Purification and Characterization of a Lipoxygenase from Immature English Peas

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Both disk gel electrophoresis and DEAE–cellulose chromatography showed at least three lipoxygenase (linoleate:oxygen oxidoreductase; EC 1.13.11.12) isoenzymes in extracts of immature English peas (*Pisum sativum* L. var. Dark Skinned Perfection). The major isoenzyme was purified 199-fold to electrophoretic homogeneity by ammonium sulfate fractionation and by DEAE–cellulose, Sepharose 6B-100, and polybuffer exchangers 94 column chromatographies with 10.2% recovery of activity. Its optimum pH was 6.5–7.0, with a single polypeptide chain of molecular weight 100 000. It was quite stable from pH 4.5 to 8.0 at 25 °C but was completely inactivated after 30 s at 80 °C and after 4 min at 65 °C. Activation energies were 24.6 kcal/mol for denaturation and 4.53 kcal/mol for conversion of substrate to product. Steady-state kinetics on linoleic acid by the pea isoenzyme and by soybean lipoxygenase-1 showed a sequential reaction mechanism. The pea isoenzyme acted on both linoleic acid and derivatives in which the carboxyl group was modified at similar rates while soybean lipoxygenase-1 preferred substrates with a free carboxyl group and had almost no activity on substrates with modified carboxyl groups. Peroxidation of linoleic acid occurred in equal amounts (50.3:49.7) at the 13- and 9-positions with the pea isoenzyme, while soybean lipoxygenase-1 gave an 87.7:12.3 ratio of the 13- and 9-hydroperoxides. Therefore, the major isoenzyme of immature English peas (*P. sativum* L. var. Dark Skinned Perfection) is similar to the type-2 soybean lipoxygenase. The major isoenzyme of immature green peas appears to be identical with the major lipoxygenase isoenzyme in dry pea seeds.

INTRODUCTION

Inactivation of enzymes present in vegetables by blanching prior to freezing is one of the major steps in the freezing process for vegetables. Peroxidase has generally been used as the index of blanching adequacy since the frozen food industry was established in the 1930s. Much research indicates that there is not a good correlation between peroxidase activity left and frozen food keeping quality (Burnette, 1977). Instead, some workers have suggested that lipoxygenase (linoleate:oxygen oxidoreductase; EC 1.13.11.12) should receive more attention as the index for proper heat treatment (Lee and Wagenknecht, 1958; Eskin and Grossman, 1977; Schwimmer, 1981). On the basis of results from our laboratory (Williams et al., 1986), we find lipoxygenase to be more important than peroxidase in the flavor deterioration of frozen English peas. One criterion for choosing the enzyme used as the index for proper heat treatment is that it have a major role in the quality deterioration of frozen foods.

Lipoxygenase is present in a wide variety of vegetables including legumes, corn, potatoes, and peas. Soybean lipoxygenase has been well studied since Theorell et al. (1947) crystallized the first soybean lipoxygenase. Lipoxygenase from dried English pea seeds (*Pisum sativum*, several varieties used) has been purified by several workers (Dillard et al., 1961; Eriksson, 1967; Erikson and Svensson, 1970; Arens et al., 1973; Haydar and Hadziyev, 1973; Haydar et al., 1975; Walker, 1975; Klein, 1976; Reynolds, 1982; Reynolds and Klein, 1982a,b). However, only limited work has been done on immature English peas, as used by the frozen food industry. Siddiqi and Tappel (1956) extracted lipoxygenase from immature English peas (var. Thomas Laxton) and concentrated it by barium acetate, acetone, and lead acetate precipitation. They indicated that 80% of the activity of the frozen concentrate was lost in 2 weeks at −18 °C. Wagenknecht and Lee (1956) achieved a 35-fold purification with 49% recovery of pea lipoxygenase by using ammonium sulfate precipitation (30–60% saturation) and heating (62 °C, 5 min). Klein (1976) reported a 9-fold purification of pea lipoxygenase with 3% recovery by using ammonium sulfate precipitation (30–60% saturation) and Sephadex G-150 and DEAE–Sephadex chromatography. The specific activity achieved was 17 100 units/mg of protein compared to 287 200 units/mg of protein when dried English pea seeds were used. Attempts to stabilize the enzyme with Ca^{2+} were largely unsuccessful.

Since there is no published work on the successful purification of lipoxygenase from immature English peas as used in the frozen food industry, the present study was undertaken to purify and characterize the major lipoxygenase isoenzyme from immature English peas. Hopefully, results obtained directly on the peas used by the industry may help in understanding the role of lipoxygenase in quality deterioration of the frozen stored peas.

