Table II. Values of the Compositional Difference Index Calculated According to Cornish-Bowden (1979) for Binary Comparisons of Some Low Molecular Weight Prolamins[a]

	$S\Delta n$ (0.42N_B)			
	LMW hordein-1	LMW gliadin-2[b]	A_4-secalin[c]	zein 13.5[d]
LMW hordein-1	0	54.3 (60.5)	38.9 (60.5)	322.3 (52.9)
LMW gliadin-2		0	31.1 (61.3)	341.1 (52.9)
A_4-secalin			0	293.7 (52.9)
zein 13.5				0

[a] Compositional difference index: $S\Delta n$. $S\Delta n = {}^1/_2\Sigma(n_{i,A} - n_{i,B})^2 - 0.035(N_A - N_B)^2 + 0.535|N_A - N_B|$, in which $n_{i,A}$ and $n_{i,B}$ are the numbers of amino acid residues of the ith type, N_A is the total number of residues in the bigger protein, and N_B is that of the smaller one. A value for $S\Delta n < 0.42N_B$ indicates a high degree of sequence homology between the two proteins. [b] From Prada et al. (1982). [c] Amino acid analysis from Charbonnier et al. (1981) corrected for $N = 146$ (corresponding to M_r 16 000). [d] Amino acid analysis from Gianazza et al. (1977) corrected for $N = 126$ (corresponding to M_r 13 500).

rye [see Miflin et al. (1981), García-Olmedo et al. (1982), and Salcedo et al. (1982)].

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Purification and Characterization of a Type-1 Lipoxygenase from Pea Seeds

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A type-1 lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12) was purified from pea seeds by a combination of ammonium sulfate fractionation, gel filtration, ion-exchange chromatography, and preparative isoelectric focusing in a granulated gel. Lipoxygenase-1 was very unstable, especially at pH values below 6, and extensive loss of enzyme activity occurred during preparative isoelectric focusing. Partially purified lipoxygenase-1 focused into enzyme-active bands at pH 4.05 and 4.20. This preparation effectively catalyzed the oxidation of linoleate, linolenate, methyl linoleate, and trilinolein substrates but exhibited much lower activity than type-2 pea lipoxygenases. Highest activity occurred with linoleic acid, with maximum activity in the 9.0–10.0 range and an apparent K_m of 0.20 mM at pH 9.0. Apparent molecular weights of 64 000 and 65 000 were obtained by gel filtration chromatography and sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the isoenzyme with pI = 4.05, respectively. Like soybean lipoxygenase-1, pea lipoxygenase-1 was not as effective in carotene cooxidation as the type-2 enzymes, and production of 280 nm absorbing compounds occurred only after the system became anaerobic. Lipoxygenase-1 was ineffective in bleaching chlorophyll.

Lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12) is present in a wide variety of plants, especially legumes, and is believed to be one of the main oxidative catalysts in vegetables. The primary physiological role of the lipoxygenase reaction in plants is unclear, although it has been associated with ripening, abscission, and germination (Axelrod, 1974; Veldink et al., 1977; Pattee, 1979).

Lipoxygenase catalyzes the oxidation of unsaturated

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long-chain fatty acids and esters containing a *cis,cis*-1,4-pentadiene system. The resultant cis,trans conjugated diene hydroperoxides can be degraded to volatile compounds, such as alcohols, acids, ketones, and aldehydes, responsible for off-odors and off-flavors; bleaching of carotenoid and chlorophyll pigments that cause undesirable color changes can also occur (Rackis et al., 1979; Gardner, 1979).

Lipoxygenase isoenzymes are classified as type-1 or type-2 enzymes on the basis of their pH of optimum activity and the positional specificity of the hydroperoxides formed (Grosch and Laskawy, 1979; Grossman and Zakut, 1979; Van Os et al., 1979; Galliard and Chan, 1980). Type-2 lipoxygenases generally have pH optima of 6–7, and therefore, most of the lipoxygenases found in plants are considered as type-2 enzymes. Type-1 lipoxygenase has optimum activity at pH 9–10 and is the original enzyme crystallized from soybeans by Theorell et al. (1947), later designated lipoxygenase-1 (Christopher et al., 1970). Since the pH optimum for most of the plant lipoxygenases ranges from 5.5 to 7.0, soybean lipoxygenase-1 is thought to be anomalous (Galliard and Chan, 1980).

In 1979, Yoon and Klein purified and characterized two type-2 lipoxygenase isoenzymes from pea seeds. A third isoenzyme was isolated that, on the basis of elution from DEAE-Sephadex A-50 and pH 9 activity, appeared to correspond to the Theorell enzyme in soybeans. It had previously been assumed that this form was totally absent in peas (Weber et al., 1973a). The objective of the present study was to purify and more fully characterize pea lipoxygenase-1.

