount of 0.05 M Tris-HCl buffer, pH 8.0, containing 0.05 M NaCl, and dialyzed for several hours against the same buffer.

DEAE-Sephadex A-50 Chromatography. The dialysate containing about 326 mg of protein was applied to a DEAE-Sephadex column equilibrated with 0.05 M Tris-HCl buffer, pH 8.0, containing 0.05 M NaCl. The enzyme was then eluted with a linear NaCl gradient, 0.05–0.35 M. Elution was made at 32 mL/h and 5-mL fractions were collected. Fractions corresponding to lipoxygenase isoenzymes 1 and 2 were pooled separately. The two enzyme preparations were concentrated by ultrafiltration, dialyzed against distilled water, and lyophilized.

Hydroxylapatite Chromatography. In an initial experiment, the lipoxygenase 1 and 2 fractions from the DEAE-Sephadex column were pooled together, concentrated by ultrafiltration, and dialyzed against 0.01 M phosphate buffer, pH 6.8. The dialysate was applied to a hydroxylapatite column (2.2 × 18 cm) equilibrated with a linear gradient of 0.01–0.3 M sodium phosphate buffer, pH 6.8. The active fractions were pooled, dialyzed against distilled water, and lyophilized.

Assay Procedures. Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard, $E_{280}^{0.1\%} = 0.632$. The absorbance of protein at 280 nm was used as an index of protein concentration in the column eluants.

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Lipoxygenase activity was assayed spectrophotometrically at 234 nm as described in the previous paper (Truong et al., 1979). The reaction mixture was composed of 1.6×10^{-3} M linoleic acid and 0.05% Tween 20 dispersed in 0.1 M phosphate buffer pH 6.2. With 0.05% Tween 20, the substrate completely dissolved in a wide range of pHs including pH 4 and below. This concentration of Tween 20 has been shown to be optimal for cowpea lipoxygenase, and higher concentrations inhibited the enzyme activity (unpublished data). The enzyme was assayed at 30 °C by using a Beckman recording spectrophotometer, Model 24. One unit of lipoxygenase activity is defined as the amount of enzyme which produces a change of 1 unit of absorbance at 234 nm per min.

Substrate Preparation. Linoleic acid was prepared as described by Rackis et al. (1972). Linoleic acid and oleic acid were prepared in the same manner and had final concentrations of 1.64×10^{-3} and 1.58×10^{-3} M in the assay mixture, respectively.

Monolinolein (0.1 mL) was mixed with 2 mL of ethanol, 0.1 mL of Tween 20, and 6.5 mL of 0.1 N NaOH until a clear solution was obtained. The solution was diluted to 20 mL with water.

Trilinolein (0.1 mL) was dispersed in 3.3 mL of 0.1 N NaOH and 0.125 mL of Tween 20 and diluted to 20 mL with ethanol.

Monolinolein and trilinolein had a final concentration in the assay mixture of 1.28×10^{-3} and 0.55×10^{-3} M, respectively. For assays with trilinolein, the reaction mixture included 8.4% ethanol.

Effects of Metals, Antioxidants, and Other Substances. Lipoxygenase solution was incubated with 1 mM of different metals and various concentrations of cyanide for 30 and 5 min, respectively, at 25 °C. The treated enzyme was assayed as described above.

Antioxidants and ethanol were added to the assay mixture before addition of the enzyme to start the reaction.

Polyacrylamide Gel Electrophoresis. Disc gel electrophoresis was performed at pH 9.0 by the method of Davis (1964) using a Buchler electrophoresis apparatus at 3 mA/tube for 2 h at 4 °C. Protein was stained by immersing the gels in 1% amido black 10 B. Destaining was performed in a Bio-Rad diffusion destainer. Gels were scanned at 600 nm by using a Gilford spectrophotometer, Model 250, with a gel scanner attachment.

Estimates of molecular weights of lipoxygenase isoenzymes were done by $NaDodSO_4$ gel electrophoresis (Weber and Osborn, 1969) using 10% acrylamide. The standard proteins used were lysozyme, β-lactoglobulin, trypsinogen, pepsin, ovalbumin, and bovine plasma albumin (Sigma Chemical Co).