EXPERIMENTAL PROCEDURES

Materials and Reagents. Immature English peas (*P. sativum* L. var. Dark Skinned Perfection) grown in Patterson, CA, were harvested by hand, shelled immediately, and placed in plastic bags over dry ice in ice chests. The peas selected were those judged to give a premium-quality frozen product. They were frozen within 3 h after harvest, stored at −23 °C, and used within 6 months of frozen storage.

Polybuffer exchangers 94, polybuffer 74, and Blue Dextran-2000 were from Pharmacia Fine Chemicals Co. Sepharose 6B-100, DEAE–cellulose, acrylamide, and bisacrylamide were from Sigma Chemical Co. Chicken ovalbumin and ovotransferrin were gifts from the laboratory of Dr. R. E. Feeney. Bovine serum albumin, soybean lipoxygenase-1, and soybean trypsin inhibitor were from Sigma Chemical Co.

Linoleic acid, linoleyl alcohol, linoleyl acetate, methyl linoleate, ethyl linoleate, trilinolein, and sodium linoleate were from Sigma Chemical Co. and were used without

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further purification. O_2 and N_2 were from Liquid Carbonics Co. Tween-20, Coomassie Brilliant Blue R-250, Blue Dextran-2000, and buffer salts of reagent grade were from Sigma Chemical Co. Deionized distilled water was used in all experiments.

Methods. Activity. Lipoxygenase activity was determined spectrophotometrically at 234 nm by the methods of Surrey (1964) and Al-Obaidy and Siddiqi (1981) with some modifications. The substrate stock mixture, consisting of 157.2 μL of pure linoleic acid, 157.2 μL of Tween-20, and 10 mL of deionized distilled water, prepared fresh daily, was clarified by adding 1.0 mL of 1.0 N NaOH and made to 50 mL with deionized distilled water. Before assay, this substrate stock solution (0.01 M) was diluted 4-fold with 0.2 M phosphate buffer, pH 7.0. The substrate–buffer solution (2.5×10^{-3} M substrate) was flushed with oxygen for 2 min and allowed to equilibrate at 25 °C for 10 min before adding the enzyme. The total reaction volume was 3.0 mL.

A unit of enzyme activity was defined as that amount of enzyme that produced a change in o.d. of 1.0/min at 234 nm, under the assay conditions. ϵ_m for diene conjugation of linoleic acid was 2.74×10^4 M^{-1} cm^{-1} (Tappel et al., 1952). For optimal reproducibility and linearity between enzyme concentration and observed activity, the quantity of enzyme tested was kept in the range of 1 ± 0.5 units/mL.

Protein Determination. Protein was determined by the Lowry method (Lowry et al., 1951) using crystalline bovine serum albumin as a standard and by absorbance at 280 nm.

Purification. All purification steps were carried out at 4 °C. Frozen immature English peas were weighed and blended in a Waring blender with cold acetone (-25 °C) in the ratio of 1:2.5 (w/v). The acetone powder was made by filtering the blended slurry in a Buchner funnel with suction and washing with another 2 volumes of cold acetone. The acetone powder then was extracted with 0.2 M sodium phosphate buffer (1:4, w/v), pH 7.0, containing 5% NaCl for 1–2 h. The extract was centrifuged at 17300*g* for 20 min. The pellet was discarded, and the supernatant was adjusted to pH 7.0. Solid ammonium sulfate sufficient to give 25% saturation at 4 °C was added to the solution with continuous stirring for 1 h. After centrifugation at 17300*g* for 20 min, the supernatant was brought to 50% saturation of ammonium sulfate with stirring for 1 h. A yellowish white precipitate formed; the preparation was centrifuged as described above, and the supernatant was discarded. The pellet was dissolved in 5 mM sodium phosphate buffer, pH 7.4, and dialyzed against the same buffer with several changes of buffer until the conductivity of the dialyzing fluid was the same as the original buffer.

The dialyzed solution was centrifuged as described above, and the pellet was discarded. The supernatant was applied to the top of a DEAE–cellulose column (2.6 × 40 cm) previously equilibrated with 5 mM sodium phosphate buffer, pH 7.4. The column was washed with the same buffer, and the protein eluted was monitored by absorbance at 280 nm. After the absorbance returned to the base line, further elution was carried out with a linear salt gradient of 0–0.1 M NaCl (or 0–0.2 M NaCl or 0–0.5 M NaCl) in 5 mM sodium phosphate buffer, pH 7.4.