MATERIALS AND METHODS

Pea Seeds and Other Materials. Pea seeds (*Pisum sativum* cv. Little Marvel) were obtained from Northrup King Co. (Minneapolis, MN). Linoleic acid was purchased from Nu-Chek Prep, Inc. (Elysian, MN). Methyl linoleate, linolenic acid, trilinolein, β-carotene, chlorophyll *a*, dithiothreitol, Coomassie Brilliant Blue R-250, chloride diagnostic kit no. 830, and proteins were purchased from Sigma Chemical Co. (St. Louis, MO). Ultrodex and Ampholines were obtained from LKB-Produkter AB (Bromma, Sweden). Bio-Rad Protein Assay Kit was obtained from Bio-Rad Laboratories, Richmond, CA. Acrylamide (enzyme grade), *N,N'*-methylenebis(acrylamide) (Bis), *N,N,N',N'*-tetramethylethylenediamine (Temed), 3,3'-dimethoxybenzidine dihydrochloride (DBH), and sodium dodecyl sulfate ($NaDodSO_4$) were obtained from Eastman Kodak Co., Rochester, NY. Deionized, distilled (NANOpure) water was used to prepare all solutions.

Enzyme Purification. Pea lipoxygenase isoenzymes were isolated and purified by a modification of the procedure of Yoon and Klein (1979). The initial extrction was done for 30 min in 50 mM sodium acetate buffer (pH 4.5), and a 0–400 mM NaCl gradient was used in the ion-exchange chromatography on DEAE-Sephadex A-50. The lipoxygenase-1 fraction (elution volume 470 mL) having the highest specific activity, 4.8×10^3 units/mg of protein (assayed spectrophotometrically at pH 9.0), was used for K_m determination, pH profile, carbonyl production, and pigment bleaching studies. Lipoxygenase-1 was further purified by preparative flat-bed isoelectric focusing (IEF) in a granulated gel (Ultrodex) of pH range 3.5–5.5 (Winter et al., 1975). Carrier ampholytes were removed from the purified lipoxygenase by passage through a Sephadex G-50 column (2.5 × 12 cm) by using 50 mM sodium phosphate buffer (pH 6.8) as the eluent. Because of the instability of this enzyme, the purified lipoxygenase-1 ($pI = 4.05$) was used only in analytical IEF and molecular weight determinations.

Assay Procedures. Lipoxygenase activities were determined spectrophotometrically and polarographically at room temperature (25 ± 2 °C) as outlined by Yoon and Klein (1979). Throughout the purification, a linoleate–Tween 20 substrate was used (Yoon and Klein, 1979). The stock solution was diluted immediately prior to the assay with 4 volumes of 50 mM Tris-HCl buffer (pH 9.0) and saturated with oxygen prior to enzyme addition. Concentrations in the reaction cell were 2.56 mM linoleate and 0.08% Tween 20. For the polarographic measurement of oxygen uptake, the YSI 5304 Micro Field Conversion Kit accessory for the YSI Model 53 biological oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, OH) was used. Lipoxygenase activity was calculated from the initial rate of oxygen absorption by assuming an initial dissolved oxygen concentration of 1.13 μmol/mL (Chappell, 1964).

Absorbance at 280 nm was used as an index of protein concentrations in column chromatography fractions. Protein was determined by the method of Lowry et al. (1951) and, in the instances where residual ampholytes might have been present, by a Coomassie Brilliant Blue G-250 binding assay (Bio-Rad Laboratories, 1979).

Carbonyl production activity was determined spectrophotometrically by measuring the increase in absorbance at 280 nm. The reaction mixture contained 0.26 mM linoleate–Tween 20 substrate, 50 mM Tris-HCl buffer, pH 9.0, and 0.1 mL of enzyme preparation, about 500 units of lipoxygenase activity.

Carotene and chlorophyll bleaching activities of lipoxygenase-1 were determined spectrophotometrically by using modified methods of Ben-Aziz et al. (1971) and Arens et al. (1973). The carotene solution was prepared by mixing 2 mg of β-carotene with 0.09 g of Tween 80, dissolving in 2.0 mL of chloroform, which was subsequently evaporated under a stream of nitrogen, and dissolving the resulting residue in 4 mL of water. The chlorophyll solution was prepared by dissolving 1 mg of chlorophyll *a* and 0.036 mL of Tween 80 in 10 mL of acetone. This solution was subsequently evaporated to dryness under a stream of nitrogen and the resulting residue dissolved in 2 mL of water.

Immediately prior to assay, 0.02 mL of aqueous carotene or chlorophyll solution was added to 2 mL of oxygenated, buffered (50 mM Tris-HCl, pH 9.0) linoleate–Tween 20 substrate. Immediately after the addition of 0.1 mL of the lipoxygenase-1, the decrease in absorbance at 460 nm (carotene bleaching) or 430 nm (chlorophyll bleaching) was recorded. Initial assay mixtures contained 0.26 mM linoleate, 8.8 μM β-carotene or 5.3 μM chlorophyll *a*, and 0.02% Tween 80.