RESULTS AND DISCUSSION

Lipoxygenase Activity in Cowpea Varieties/Lines. Screening for the lipoxygenase activity of 89 cowpea varieties with varying performance in yield and pest and disease resistance indicates a large variation of enzyme activity. Table I shows part of the data obtained. The TVu-373-1-1 variety possesses the highest specific activity of 397 units/mg of protein whereas PI-293455 has only 30 units/mg of protein. UPL Cp2 was found to have 271 units/mg of protein lipoxygenase activity. This wide range of lipoxygenase activity among the various varieties/lines assayed suggests the existence of a germ plasm pool that can be used for selecting cultivars of the desired enzyme activity. Chapman et al. (1976) also reported a wide variation of lipoxygenase content in soybean genotypes and have noted that the enzyme activity is genetically controlled. A reduction of 50% of the original lipoxygenase activity in soybeans through breeding was achieved without any difficulty (Hammond et al., 1972). More recently, two soybean lines lacking lipoxygenase activity were identified (Hildebrand and Hymowitz, 1981).

Table I. Lipoxygenase Activity in Different Varieties/Lines of Cowpea[a]

variety/line	sp act.,[b] units/mg of protein
TVu-373-1-1	397
TVu-1566	386
PI-181833	281
UPL Cp2	271
TVu-702-1-1	179
TVu-461	141
PI-255781	92
TVu-131	86
PI-269664	52
TVu-356	36
PI-293455	30

[a] Enzyme was extracted with 0.1 M phosphate buffer, pH 5.8, 4 °C, in a chilled mortar and pestle for 5 min and centrifuged at 12000g for 20 min at 4 °C. The clear supernatant was used for the enzyme assay. [b] Average of two replications.

Table II. Distribution of Lipoxygenase Activity in Cowpea Seeds (var. UPL Cp2)

part	% of seed	lipoxygenase act.[a] total act. in 1 g of sample	act. in 1 g of seeds	% act. in seed part
whole seed	100	27 100	27 100	100
seed coat	7.5	4 700	350	1.3
cotyledon	90	28 800	25 920	94.0
hypocotyl	2.5	52 200	1 300	4.7

[a] Average of two replications. Enzyme extraction was as described in Table I.

However, the performance of these lines in terms of yield, pest and disease resistance, seed viability, etc. should first be evaluated because the physiological role of lipoxygenase in plants is still unknown. In cottonseed, the reduction of gossypol made the plant susceptible not only to common cotton pathogens but also to uncommon ones. This is an example of the deterrent effect of manipulating the biological components in plant without the basic knowledge of their functions.

Lipoxygenase Distribution in Cowpea Seeds. The lipoxygenase activity in different parts of the cowpea seeds is shown in Table II. Ninety four percent of total activity was localized in the cotyledon which was 90% of the whole seed. Yamamoto et al. (1970) reported that rice lipoxygenase is localized mostly, if not entirely, in the germ fraction. Svensson and Eriksson (1974) found that the outer cotyledon of pea seeds contained a considerably higher lipoxygenase activity than the inner cotyledon and the skin.

Lipoxygenase Purification. *Enzyme Extraction and Ammonium Sulfate Fractionation.* The lipoxygenase specific activity of water extract was 168 units/mg of protein (Table III) which is about half of the value obtained from crude extract by using phosphate buffer, pH 5.8, as the extractant as reported in the previous paper using the same cowpea variety (Truong et al., 1979). The 134% increase in total protein and only 30% increase in total lipoxygenase activity in the water extract as compared to the phosphate buffer, pH 5.8 extract contributed to the decrease in the specific activity of the former. Water extracts not only albumin but also globulin due to the presence of salt in the seeds. Sefa-Dedeh and Stanley

Table III. Summary of Purification of Cowpea Lipoxygenases[a]

fraction	vol, mL	total act., units	total protein, mg	sp act., units/mg of protein	degree of purification	% recovery
water extract	176	348 194	2082	168	1.0	100
albumin	202	233 570	587	404	2.4	67
40% $(NH_4)_2SO_4$	227	236 583	430	548	3.3	68
40-60% $(NH_4)_2SO_4$	15	217 000	326	664	4.0	62
DEAE pooled fraction						
L-1	105	63 985	92	720	4.3	18
L-2	69	14 400	40	336	2.0	4
hydroxylapatite pooled fraction						
L-1	46	7935	5	1568	9.3	2

[a] Values are means of at least three replications except for the hydroxylapatite pooled fraction with two determinations.