The fractions from the DEAE–cellulose column containing more than 10% of the maximum lipoxygenase activity of the peak tube were pooled, and the enzyme was precipitated by ammonium sulfate fractionation between 50% and 90% saturation. The supernatant, with no lipoxygenase activity, was discarded, and the pellet was redissolved in 5 mM sodium phosphate buffer, pH 7.2, containing 0.03 M NaCl and applied directly to the top of a Sepharose 6B-100 column (1.6 × 98 cm) preequilibrated with the same buffer. Only one peak with lipoxygenase activity was observed. The fractions were pooled and concentrated to 2 mL by ultrafiltration on a Millipore membrane. The concentrated enzyme solution was then applied to the top of a polybuffer exchangers 94 column (0.9 × 48 cm) preequilibrated with 0.025 M imidazole–HCl buffer, pH 7.4. The enzyme was eluted by applying a polybuffer 74 solution, pH 4.0, to form a pH 7.0–4.0 gradient. The peak containing the major lipoxygenase activity was pooled and precipitated by 90% saturation of ammonium sulfate to remove the polybuffer 74, desalted, lyophilized, and stored in the freezer.

Disk gel electrophoresis was performed according to the method of Davis (1964) with some modifications. Duplicate gels for each sample were run; one was stained for protein with Coomassie Brilliant Blue R-250 and the other for activity by using the method of Guss et al. (1967) with modification (see below). The 7.5% gel system used, containing 1% soluble starch, stacked at pH 8.9 and ran at pH 9.5. Electrophoresis was carried out at 4 °C. Samples containing 10–30 μg of protein and 3 drops of 0.25% bromophenol blue in the electrode buffer (glycine–Tris buffer), pH 8.3, were layered on top of the gels. The current used was 2 mA/gel initially for 20 min to stack the proteins; it was then increased to 3 mA/gel for 1.5 h.

For protein staining, the gels were first fixed for 1 h in 11.4% trichloroacetic acid in 30% methanol containing 3.4% sulfosalicylic acid and then immersed for 2–6 h in 0.25% Coomassie Brilliant Blue R-250 in 50% methanol containing 9.2% acetic acid. The gels were destained with 5% methanol containing 7.5% acetic acid.

For activity staining, the gels were immersed for 30 min at 24 °C in the substrate solution (2.5×10^{-3} M linoleic acid in 0.2 M phosphate buffer, pH 7.0). After they were washed with deionized distilled water several times, the gels were treated with 5% KI in 15% acetic acid in carefully cleaned test tubes covered with cork stoppers for 15–30 min or until the white band(s), indicating lipoxygenase activity, appeared. The gels were transferred to other test tubes containing deionized distilled water and left overnight. The gels were exposed to short-wavelength UV light (Model UVGL-25, U-V Products) for 1 min. The white band(s) changed to a blue color for easy visualization.

Molecular Weight Determination. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed in 7.5% polyacrylamide gels at pH 7.2 in the presence of 2-mercaptoethanol as described by Weber and Osborn (1975). The molecular weight protein standards for SDS gel electrophoresis were soybean lipoxygenase (M_r 100 000), bovine serum albumin (M_r 66 200), chicken ovalbumin (M_r 43 000), Kunitz soybean trypsin inhibitor (M_r 21 500), and chicken lysozyme (M_r 14 400).

The molecular weight of the purified enzyme was also determined on a calibrated Sepharose 6B-100 column (1.6 × 98 cm) equilibrated with 0.03 M NaCl in 5 mM sodium phosphate buffer, pH 7.0, at 4 °C. Soybean lipoxygenase, bovine serum albumin, chicken ovalbumin, α-chymotrypsinogen (M_r 25 000), and chicken lysozyme were used as molecular weight protein standards.

Amino Acid Analysis. A sample of the isolated pea lipoxygenase isoenzyme was hydrolyzed in 6 N HCl at 110 °C for 24 h. Analyses of the hydrolyzed samples were carried out on a Dionex amino acid analyzer, Model D-500. Cystine/cysteine and methionine were determined by performic acid oxidation of the sample prior to hydrolysis

Table I. Purification of Lipoxygenase from Immature English Peas

treatment	units of act./100 g[a]	protein,[a] abs units at 280 nm/100 g	sp act., units of act./units of protein	purificn,[b] fold	rec, %
extract of acetone powder	31100	6370	4.88	1.00	100
25% $(NH_4)_2SO_4$ supnt	31400	4810	6.53	1.34	101
25–50% $(NH_4)_2SO_4$ ppt	26600	1680	15.8	3.24	85.5
dialysis	23200	766	30.3	6.21	74.6
DEAE-cellulose	8910	41.4	215	44.1	28.6
Sepharose 6B-100	8670	31.8	273	55.9	27.9
PBE[c]					
peak 1	2510	2.59	969	199	8.07
peak 2	672	0.8	840	172	2.16

[a] 100 g of English peaks. [b] Based on specific activity of the original extract. The specific activity of each isoenzyme in the original extract is not known. [c] Polybuffer exchange chromatography.

and quantitation as cysteic acid and methionine sulfone on the analyzer (Hirs, 1967). The method of Edelhoch (1967) was used to determine tryptophan and tyrosine.