Analytical Polyacrylamide Gel Electrophoresis. Analytical polyacrylamide gel electrophoresis was performed in 7.5% polyacrylamide gels at pH 8.9 according to the method of Davis (1964) and at pH 7.5 as described by Gabriel (1971). The formulas for the resolving gels were modified to contain 1% soluble starch. For general protein staining, the gels were immersed in 0.25% Coomassie Brilliant Blue R-250 in 45% methanol–9% acetic acid in water overnight and then destained by diffusion in 30% ethanol–10% acetic acid in water.

Lipoxygenase activity on the gels was visualized by a modified starch–KI method (Guss et al., 1967) with a substrate of 2.56 mM linoleic acid in 50 mM Tris-HCl buffer, pH 9.0. Gels were also stained by the method described by de Lumen and Kazeniac (1976), which is based on the oxidation of the dye, 3,3'-dimethoxybenzidine

hydrochloride (DBH) by hydroperoxides.

Analytical Isoelectric Focusing. Analytical isoelectric focusing (IEF) was carried out in horizontal thin layers of polyacrylamide gel containing 1% soluble starch by using the LKB 2117 multiphor system and following the procedure described in LKB Application Note 250 (Winter et al., 1977). For staining, gels were cut in two, and one part was stained for protein with Coomassie Brilliant Blue R-250 (Winter et al., 1977). The activity of lipoxygenase isoenzymes after isoelectric focusing was detected in the other part by the method of Guss et al. (1967).

Molecular Weight Determination. For determination of the molecular weight of the enzyme, analytical polyacrylamide gel electrophoresis in the presence of NaDod-SO_4 was carried out in horizontal thin layers of polyacrylamide gel (Fehrnstrom and Moberg, 1977) and the standard NaDodSO_4–phosphate system of Weber and Osborn (1969). The addition of 2-mercaptoethanol to some samples was omitted to obtain the NaDodSO_4 gel pattern of nonreduced enzyme. Equine skeletal muscle myoglobin (M_r 17 200), ovalbumin (M_r 45 000), bovine serum albumin (M_r 67 000), human transferrin (M_r 77 000), and rabbit muscle phosphorylase *b* (M_r 94 000) were run simultaneously as standards. Apparent molecular weights of the isoenzymes were interpolated from the semilogarthmic plot of known molecular weight vs. relative mobility.

Apparent molecular weight was also determined by gel filtration chromatography under nondenaturing conditions according to the procedure of Andrews (1964) using a calibrated column (5 × 100 cm) of Sephadex G-150. Bovine pancreas ribonuclease *a* (M_r 13 700), bovine pancreas chymotrypsinogen A (M_r 25 000), ovalbumin (M_r 45 000), bovine serum albumin (M_r 67 000), and rabbit muscle aldolase (M_r 158 000) were used as standards. Lipoxygenase-1 samples were eluted from the column with 50 mM sodium phosphate buffer, pH 6.8, at a flow rate of 65 mL/h, and eluate fractions were assayed at pH 9.0. The apparent molecular weight of lipoxygenase-1 was interpolated from the calculated K_{av} from a semilogarthmic plot of the known molecular weight vs. the K_{av} value for each protein standard.

pH–Activity Profile. The pH–activity profile of lipoxygenase-1 was determined spectrophotometrically at various pH values under conditions of constant ionic strength with linoleate–Tween 20 substrate. The buffer systems (50 mM) used were as follows: citrate–phosphate, pH 4.5–7.0; sodium phosphate, pH 6.0–8.0; Tris-HCl, pH 7.5–9.0; borax–NaOH, pH 9.4–10.0; sodium phosphate–NaOH, pH 11.5–12.0; and KCl–NaOH, pH 12.5–13.0. Stock linoleate–Tween 20 substrate was diluted with 4 volumes of the appropriate buffer immediately prior to assay. The initial substrate concentration was 2.56 mM linoleate, and 0.1 mL of enzyme preparation was used.

The stability of lipoxygenase-1 was investigated by incubating 0.25-mL aliquots of enzyme preparation in 2.5 mL of 50 mM buffer solutions of various pH values (described above). After 1-h incubation at 4 °C, 1-mL samples were removed and assayed spectrophotometrically at pH 9.0 for remaining lipoxygenase activity.

K_m Determination. For determination of apparent K_m values for lipoxygenase-1 at pH 9.0, linoleic acid concentrations were varied from 0.05 to 10.3 mM by diluting stock linoleate–Tween 20 substrate (Yoon and Klein, 1979) with water before diluting with buffer for assay. The data were transformed according to Lineweaver–Burk, and the straight lines best fitting the measured values obtained under conditions without a lag phase were determined by the method of least squares (Michal, 1978). In the instances where marked deviations from Michaelis–Menten kinetics occurred at higher linoleate concentrations, K_m values could be calculated by extrapolation of the linear portions of the double-reciprocal plots.