(1979) found that cowpea proteins had a higher solubility in water than in 0.5 M sodium chloride solution. Removal of salts from the extract by dialysis against distilled water resulted in the precipitation of globulin. The supernatant, albumin, was about 28% of the water-extracted proteins or 12% of the seed proteins. Lipoxygenase is localized mostly in the albumin fraction. The discarded globulin contained only about 4% of the total activity of the extract.

A recovery of 67% lipoxygenase in the albumin fraction as compared to the water extract may be due to the effect of dialysis and/or the stability of the enzyme with time. The former relates to the difference in responses of the isoenzymes present in the water extract to ions. Some isoenzymes lost their activity when specific ions are removed by dialysis. The crude extract of barley lipoxygenase lost activity when dialyzed against distilled water but not with tap water (Franke and Frehse, 1953). The latter is more possible because the water extract lost more than 50% of the total lipoxygenase activity after standing for 2 days at 4 °C. However, removal of globulin by dialysis resulted in a 2.4-fold purification as compared to that of the water extract (Table III).

Ammonium sulfate fractionation did not result in further decrease in percent recovery as indicated in Table III. The 40–60% $(NH_4)_2SO_4$ precipitate possessed a specific activity of 664 units/mg of protein which is 4- and 1.6-fold purification as compared to that of the water extract and albumin fraction, respectively.

DEAE-Sephadex A-50 Ion-Exchange Chromatography. On the DEAE-Sephadex A-50 column, the 40–60% $(NH_4)_2SO_4$ precipitate was separated into two major protein peaks which contained lipoxygenase activity (Figure 1). There are, therefore, two isozymes which can be numbered lipoxygenases 2 and 1 (L-2 and L-1), in the reverse order of their elution from the ion-exchange column, similar to the case of the soybean lipoxygenase isoenzymes (Christopher et al., 1972).

The pooled fraction of L-1 contained an average of 92 mg of protein with 63 985 lipoxygenase activity units or 18% of the original activity. This had a specific activity of 720 units/mg of protein or 4.3-fold purification as compared to that of the water extract (Table III). On the other hand, the pooled fraction of L-2 possessed a lower lipoxygenase activity, about one-fourth that of the L-1 fraction. The low specific activity and recovery (336 units/mg of protein and 4%) obtained for L-2 may be due to its lesser stability upon purification. Passing the DEAE-Sephadex pooled fraction of L-2 through a Sephadex G-200 column resulted in only a 10% recovery of the applied total activity while that of L-1 showed a recovery of 60% or more. When L-1 was allowed to stand at 4 °C, no decrease in activity of L-1 was noted, even for at least 1 week, while L-2 lost about 65% of its activity. However,

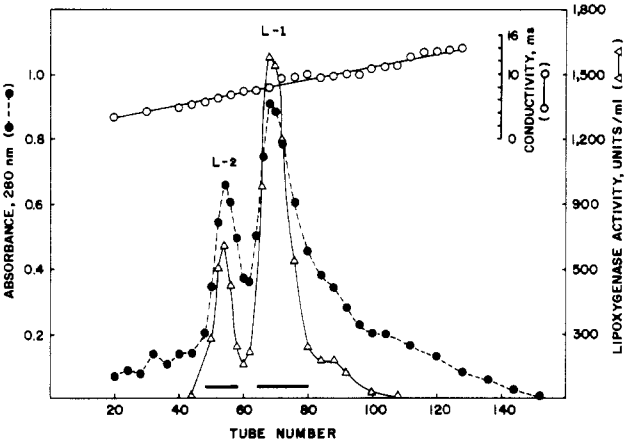

Figure 1. Separation of cowpea lipoxygenase isoenzymes on the DEAE-Sephadex A-50 column (2.5 × 30 cm). Five-milliliter fractions were collected at the rate of 32 mL/h by using Tris-HCl buffer, pH 8.0, with a NaCl linear gradient of 0.05–0.35 M.

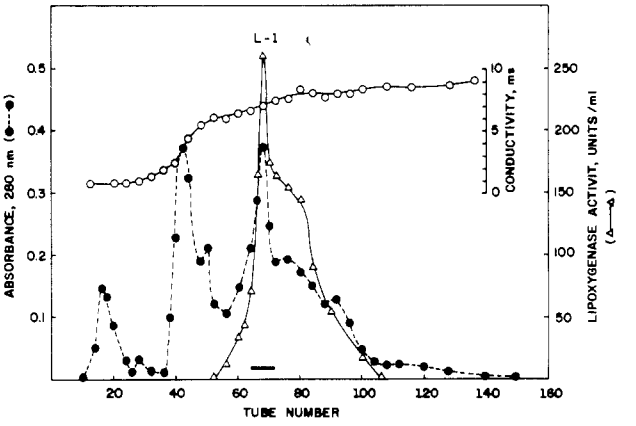

Figure 2. Hydroxylapatite column chromatography of DEAE-Sephadex A-50 pooled fractions. Column size: 2.2 × 18 cm. Three-milliliter fractions were collected by using a linear gradient of 0.01–0.3 M phosphate buffer, pH 6.8, at the rate of 8 mL/h.

the two isoenzymes were stable for several months in the form of lyophilized powder.