Kinetic Studies. Steady-state kinetics of hydroperoxidation of linoleic acid catalyzed by the isolated pea lipoxygenase isoenzyme were determined with different O_2 concentrations made by mixing various known amounts of O_2 and N_2 with a gas mixing pump (Calibrated Instruments, Inc.). A YSI Model 5331 oxygen probe, YSI Model 5301 standard bath assembly (Yellow Springs Instrument Co.), and chemical microsensor, Model 1201 (Transidyne General Co.), were used to determine the O_2 concentration in all of the reactions.

Spectrophotometric measurements were carried out in specially designed cuvettes (1-cm light path) closed with a seal cap and continuously flushed with gas mixtures of O_2 and N_2 at the desired O_2 concentration (monitored continuously) through Teflon tubing of 0.011 in. i.d. Linoleic acid solutions were also flushed with the gas mixture until the O_2 electrode showed equilibration of O_2 concentration before being pipetted into the cuvettes. The linoleic acid solutions were made from the stock solution (0.01 M) by dilution with 0.2 M phosphate buffer, pH 7.0. The enzyme was added to the reaction at 24 °C with a syringe, and the absorbance at 234 nm was measured with a Cary-219 spectrophotometer. Rapid mixing was achieved by a stirrer installed under the cuvette holder.

An LSI-11/2 microcomputer (Digital Equipment Corp., DEC) with Tektronic plotter and DEC writer IV printer (DEC) was connected to the Cary-219 spectrophotometer for data acquisition, data storage, plotting, and print out of the results. During the experiments, a chart recorder was also used to collect the data for comparison and checking.

The double-reciprocal plot method of Lineweaver–Burk (Lineweaver and Burk, 1934) was used to determine K_m and V_{max} for both substrates, O_2, and linoleic acid.

Substrate Specificity. The compounds used for study of substrate specificity of the enzyme were linoleic acid, linoleyl alcohol, linoleyl acetate, methyl linoleate, ethyl linoleate, propyl linoleate, and trilinolein. Due to different solubilities in the different solvent systems, comparison of enzyme activity on the different substrates was based on the substrate system using ethanol as the solvent. Each compound was weighed out in an amount to give a concentration of 0.01 M and dissolved in 95% ethanol; then, it was diluted with 0.2 M phosphate, pH 7.0, and ultrasonicated for 1 min to give the final working concentration of 0.2 mM, which was the maximum concentration possible with some substrates without having any turbidity. The temperature of the reaction was 24 °C.

Product Specificity. Product specificity of linoleate hydroperoxidation catalyzed by purified soybean lipoxygenase-1 and isolated English pea lipoxygenase isoenzyme was studied by using radioactive linoleic acid [1-^{14}C] (NEC-501, 0.05 mCi, 52.6 mCi/mmol; NEN, Boston, MA). [1-^{14}C]Linoleic acid was diluted with unlabeled material to a specific activity of 16 μCi/mmol (Hamberg, 1971). Twenty microliters of [1-^{14}C]linoleic acid was added to each of two flasks containing 62.5 mL of 2 mM unlabeled linoleic acid; one flask contained 0.2 M borate buffer, pH 9.0, the other flask 0.2 M sodium phosphate buffer, pH 7.0, respectively. After 1 h of incubation at 0 °C (Roza and Francke, 1973) for equlibration, 0.1 mL of soybean lipoxygenase-1 solution (1 mg/0.25 mL of H_2O) was added to 20 mL of the buffer at pH 9.0 and 0.1 mL of isolated English pea lipoxygenase isoenzyme solution (2 mg/0.25 mL of H_2O) was added to 20 mL of the buffer at pH 7.0. Controls at the two different pHs were also prepared with no enzyme added. The four flasks were incubated in an ice bath under O_2 for 1 h. The conversion of the substrate to product, as measured by absorbance at 234 nm, was 90% for soybean lipoxygenase-1 and 40% for pea lipoxygenase isoenzyme.