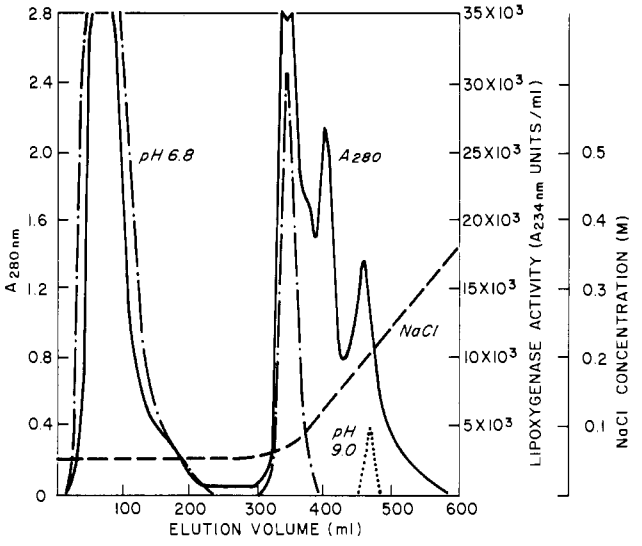

Figure 1. Separation of pea seed lipoxygenase isoenzymes on DEAE-Sephadex A-50. Absorbance was measured at 280 nm (—), and lipoxygenase activity (A_{234nm} units per milliliter) was assayed at pH 6.8 (-·-) and pH 9.0 (···) as described in the text.

Substrate Specificity. Lipoxygenase activity was assayed polarographically by using the lipoxygenase-1 preparation from anion-exchange chromatography. Stock substrate solutions were prepared in the following manner. Tween 20 substrates were made by mixing 0.4 mL of linoleic or linolenic acid with an equal volume of Tween 20, adding 1.2 mL of 1 N KOH, and diluting to 100 mL with water (Yoon and Klein, 1979). Linoleic acid–ethanol substrate was prepared by dissolving 0.1 mL of linoleic acid in 15 mL of 95% ethanol and bringing the volume to 25 mL with water; for linolenic acid, 100 mg was dissolved in the ethanol. Methyl linoleate was made by dissolving 0.108 mL in 25 mL of a 40:60 (v/v) acetone–95% ethanol mixture. Trilinolein substrate was prepared by dissolving 100 mg in 8.9 mL of a 40:60 (v/v) acetone–95% ethanol mixture. Before the addition of buffer, 0.04 mL of stock substrate solution was diluted with 0.36 mL of water in the reaction cuvette. Initial reaction concentrations for linoleic, linolenic, methyl linoleate, and trilinolein substrates were 0.257, 0.287, 0.261, and 0.256 mM, respectively.

Replication. The enzyme purification was repeated 3 times. All assays were run in triplicate. Values given in the text are representative of those obtained in all replications.

RESULTS AND DISCUSSION

Preparation of Pea Lipoxygenase-1. Adequate quantities of pea lipoxygenase-1 for characterization studies were obtained by making several modifications in the isolation procedure used by Yoon and Klein (1979). By use of an initial pH 4.5 extraction instead of pH 6.8, extraneous high molecular weight proteins were eliminated. Sufficient quantities of lipoxygenase-1, a minor protein component, for characterization were thus obtained without overloading the Sephadex G-150 gel when applying the lipoxygenase-active fraction from ammonium sulfate fractionation. The specific activity (assayed spectrophotometrically at pH 6.8) of lipoxygenase in the crude pH 4.5 extract (4.5×10^4 units/mg of protein) was approximately twice that obtained from a pH 6.8 extraction.

A change in the linear NaCl gradient from 50–400 mM to 0–400 mM resulted in more distinct separation of the isoenzymes. The elution profile with the 0–400 mM NaCl gradient is shown in Figure 1. The isoenzymes were numbered in reverse of their order of elution to conform to the procedure used to number the soybean isoenzymes (Christopher et al., 1972). The two predominant isoenzymes, lipoxygenase-2 (elution volume 330 mL) and -3 (elution void volume 100 mL), were eluted first and correspond to fractions II and I, respectively, while lipoxygenase-1 (elution volume 470 mL) corresponded to fraction IV isolated by Yoon and Klein (1979).

Polyacrylamide gel electrophoresis of the lipoxygenase-1 fraction from ion-exchange chromatography exhibiting the highest specific activity resulted in six protein bands. Gels stained using the KI procedure exhibited a dark brown band with $R_f = 0.55$, while gels stained with DBH exhibited an orange band of the same relative mobility; lipoxygenase-1 was the most anionic protein in the fraction and appeared to be the main protein based on the intensity of the protein band. The R_f (0.55) for the lipoxygenase-active band was comparable to that reported for fraction IV (R_f = 0.54) by Yoon and Klein (1979). A lipoxygenase-active band of 0.55 relative mobility was also obtained after polyacrylamide gel electrophoresis of crude extracts and the pooled lipoxygenase-active fraction from gel filtration chromatography. Throughout the study, with equal protein concentrations and on both polyacrylamide gel electrophoresis and IEF gels, DBH dye was not nearly as sensitive as the KI procedure for staining lipoxygenase-1.