Hydroxylapatite Chromatography. The elution pattern of DEAE-pooled fractions of L-1 and L-2 on the hydroxylapatite column is shown in Figure 2. The separation revealed three major protein peaks among which only one possessed lipoxygenase activity. It is identified as L-1 because the pooled fraction exhibited an almost homogeneous protein band as determined by electrophoresis with an R_f value corresponding to that of L-1 obtained from the DEAE-Sephadex column (Figure 3). The other two major protein peaks eluted before L-1 showed no lipoxygenase activity. Apparently, the more labile L-2 isozyme was

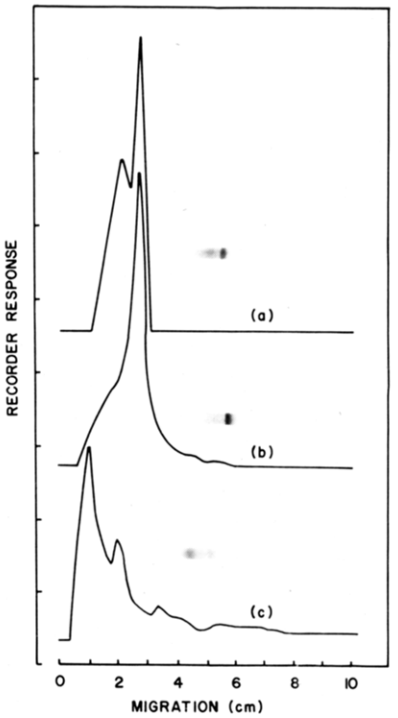

Figure 3. Disc gel electrophoresis protein patterns of cowpea lipoxygenase isoenzymes and their densitometer scans: (a) DEAE-Sephadex A-50 pooled fraction of L-1; (b) hydroxylapatite pooled fraction of L-1; (c) DEAE-Sephadex A-50 pooled fraction of L-2.

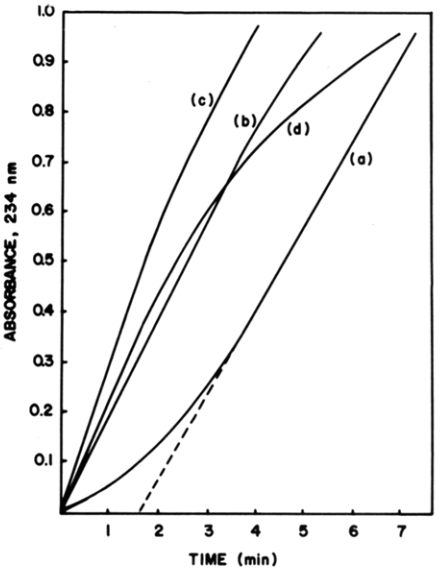

Figure 4. Progress curves of the oxidation of linoleic acid catalyzed by cowpea L-1 and L-2. (a and b) L-1 and L-2 with new linoleic acid solution; respectively; (c and d) L-1 and L-2 with old linoleic acid solution, respectively.

inactivated during passage through this column. A repeat of this experiment confirmed the loss of L-2 activity on passage through the hydroxylapatite column. Subsequently, only L-1 active pooled fraction from the DEAE-Sephadex step was applied on hydroxylapatite for further purification.

Hydroxylapatite chromatography resulted in an increase in specific activity up to 1568 units/mg of protein or 9.3-fold purification and a 2% recovery of the original total activity (Table III). Christopher et al. (1970) obtained a homogeneous soybean lipoxygenase 2 with a 50-fold purification and 0.2% recovery in a purification procedure in which hydroxylapatite was the last step. Recently, Truong et al. (1981a) reported a further increase in the degree of purification of two winged bean lipoxygenase isoenzymes eluted from the DEAE-Sephadex column by passing through hydroxylapatite columns.