The hydroperoxide (product) was extracted from the reaction mixture with ether (three times with 10 mL) by the method of Roza and Francke (1973). The combined ether extract was washed twice with water, dried over anhydrous sodium sulfate, and concentrated in a 50 °C water bath under the hood. The free fatty acids were esterified with diazomethane, and the solvent was removed. The residue was redissolved in a small amount of petroleum ether (bp 35–60 °C). The methyl esters of the hydroperoxides were separated on precoated 0.25-mm-thick silica gel plates (Polygram Sil G/UV254, Macherey-Nagel) by developing in a mixture of petroleum ether–chloroform–ethyl acetate (45:45:10, v/v). The hydroperoxides (LOOH) were located by using short-wavelength UV light (Model UVGL-25, U-V Products). Quantitative determination of the ratio of 13-LOOH and 9-LOOH was done by cutting the silica gel plates into 1-cm-long pieces and using liquid scintillation counting.

RESULTS AND DISCUSSION

Purification of Pea Lipoxygenase Isoenzyme. The procedure used to purify the major isoenzyme of immature English pea lipoxygenase is summarized in Table I.

The frozen peas were homogenized in 2.5 volumes of cold acetone (−25 °C) in order to remove chlorophyll, lipids, and phenols to prevent enzymatic browning. This greatly improved the recovery of activity in subsequent steps. Disk gel electrophoresis, followed by protein and activity staining, on crude extracts before and after acetone treatment did not show any significant changes in number or amounts of the isoenzyme bands.

Various purification methods were tried in order to attain maximum purification with minimum loss of activity. Addition of 5% NaCl in the extracting buffer (0.2

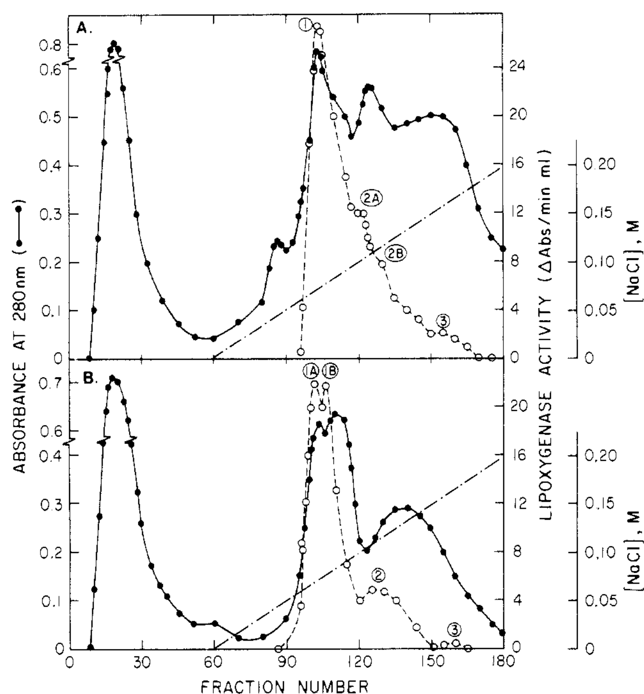

Figure 1. DEAE-cellulose chromatographic purification of immature English pea lipoxygenase. After ammonium sulfate fractionation, the precipitated enzyme was dissolved in 5 mM sodium phosphate buffer, pH 7.4, dialyzed against the same buffer for 24 h, and applied to the top of a DEAE-cellulose column (2.6 × 40 cm) previously equilibrated with the same buffer. Elution was carried out at 4 °C with a linear salt gradient from 0 to 0.2 M NaCl in 5 mM sodium phosphate buffer, pH 7.4: ●, absorbance at 280 nm; ○, lipoxygenase assay; ·-·, sodium chloride gradient. Key: A, ammonium sulfate cut applied was 25–50% fraction; B, ammonium sulfate cut applied was 20–50% fraction.

M phosphate, pH 7.0) increased total activity extracted by 30–40%. The highest activity recovery was obtained by fractionation between 25 and 50% ammonium sulfate. Eriksson and Svensson (1970) used the same range of ammonium sulfate concentration for dried English split pea seed lipoxygenase purification. Klein (1976) precipitated the protein at 60% ammonium sulfate saturation instead of 50% since she found that further purification of the 50% ammonium sulfate precipitate from the pea extract by gel and ion-exchange chromatography resulted in almost total loss of activity.