Lipoxygenase-1 eluted from the ion exchanger (elution volume 470 mL) did not exhibit characteristic heme absorbance at 400–430 nm. In this study, a fraction corresponding to fraction III isolated by Yoon and Klein (1979) was also identified, eluting between lipoxygenases-1 and -2. This fraction (elution volume 420 mL) had lower lipoxygenase activity (measured spectrophotometrically at pH 9) than did lipoxygenase-1, but oxygen uptake was greater than that catalyzed by lipoxygenase-1. This fraction exhibited a sharp absorbance peak at 410 nm, so it is probable that the linoleate oxidation activity was due to heme protein catalysis. Like lipoxygenase, heme proteins also catalyze the peroxidation of unsaturated lipids, although the main reaction catalyzed by heme protein is thought to be homolytic scission of preformed hydroperoxides (Grossman and Zakut, 1979).

Analytical IEF of the lipoxygenase-1 from the anion exchanger indicated the presence of two lipoxygenase-active bands with pI values of 4.05 and 4.20. For elimination of enzyme-active complexes that formed during IEF, pH gradients were prefocused (Righetti, 1979). It is of interest that in preliminary experiments lipoxygenase-1 was more stable and exhibited higher activity when it was complexed with ampholytes. The apparent pI values obtained for lipoxygenase-1 were lower than the 5.5–5.68 values reported in the literature for soybean lipoxygenase-1 (Catsimpoolas, 1969; Christopher et al., 1972; Verhue and Francke, 1972; Grosch et al., 1977; Diel and Stan, 1978), suggesting that the enzymes from the two sources differ structurally.

After preparative IEF, the pI = 4.05 and 4.20 lipoxygenase-active bands were eluted from the granulated gel. A single protein band was obtained after polyacrylamide gel electrophoresis of the pI = 4.05 lipoxygenase under dissociating conditions in $NaDodSO_4$–polyacrylamide gels. Also, single protein bands were obtained after electrophoresis under nondenaturing conditions at pHs 8.9 and 7.5, R_f = 0.55 and 0.40, respectively. The relative mobilities of the protein bands corresponded to those of the lipoxygenase-active bands. Gels loaded with as much as 320 μg of purified lipoxygenase-1 in attempts to detect minor contaminants still exhibited only one band. In addition, lipoxygenase-1 refocused as a single protein band at pI = 4.05 on analytical electrofocusing polyacrylamide gels.

The lipoxygenase-1 isoenzymes obtained from preparative IEF were very unstable, with activity decreasing more than 50% after 24 h at 4 °C. Attempts to increase their stability by the addition of dithiothreitol to reduce thiol groups were unsuccessful. The extensive loss of activity that occurred is not unusual for enzymes focusing at extreme pHs, because of irreversible denaturation (Tipton and Dixon, 1979). Chepurenko et al. (1978) also reported decreased pea lipoxygenase activity after IEF that they attributed to the complexing of ampholytes with iron in the enzyme.

The instability of pea lipoxygenase-1 probably accounts for its going unnoticed until Yoon and Klein (1979) reported the presence of this isoenzyme in pea seeds. Arens et al. (1973) did not report pH 9 activity for the labile pea lipoxygenase isoenzyme that they detected in early stages of purification. The pea lipoxygenase-4 identified on alkaline polyacrylamide gels by Borisova et al. (1977) had a similar elution profile to that of lipoxygenase-1 on DEAE-Sephadex A-50 and exhibited a relative mobility (R_f = 0.58) on polyacrylamide gel electrophoresis similar to that for pea lipoxygenase-1 (R_f = 0.55) in the present study. In a preliminary study done in our laboratory with another variety of pea seeds (cv. Alderman Tall Telephone), lipoxygenase-1 was isolated as were lipoxygenases-2 and -3, indicating that lipoxygenase-1 may be more universal than previously believed. The presence of an alkaline pH optimum isoenzyme has also been identified in peanuts; like pea and soybean lipoxygenases-1, peanut lipoxygenase-1 also eluted from DEAE-Sephadex A-50 after the two neutral pH optima peanut isoenzymes (Sanders et al., 1975). Hildebrand and Hymowitz (1981) reported that only two soybean genotypes lacked lipoxygenase-1, suggesting that the type-1 enzyme is usually present in that legume.

pH–Activity Profile. Lipoxygenase-1 exhibited a broad pH optimum extending from 8.5 to 11.0 with maximal activity in the 9.0–10.0 range. This is similar to the pH 8–9.5 optimum reported for soybean lipoxygenase-1 (Christopher et al., 1972; Diel and Stan, 1978). Because of the observed instability of lipoxygenase-1 after ion-exchange chromatography and preparative isoelectric focusing, its pH stability was examined. Lipoxygenase-1 was more stable at pH values between 6.8 and 11.5 than at pH values below 6. At pH 4.5, lipoxygenase-1 had less than 50% of the activity found at pH 6.8–11.5, which accounts for the large loss in activity observed after preparative isoelectric focusing. Because of the instability at pH 4.5, exposure to this pH was reduced as much as possible during the extraction procedure.