Characterization of L-1 and L-2. *Disc Gel Electrophoretic Pattern of L-1 and L-2.* The polyacrylamide gel electrophoresis of L-1 and L-2 are shown in Figure 3. L-1 obtained from the DEAE-Sephadex column exhibited one major and one diffused broad protein band with R_f values of 0.25 (70% purity) and 0.13–0.21, respectively. The diffused band was removed by chromatography on hydroxylapatite, giving an almost homogenous protein of L-1 (Figure 3, gel b). L-2 revealed three minor bands and a major band with an $R_f = 0.11$ (80% purity). On the basis of the R_f value, L-1 and L-2, respectively, corresponded to the major and minor lipoxygenase isoenzymes of the zymogram reported by Truong et al. (1979) using ammonium sulfate precipitation of the cowpea crude extract as an enzyme source. The major lipoxygenase isoenzyme in many legumes had an R_f value in the range of 0.22–0.27 (Eskin and Henderson, 1974, 1976; Yoon and Klein, 1979; Truong et al., 1981a).

Lipoxygenase Reaction Progress Curves. The two cowpea isoenzymes exhibited different behavior in their action toward linoleic acid as indicated in the progress curves (Figure 4). With fresh linoleic acid, L-1 had a lag period of almost 2 min (Figure 4a), while with L-2, the initial velocity can be obtained right after the addition of the enzyme to the reaction mixture (Figure 4b). This means that the initial quantity of the hydroperoxides in the solution was always enough for L-2 to have its maximal rate. This is evident when the old linoleic acid solution (1 week at 4 °C) was used as the substrate for both L-1 and L-2 (parts c and d of Figure 4). Egmond et al. (1976) explained this phenomenon in soybean lipoxygenase by proposing a product binding site on the enzyme molecule which regulates the enzyme in its action. The lag period is usually associated with lipoxygenase (Wheeler and Wallace, 1978). However, Nicolas and Drapron (1977) did not observe any lag period with horse bean lipoxygenase.

pH–Activity Profile. Both cowpea L-1 and cowpea L-2 exhibited a narrow range of pH–activity with a maximum at 6.2 which is the same as that of the cowpea crude extract reported earlier (Truong et al., 1979). The two isoenzymes had no activity below pH 4 or above pH 7.5. The profiles are similar to those of peanut L-2 and L-3, soybean L-2, and pea and winged bean lipoxygenase (Sanders et al., 1975; Diel and Stan, 1978; Yoon and Klein, 1979; Truong et al., 1981b).

Kinetic Constant. L-1 and L-2 differ markedly in their K_m values as determined by a Lineweaver–Burk plot. Apparent K_m values of 0.8×10^{-3} M and 0.55×10^{-4} M linoleic acid was obtained for L-1 and L-2, respectively. The apparent K_m of crude cowpea lipoxygenase is 0.195×10^{-3} M linoleic acid (Truong et al., 1979). Ben-Aziz et al. (1970) reported different K_m values for soybean lipoxygenase of 1.1×10^{-4} M and 6.6×10^{-4} M at Tween 20 concentrations of 0.017 and 0.25%, respectively, which are comparable to that of cowpea L-2. Pea seed lipoxygenase had a K_m of 3.08×10^{-4} M (Klein, 1976). K_m values close to that of cowpea L-1 were reported for lipoxygenase from rice bran, small faba bean, and winged bean (Eskin and Henderson, 1974; Shastry and Rao, 1975; Truong et al., 1981b).

Molecular Weights. Molecular weights of cowpea lipoxygenase iosenzymes obtained from DEAE-Sephadex chromatography were determined by the NaDodSO$_4$–

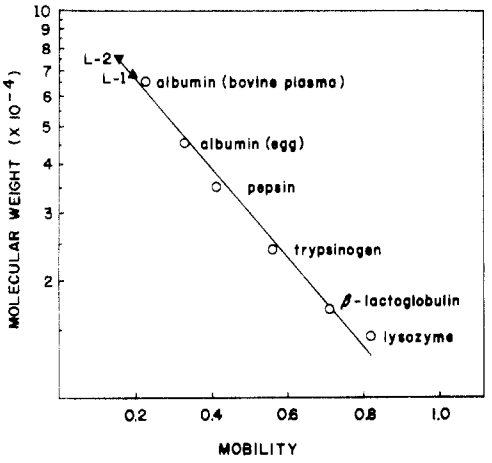

Figure 5. Calibration curve for determination of the molecular weight of cowpea L-1 and L-2 using $NaDodSO_4$–polyacrylamide gel electrophoresis.