Further purification of the ammonium sulfate fraction was done by ion-exchange chromatography on DEAE-cellulose. Initially, DEAE-cellulose chromatography was performed by using a NaCl gradient in 5 mM phosphate buffer, pH 7.4, from 0 to 0.5 M. All lipoxygenase activity was eluted by ionic strength 0.2. Therefore, chromatography was repeated with a 0–0.2 M NaCl gradient (Figure 1A). The results (Figure 1A) indicated roughly the presence of three or four isoenzymes (in agreement with the disk gel electrophoresis results; not shown). The major peak eluted at μ 0.10, with shoulders at μ 0.11 and 0.13. A very small peak of activity eluted at μ 0.16. Improvement of the chromatography, by using a different ammonium sulfate fractionation range, slower flow rates, and less sample added to the column, was accomplished (Figure 1B). The isoenzyme isolated (Table I) is that labeled peak 1A (Figure 1B). It accounted for at least 40% of the total activity recovered at this step (activity of peaks 1A and 1B combined is 65% of total activity; in Figure 1A, peak 1 is 66% of total activity). Yoon and Klein (1979) reported four isoenzymes in dry English pea seeds (var. Little Marvel). The major isoenzyme eluted from a DEAE-

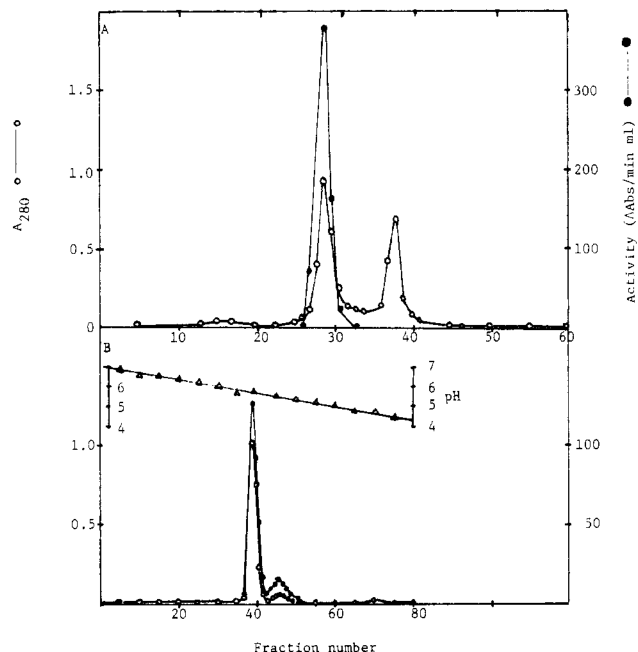

Figure 2. (A) Purification of pea lipoxygenase by Sepharose 6B-100 gel filtration. Enzyme from the DEAE-cellulose column (Figure 1B, fractions 95–120) was precipitated by ammonium sulfate fractionation (50–90% saturation). The precipitated enzyme was dissolved in 5 mM sodium phosphate buffer, pH 7.2, containing 30 mM NaCl and applied to the top of a Sepharose 6B-100 column (1.6 × 98 cm) preequilibrated with the same buffer at 4 °C. Five-milliliter fractions were collected. (B) Purification of pea lipoxygenase by chromatofocusing chromatography. The enzyme solution from the Sepharose 6B-100 column (fractions 26–31) was concentrated to 2 mL by ultrafiltration on a Millipore membrane. The concentrated enzyme solution was applied to the top of a polybuffer exchangers 94 column (0.9 × 48 cm) preequilibrated with 25 mM imidazole-HCl buffer, pH 7.4, at 4 °C. The enzyme was eluted by applying a polybuffer 74 solution, pH 4.0, to form a pH 7.0–4.0 gradient. Five-milliliter fractions were collected.

Sephadex column at pH 6.8 at μ 0.14. The other, much smaller, eluted peaks were at μ 0.20, 0.22, and 0.30. Eriksson and Svensson (1970) reported that lipoxygenase extracted from ripe dry English pea seeds (var. Whitham Wonder, a wrinkled type) eluted from a DEAE-cellulose column at pH 6.5 at μ 0.11.

Fractions marked 1A and 1B (Figure 1B; fractions 95–120) having activity of 25 units or higher were pooled. At this step, there was a decrease in total activity recovery (Table I; recovery after dialysis was 74.7%; after DEAE-chromatography the total recovery (including all fractions with activity) from the column was 37.1%). Several factors may be responsible for this decrease including enzyme instability or removal of unknown activators. Eriksson and Svensson (1970) noted that hemoproteins such as peroxidase, catalase, and cytochromes are strong lipid peroxidizers and that these enzymes and proteins were separated from lipoxygenase in the DEAE-cellulose step. In the present work it was found the total loss in activity was low when activity was measured immediately; however, after the solution was allowed to stand overnight at 4 °C, the total activity recovery was lower, as shown in Table I.

The two isoenzymes eluted as a single peak from a Sepharose 6B-100 column (Figure 2A) but were separated from other protein(s). This indicates that the isoenzymes have similar molecular weights.