Molecular Weight. On the basis of gel filtration, lipoxygenase-1 (pI = 4.05) exhibited an apparent molecular weight of 64 000. The relative mobility of lipoxygenase-1 (pI = 4.05) on 7.5% $NaDodSO_4$–polyacrylamide gels was characteristic of a protein having a molecular weight of 65 000. Identical results were obtained in the absence of mercaptoethanol, so lipoxygenase-1 probably does not consist of subunits connected by disulfide linkages. Also, the similarity of the values obtained by the two different methods makes it likely that the isoenzyme consists of only a single polypeptide chain.

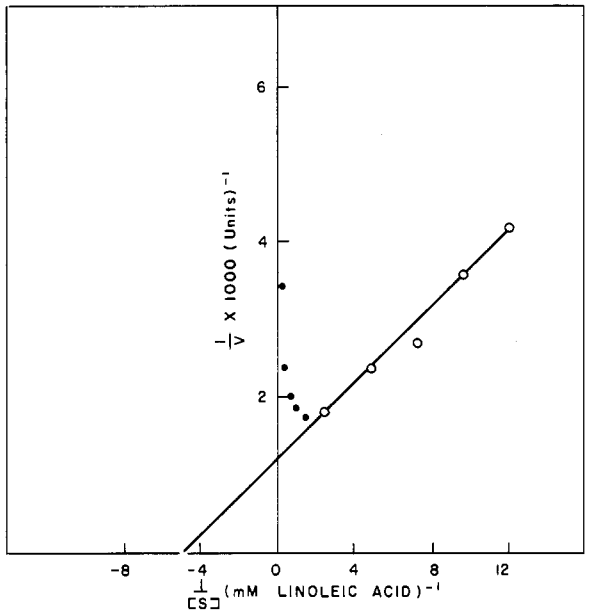

Figure 2. Double-reciprocal plot of the linoleic acid concentration vs. the initial rate of conjugated diene formation catalyzed by pea lipoxygenase-1 at pH 9.0 with 50 mM Tris-HCl buffer. Assay conditions are described under Materials and Methods. Closed circles indicate substrate inhibition.

The estimated molecular weights for pea lipoxygenase-1 are close to the 67 000 obtained by ultracentrifugation (Eriksson and Svensson, 1970), the 78 000 obtained by gel filtration (Arens et al., 1973), the 74 000 and 76 000 obtained by $NaDodSO_4$–polyacrylamide gel electrophoresis (Haydar and Hadziyev, 1973; Haydar et al., 1975), the 70 000 estimate obtained by gel filtration (Borisova et al., 1977) for pea lipoxygenases, and the 73 000 estimate obtained by gel filtration for peanut lipoxygenases-1, -2, and -3 (Sanders et al., 1975). Haydar et al. (1975) reported that the pea lipoxygenase with an apparent molecular weight of 76 000 did not retain enzyme activity after storage of the pea seeds. The 64 000 and 65 000 estimates for pea lipoxygenase-1 are less than the 100 000 and 108 000 values reported for soybean lipoxygenases-1, -2, and -3 (Stevens et al., 1970; Christopher et al., 1972; Diel and Stan, 1978).

Michaelis Constants. Apparent K_m values for lipoxygenase-1 obtained for linoleic acid at pH 9.0 by the polarographic and spectrophotometric (Figure 2) assays were 0.18 mM and 0.20 mM, respectively. Substrate inhibition (note closed circles in Figure 2) occurred at linoleate concentrations greater than 0.5 mM as indicated by the existence of a lag period that was abolished by addition of more enzyme. Similar substrate inhibition was observed by Egmond et al. (1976) with soybean lipoxygenase-1. Earlier, Smith and Lands (1972) suggested that the enzyme had two binding sites, one to which only substrate could bind and the other for which product and substate competed. Excess substrate would prevent hydroperoxide formation by flooding both sites and forming an abortive enzyme–substrate complex.

The K_m values obtained for pea lipoxygenase-1 in the present study are greater than the 8.5×10^{-5} M reported for soybean lipoxygenase-1 at pH 9.0 (Bild et al., 1977) and lower than the K_m values obtained for type-2 pea lipoxygenases at pH 6.8, which ranged from 2.22 to 3.62 mM (Reynolds, 1981).

Attempts to determine K_m values for linoleic acid dissolved in ethanol were not successful due to inhibition of lipoxygenase activity by the levels of alcohol necessary to solubilize high concentrations of linoleic acid. For the same reason, K_m values were not successfully determined for methyl linoleate and trilinolein substrates.