Table IV. Substrate Specificity of Cowpea Lipoxygenase Isoenzymes

substrate	rel act., % L-1	rel act., % L-2
oleic acid	0	0
linoleic acid	100	100
linolenic acid	0	45
monolinolein	0	0
trilinolein	0	0

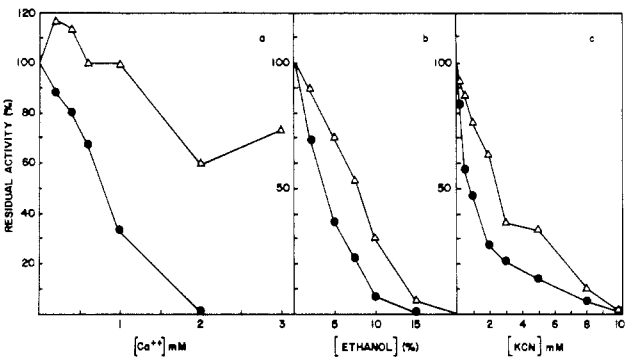

Figure 6. Effect of calcium (a), ethanol (b), and KCN (c) on cowpea lipoxygenase (Δ) L-1 and (●) L-2.

Table V. Effect of Different Ions on the Activity of Cowpea Lipoxygenase Isoenzymes[a]

ions (1 mM)	rel act., % L-1	rel act., % L-2
none	100	100
Ba^{2+}	20	75
Hg^{2+}	0	0
Mn^{2+}	15	55
Mg^{2+}	50	65
Cu^{2+}	63	70
Zn^{2+}	65	48
Fe^{2+}	34	28
Fe^{3+}	0	53

[a] Mean of two replications.

polyacrylamide gel electrophoresis method. The molecular weight was estimated from a plot of mobility vs. molecular weight of standard proteins (Figure 5). The values of 68 000 and 74 000 were obtained for cowpea L-1 and L-2, respectively, close to that reported in the previous paper using Sephadex G-150 gel filtration (Truong et al., 1979). Peanut, pea, winged bean, and soybean lipoxygenase isoenzymes exhibited molecular weights of 73 000, 74 000, 80 000, and 105 000, respectively (Diel and Stan, 1978; Haydar and Hadziyev, 1973; Sanders et al., 1975; Truong et al., 1981b).

Substrate Specificity. Among the common substrates tested, linoleic acid is the best substrate for both cowpea L-1 and cowpea L-2 (Table IV). The lack of reaction of the two isoenzymes toward oleic acid (1.58×10^{-3} M) confirmed the lipoxygenase type of the isolated enzymes. Only L-2 can oxidize linolenic acid (1.64×10^{-3} M) with a relative activity of 45% compared to its activity toward linoleic acid (1.61×10^{-3} M). L-1 was unreactive toward linolenic acid, at least under the conditions used. Because the two substrates were prepared in the same manner and their concentrations in the reaction mixture were similar, the difference in reactivity of the two isozymes might be due to a difference in their optimal substrate requirements. High substrate concentrations inhibit mungbean and barley lipoxygenase (Surrey, 1964; Lulai and Baker, 1976). A further experiment on the effect of varying linolenic acid concentrations as well as pH of the reaction mixture on the activity of L-1 and L-2 should be conducted to obtain definite conclusions on the cowpea lipoxygenase specificity for linolenic acid.

The unreactivity of L-1 and L-2 toward monolinolein (1.28×10^{-3} M) and trilinolein (0.55×10^{-3} M) may be due to either the orientation of the hydroxyl groups at the substrate interface hindering substrate–enzyme interaction (Guss et al., 1968) or the sensitivity of the enzymes toward the solvent ethanol used to disperse the substrate as in the case of trilinolein or both. The final concentration of ethanol (8.4%) in the reaction mixture using trilinolein as the substrate could cause 75% and 40% inactivation of L-2 and L-1, respectively (Figure 6). Guss et al. (1968) reported that neither mono- nor dilinolein was a good substrate for wheat and soybean lipoxygenase. However, soybean lipoxygenase and pea lipoxygenase were very reactive toward trilinolein (Haydar et al., 1975; Verhue and Franke, 1972) while wheat lipoxygenase was almost unreactive (Guss et al., 1968) to the same substrate.