When run on a chromatofocusing polybuffer exchanger column, the enzyme fraction from the Sepharose 6B-100

column was separated into a major and a minor peak (Figure 2B; Table I) which had pI values of 5.8 and 5.6, respectively. We shall designate the major isoenzyme as isoenzyme-1. Eriksson and Svensson (1970) found that dry English split pea seed (var. Whitham Wonder) lipoxygenase was separated into two major and one minor peaks on isoelectrofocusing. The two major peaks had pI values of 5.80 and 5.82. Reynolds (1982) reported that there were four lipoxygenase isozymes present in dry split pea seeds (var. Little Marvel) having pI values of 4.05, 4.20, 5.82, and 6.25. Therefore, the major isoenzyme isolated in this work of pI 5.8 appears to be identical with the major lipoxygenase isoenzyme in dry pea seeds.

The overall purification procedure of isoenzyme-1 gave a 199-fold increase in specific activity with 10.2% (combined peaks from polybuffer exchange column) recovery of activity. Three reports of partial purification of lipoxygenase from immature English peas are available. Lee and Wagenknecht (1958) obtained a 35-fold increase in lipoxygenase specific activity over that of lyophilized immature English peas by ammonium sulfate fractionation and heat treatment and a recovery of 49% of the total activity originally present in the starting material. Siddiqi and Tappel (1956) prepared a partially purified lipoxygenase from immature English peas (var. Thomas Laxton) by water extraction of an acetone powder and treatment with barium acetate, acetone, and basic lead acetate. They pointed out that attempts at further purification by ammonium sulfate fractionation resulted in a considerable loss of total activity. Klein (1976) prepared lipoxygenase from the acetone powder of immature English green peas by buffer extraction containing 2 mM $CaCl_2$, 30–60% ammonium sulfate fractionation, Sephadex G-150 gel filtration, and DEAE–Sephadex chromatography; she achieved a 9-fold purification with 3% recovery of activity.

Disk Gel Electrophoresis. During the course of purification, purity of the isoenzyme-1 as a protein was monitored by disk gel electrophoresis by the method of Davis (1964) including protein staining and by the specific lipoxygenase activity staining procedure developed by Guss et al. (1967) with modifications. Since isoenzyme-1 showed no activity at pH 9.0, the activity staining system was modified by using 0.2 M phosphate buffer, pH 7.0, instead of 0.5 M Tris-HCl, pH 8.3. The position corresponding to lipoxygenase activity initially gave a white band instead of a brown band (as obtained with soybean lipoxygenase). The same phenomenon was observed by Yoon and Klein (1979), but by exposing the gel to short-wavelength ultraviolet light for 1 min, the white band turned blue, which was stable and could be visualized much more easily.

Figure 3 shows the results on disk gel electrophoresis with isoenzyme-1 eluted from the chromatofocusing polybuffer exchanger chromatography. Only one protein band was present and corresponded to the only band shown by the activity stain. Smaller amounts of enzyme added to the gel gave a sharper band; however, it was more difficult to detect lipoxygenase activity.

Molecular Weight Determination. The molecular weight of isolated isoenzyme-1 was estimated by the methods of SDS polyacrylamide disk gel electrophoresis and by exclusion gel chromatography on Sepharose 6B-100 (data not shown). Only one band was observed on SDS polyacrylamide disk gels stained for protein. The band had the same R_f as that of soybean lipoxygenase-1, with a molecular weight of approximately 100 000. In exclusion gel chromatography on a Sepharose 6B-100 column previously calibrated with proteins of known molecular weights, isoenzyme-1 eluted at the same point as the major peak of soybean lipoxygenase, indicating a molecular weight of 100 000 for the isoenzyme-1.

Figure 3. Polyacrylamide disk gel electrophoresis of isolated pea lipoxygenase isoenzyme-1. The sample size was 30 μg of protein/gel (7.5% acrylamide, 0.2% bisacrylamide). Gels were stained for protein (left) and activity (right). The current was 2.5 mA/gel for the first 20 min and then 3 mA/gel for 1 h at 4 °C.

Spaapen et al. (1977) reported that a lipoxygenase purified from dry English pea seeds (var. Bliss Abundance) had a molecular weight of 98 000. Reynolds (1982) reported that pea lipoxygenase-2 from dry pea seeds (var. Little Marvel), with an optimum pH at 7.0 and pI of 5.82, had a molecular weight of 94 000. However, the type-1 lipoxygenase with pI of 4.05 from dry English pea seeds (var. Little Marvel) was reported to have a M_r 64 000 by gel filtration and 65 000 by SDS–polyacrylamide gel electrophoresis (Reynolds and Klein, 1982a). Haydar et al. (1975) found that their purified lipoxygenase from dry English pea seeds (var. Homesteader) had a molecular weight of 106 000. However, Eriksson and Svensson (1970) and Arens et al. (1973) have reported molecular weights of 72 000 and 78 000, respectively, for lipoxygenase from dry English pea seeds (var. Whitman Wonder and Mignon, respectively).