Table I. Substrate Specificity of Pea Seed Lipoxygenase-1 at pH 9.0

substrate	μmol of O_2 min^{-1} (mg of protein)$^{-1}$
linoleic acid–Tween 20	0.93
linoleic acid–ethanol	0.90
linolenic acid–Tween 20	0.31
linolenic acid–ethanol	0.28
methyl linoleate	0.29
trilinolein	0.25

Substrate Specificity. The substrate specificity of lipoxygenase-1 (anion-exchange preparation) at pH 9.0 was determined by comparing activities with linoleic acid, linolenic acid, methyl linoleate, and trilinolein (Table I). Additionally, ethanol and Tween 20 dispersed substrates were compared for linoleic and linolenic acids.

Lipoxygenase-1 was most active with linoleic acid. This agrees with results reported for soybean lipoxygenase-1 at pH 9 (Christopher et al., 1970, 1972; Verhue and Francke, 1972; Bild et al., 1977). For both linoleic and linolenic acid substrates, the activities obtained with the ethanol and Tween 20 dispersed substrates were similar. This is in contrast to the results observed with pea lipoxygenases-2 and -3 where activities were greatly reduced with the ethanolic substrates as compared to Tween 20 dispersed substrates on an equal substrate concentration basis (Reynolds and Klein, 1982). This can be accounted for in part by the fact that, due to a lower K_m, lipoxygenase-1 assays were done with 10-fold lower substrate concentrations than those used for the lipoxygenase-2 and -3 assays. At these lower substrate concentrations, the ethanol concentration was 1.2%, a concentration where ethanol does not seem to affect lipoxygenase activity (Reynolds, 1981).

Formation of 280 nm Absorbing Material. It is known that under certain reaction conditions, lipoxygenase produces 280 nm absorbing keto and peroxy acids and hydroperoxides (Vioque and Holman, 1962; Garssen et al., 1971; Pistorius, 1974; Hurt and Axelrod, 1977; Yoon and Klein, 1979). Pistorius (1974) and Yoon and Klein (1979), however, pointed out that the formation of these carbonyl compounds was not a general property of all lipoxygenase isoenzymes.

When lipoxygenase-1 (anion-exchange preparation) was incubated with 0.26 mM linoleic acid at pH 9.0, there was rapid production of conjugated dienes that reached a maximum in approximately 3 min and then leveled off with increasing reaction time. After about 7 min, the formation of 280 nm absorbing material was apparent. The production of 280 nm absorbing material increased more rapidly after 12-min reaction time, at which time the dissolved oxygen in the reaction mixture was depleted, as indicated by parallel measurements using the Clark-type oxygen electrode. At pH 6.8, where conjugated diene activity was much less, carbonyl production was slight.

Pea lipoxygenase-1 resembles soybean lipoxygenase-1 and bush bean lipoxygenase *b* that catalyze carbonyl production only under anaerobic conditions (Pistorius, 1974; Garssen et al., 1971). Pea lipoxygenase-2 is ineffective in catalyzing carbonyl production under aerobic or anaerobic conditions at either pH 6.8 or 9.0 (Yoon and Klein, 1979). The same has also been demonstrated by Pistorius (1974) for soybean lipoxygenase-2. With pea lipoxygenase-3, carbonyl production begins simultaneously with the onset of the primary oxygenation reaction, resembling in this respect soybean lipoxygenase-3 and bush

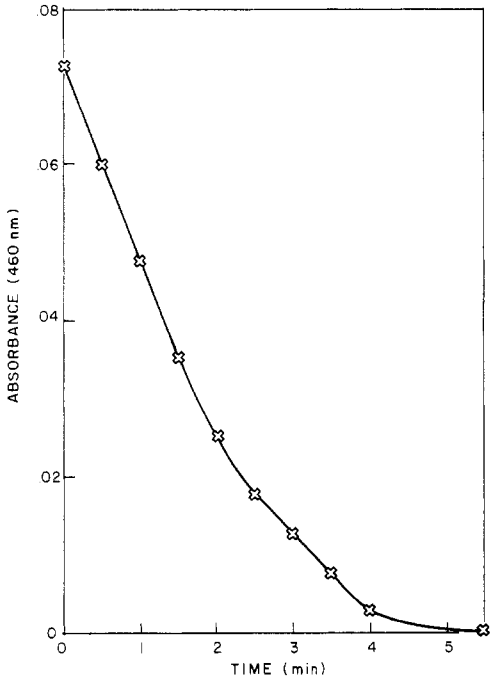

Figure 3. Carotene bleaching with pea lipoxygenase-1 at pH 9.0 using 0.26 mM linoleic acid dispersed with Tween 20 as the substrate.

bean lipoxygenase *a* (Pistorius, 1974; Hurt and Axelrod, 1977; Yoon and Klein, 1979).

The differences in the carbonyl-producing activity of the three pea lipoxygenase isoenzymes and the soybean and bush bean lipoxygenases described by Pistorius (1974) and Hurt and Axelrod (1977) suggest a slightly different mechanism of action with respect to secondary reactions for the various isoenzymes. The nature of the reaction products for the pea isoenzymes has not yet been determined.