Effect of Calcium, Ethanol, and Cyanide. Cowpea L-1 and L-2 had different responses to calcium. L-2 was progressively inhibited by increasing calcium concentrations while L-1 was activated by concentrations lower than 0.6 mM and inhibited by concentrations higher than 1 mM (Figure 6a). Calcium activated soybean L-1 and L-2 but inhibited L-3 (Restrepo et al., 1973; Zimmerman and Snyder, 1974). Sanders et al. (1975) reported that peanut L-1 was inhibited by calcium whereas L-2 and L-3 were activated by 40–400 μM calcium and inhibited by higher concentrations. The different responses of lipoxygenase preparations from faba beans and broad beans to calcium were also reported (Eskin and Henderson, 1974; Abbas et al., 1979).

Cowpea L-2 was more sensitive to inhibitory effects of ethanol and KCN than L-1 (parts b and c of Figure 6). Both isoenzymes were completely inhibited by higher than 20% ethanol and 10 mM KCN. Truong et al. (1981b) reported similar effects of ethanol and KCN on winged bean lipoxygenase isoenzymes. The inhibitory effect of KCN on various lipoxygenases has been reported (de Lumen et al., 1978; Flick et al., 1978; Sanders et al., 1975), contrary to the expected null effect of KCN on lipoxygenase which has been traditionally used to differentiate heme-catalyzed from lipoxygenase-catalyzed oxidation. The presence of iron in soybean lipoxygenase and its catalytic participation established by Pistorius and Axelrod (1974) can explain the inhibitory effect of cyanide on lipoxygenase.

Table VI. Effect of Antioxidants on Cowpea Lipoxygenase Isoenzymes[a]

effector	% inhibition		
	10^{-6} M	10^{-4} M	10^{-3} M
ascorbic acid			
L-1	27	53	100
L-2	0	16	53
butylated hydoxyanisole			
L-1	100	100	100
L-2	14	30	n.d.[b]
butylated hydoxytoluene			
L-1	100	100	100
L-2	32	52	57
propyl gallate			
L-1	10	100	n.d.
L-2	10	60	n.d.
hydroquinone			
L-1	0	100	100
L-2	10	14	82

[a] Mean of three replications. [b] Not determined because of the high absorbance at 234 nm of these substances at 10^{-3} M.

Effect of Metal Ions. The inhibitory effect of metal ions (1 mM) on cowpea lipoxygenase isoenzymes is shown in Table V. L-1 and L-2 had different responses to metal ions with less inhibition observed for L-2. Metal ions had different effects on soybean lipoxygenase isoenzymes as reported by Yamamoto et al. (1970). They observed that most of the divalent cations exert inhibitory effects on the reaction catalyzed by lipoxygenase "a" whereas marked stimulatory effects was observed on lipoxygenase "b". Activating and inhibitory effects of metal ions, i.e., Fe^{3+}, Fe^{2+}, Co^{2+}, Cu^{2+}, were observed with lipoxygenase from different sources (Shastry and Rao, 1975; Truong et al., 1981b).

Effect of Antioxidants. All of the conventional antioxidants inhibit plant lipoxygenase (Tappel, 1961; Palla and Verrier, 1974). Cowpea lipoxygenase were also inhibited by antioxidants tested (Table VI). In general, cowpea lipoxygenase L-2 was less sensitive to antioxidants than L-1. Butylated hydroxyanisole and butylated hydroxytoluene exhibited a stronger inhibitory effect on cowpea L-1 than others similar to soybean lipoxygenase (Yasumoto et al., 1970).

Thermal and pH Stability. L-1 and L-2 had marked differences in thermal stability (Figure 7). L-1 can retain about 80% of its original activity after 30 min of incubation at 50 °C while L-2 lost more than 80%. At 60 °C, L-2 had more activity after 2 min, and it took 30 min to have a similar effect for L-1. Soybean lipoxygenase isoenyzymes also had different thermal stability. Christopher et al. (1970) found half-lives of 25 and 0.7 min for lipoxygenases 1 and 2, respectively, when heated at 69 °C.

Cowpea lipoxygenase isoenzymes were stable over the pH range 4–9 (Figure 8) when incubated in the appropriate buffer for 30 min at 25 °C. The enzymes were completely denatured at pH below 3 and above 10. The pH stability and pH–activty profile of lipoxygenase could help in preventing the action of this enzyme on the natural substrates during processing.

So that the stability of the enzyme in intact seeds could be tested, seeds were soaked at pH 2.0, 4.0, and 6.0 for 10 h, after which they were blanched at 100 °C for 6 min. These conditions had earlier been shown by Okaka and Potter (1979) to reduce the beany flavor in cowpea. Results show that the soaking treatment at highly acidic pHs did not affect the lipoxygenase activity as compared to the pH 6.0 treatment (Table VII) but blanching of the soaked seeds destroyed the activity almost completely. However,

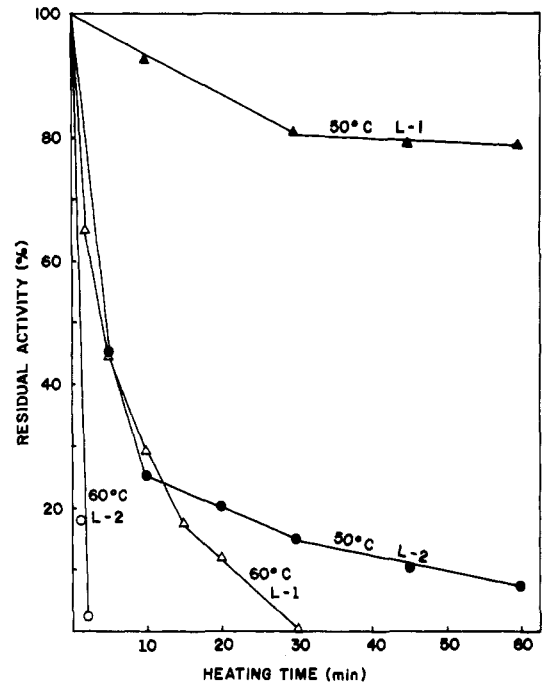

Figure 7. Thermal stability of cowpea lipoxygenase.

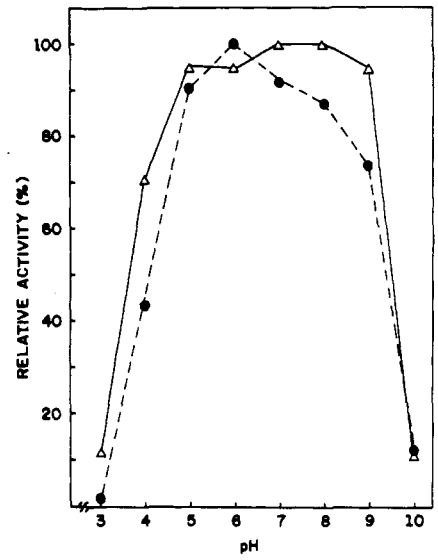

Figure 8. Effect of pH on cowpea L-1 and L-2. (Δ) L-1; (●) L-2.

it was also shown that blanching of seeds at 100 °C for 6 min without presoaking also inactivated the enzyme.

Therefore, the reduction of beany flavor of the cowpea powder produced by the process of Okaka and Potter (1979) might not be due to inactivation of lipoxygenase by soaking under acidic conditions but may be due to that by blanching. The same rate of heat inactivation of lipoxygenase in soaked and unsoaked cowpea seeds suggests that soaking can be omitted. However, before the time-consuming soaking step can be deleted, the data on the flavor scores of soaked samples are necessary. Soaking might be beneficial in this aspect if some components involved directly or indirectly in the beany flavor of the product are reduced by leaching out.

CONCLUSION

Two cowpea lipoxygenase isoenzymes, L-1 and L-2, were separated based on their difference in elution from the ion-exchange column and clearly distinguished by electrophoretic mobility. The difference in other physical and

Table VII. Effect of Soaking and Blanching on Lipoxygenase Activity of Cowpea Seeds

treatment	lipoxygenase act.,[a] units/mL
soaking for 10 h at	
pH 2.0	1391
pH 4.0	1435
pH 6.0	1430
blanching of seeds for 6 min which were soaked for 10 h at	
pH 2.0	5.2
pH 4.0	4.7
pH 6.0	5.2
blanching of unsoaked seeds at 100 °C for different times	
0 min	1091
6 min	1.1
10 min	1.5
15 min	2.6
20 min	4.0

[a] Means of two replications.

chemical properties such as kinetic constant, substrate specificity, response to ions, hydroperoxide requirements, etc. indicate that the two isoenzymes play different roles in the flavor formation as well as other physiological roles in cowpea.

In general, cowpea L-1 is similar to soybean L-2. The characteristics of purified isoenzymes could help in developing and improving the processing technique of cowpea.

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