Carotene and Chlorophyll Bleaching Activities. The pigment bleaching ability of lipoxygenase isoenzymes has been reported to differ by several investigators (Pistorius, 1974; Grosch et al., 1976; Ramadoss et al., 1978). However, reaction mechanisms for carotene and chlorophyll bleaching and the relationships between the lipoxygenase–linoleate system and pigment bleaching have not been clarified but may be related to the formation of secondary carbonyl products.

Typical carotene bleaching activity of lipoxygenase-1 at pH 9.0 in the linoleate–Tween 20 system is shown in Figure 3. A cooxidation potential of 0.006 was obtained by relating the carotene bleaching activity (change in absorbance at 460 nm) to the peroxidation activity (change in absorbance at 234 nm) at pH 9.0.

Pea lipoxygenases-2 and -3, which exhibited cooxidation potentials of 0.07 and 7.4, respectively, at pH 6.0 in 2.56 mM linoleate–Tween 20 systems, were better carotene bleachers than pea lipoxygenase-1 (Reynolds, 1981). Yoon and Klein (1979) reported ratios of carotene bleaching to peroxidation activity for pea lipoxygenases-2 and -3 of 0.23 and 0.43, respectively. They noted that the ratios were dependent on substrate, carotene, and enzyme concentrations. Pea lipoxygenase-1 was comparable to soybean lipoxygenase-1, which also exhibits only slight carotene cooxidation activity (Weber et al., 1973a,b, 1974; Grosch et al., 1977). According to Yoon and Klein (1979) and Reynolds (1981), only lipoxygenase-3 was effective in chlorophyll bleaching and carbonyl production. Thus, there appears to be a difference among isoenzymes with respect to carotene and chlorophyll bleaching that may be associated with the secondary decomposition of hydroperoxides.

CONCLUSIONS

A type-1 lipoxygenase in pea seeds has been purified and characterized. This isoenzyme, which resembles soybean lipoxygenase-1 in some respects, is present in small amounts and is extremely unstable during and after purification. Its identification in pea seeds, however, confirms that the presence of an alkaline pH optimum lipoxygenase is not confined to soybeans and may be more universal than previously believed.

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Extraction of Nitrogen from Palm Kernel Meal and Evaluation of Digestibility of Protein Isolate from the Meal by the in Vitro Method

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Techniques for maximizing the extraction of N from palm kernel meal have been investigated. Protein concentrate was also prepared from the meal and its digestibility evaluated by the in vitro method. Maximum extraction (97.78%) of N was obtained by using a meal to solvent ratio of 2 g/100 mL, 1 M NaOH solution, and an extraction time of 90 min. Other solvents such as sodium chloride and calcium hydroxide were not as efficient as NaOH. The protein concentrate was found to be more digestible than the original meal (77.1 vs. 55.2%).

The expansion of the local crushing industries has increased the availability of palm kernel meal (PKM) in Nigeria. Although palm kernel meal, with 19% protein, is cheaper than groundnut cake, which is commonly used in livestock feeds, its utilization by monogastric animals has been found to be impaired by its grittiness and high crude fiber content (Oyenuga, 1968; Fetuga, 1972; Owusu-Domfeh, 1967). Efforts have been made recently (Babatunde et al., 1975; Fetuga et al., 1977) to enhance the utilization of PKM by young and growing–fattening pigs. It was, however, felt that PKM could also be processed to yield a protein concentrate for food use. As a first step, the solubility of its nitrogenous constituents in various solvents has been studied to determine the most efficient procedure.

EXPERIMENTAL SECTION

Materials. The palm kernel meal (PKM) samples used in the present study were obtained as commercial press cakes from vegetable oil (Nigeria), Ltd., Ikeja, Lagos. These were products of oil extraction by the mechanical screw press expeller method, after being heated in kettles up to 85 °C. The meal was ground into a fine powder of particle size ≤0.2 mm. The powdered material was stored at −5 °C. Its proximate analysis (on a dry matter basis) was 18.75% ($N \times 6.25$), 6.05% crude fiber, 6.39% fat, 4.43% ash, and 64.38% nitrogen-free extract.

Extraction Procedure. The extraction techniques employed in solubilizing N from the PKM were similar to those used by Kazazis and Kalaissakis (1979) with vetch seed using NaOH as the extractant. Other extractants [deionized water, NaCl, Na_2HPO_4, KH_2PO_4, Na_2SO_4, and $Ca(OH)_2$] were tested after establishing the optimum conditions for the extraction of N from the meal using NaOH. Extractions were carried out at ambient temperature (28 °C) by using a mechanical shaker. All suspensions were centrifuged at 1400*g* for 10 min, and duplicate aliquots of the supernatant were taken for N determination.

Analyses. Analyses for the proximate constituents of the seed were carried out by using official methods of the Association of Official Analytical Chemists (1975). True nitrogen in the supernatant solutions obtained by centrifuging the suspensions was measured by the method of Lowry et al. (1951).

Protein Concentrate Preparation. Ground PKM was vortexed for 20 min with 30 volumes of 1.0 M NaOH so-

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