Similarity of the molecular weights obtained by gel filtration and by SDS–polyacrylamide disk gel electrophoresis suggests that isoenzyme-1 is a single polypeptide chain, in agreement with previous reports for lipoxygenase from dry English pea seeds (Haydar et al., 1975; Walker, 1975; Reynolds, 1982).

Amino Acid Analyses. The amino acid composition of isoenzyme-1 is shown in Table II. Values for cystine/cysteine and methionine were obtained by performic acid oxidation of the sample prior to hydrolysis and quantitation as cysteic acid and methionine sulfone on the analyzer. The enzyme contained eight half-cystine residues and no methionine/100 000 g. It is quite unusual for a protein to have no methionine, but repetitive analyses showed no methionine. The lipoxygenase purified by Eriksson and Svensson (1970) and Arens et al. (1973) from dry pea seeds contained seven methionine residues, based

Table II. Amino Acid Composition of English Pea Lipoxygenases

amino acid	no. of amino acids/M_r 100 000[b]	no. of amino acids/M_r 71 871[c]	no. of amino acids/M_r 78 000[d]
Asp	105	131 (182)[e]	78 (100)[e]
Thr[a]	47	35 (49)	37 (47)
Ser[a]	59	40 (56)	48 (61)
Glu	100	58 (81)	71 (91)
Pro	58	34 (47)	48 (61)
Gly	62	38 (53)	49 (63)
Ala	50	29 (40)	34 (44)
Val	53	28 (39)	34 (44)
Ile	53	31 (43)	34 (44)
Leu	101	61 (85)	72 (92)
Tyr[a]	43	26 (36)	29 (37)
Phe	38	23 (32)	29 (37)
His	30	25 (35)	23 (29)
Lys	61	36 (50)	49 (63)
Arg	48	28 (39)	34 (44)
Met[f]	0	7 (10)	7 (9)
$^1/_2$ Cys[f]	8	7 (10)	4 (5)
Trp	13	?	11 (14)

[a] Not corrected for partial degradation during acid hydrolysis. The hydrolysis time was 24 h. [b] Present work for isoenzyme-1 from immature peas (var. Dark Skinned Perfection). [c] From Eriksson and Svensson (1970) for dry pea seeds (var. Whitham Wonder). [d] From Arens et al. (1973) for dry peak seeds (var. Mignon). [e] Number of amino acids based on M_r 100 000 for comparative purposes. [f] Determined after performic acid treatment.

on their determined molecular weight. The enzyme was rich in aspartic and glutamic acids, leucine, glycine, lysine, serine, and proline in agreement with the results of Eriksson and Svensson (1970; except proline) and of Arens et al. (1973). There is general agreement between the present results and those of Arens et al. (1973) when compared at M_r 100 000 (Table II; except for methionine and half-cystine), but in less agreement with those of Eriksson and Svensson (1970).

Optimum pH of Isoenzyme-1. Effect of pH on activity was studied by using the buffer system of 0.05 M piperazine–0.05 M glycylglycine solution adjusted to the desired pH with 0.1 M HCl or 0.1 M NaOH, and containing 0.2 M NaCl at 25 °C. Assay solutions contained 5 mL of 0.01 M linoleic acid plus 20 mL of the buffer at various pHs; the solutions at pH 6.5 and lower were slightly turbid. The solution at pH 5.5 was more turbid than solutions at lower or higher pH values. The turbidity made the spectrophotometric assay somewhat difficult. Under these conditions, the optimum pH for isoenzyme-1 was pH 6.5–7.0 (Figure 4), quite different from soybean lipoxygenase-1 with an optimum pH of 9.0. Almost all plant lipoxygenases, except soybean lipoxygenase-1, have optimum activities at pH 6.5–7.0 (Siddiqi and Tappel, 1956; Eriksson and Svensson, 1970; Gardner and Weisleder, 1970; Galliard and Phillips, 1971; Arens et al., 1973; Weber et al., 1973; Klein, 1976; Allen et al., 1977; Reynolds and Klein, 1982a). Reynolds and Klein (1982a) have shown that dry English pea seeds (var. Little Marvel) contain a small amount of a type-1 lipoxygenase with an optimum pH of 9–10.

pH Stability of Isoenzyme-1. The effect of pH on enzyme stability was studied by using the same buffer system as described above for determining the optimum pH. The pH was adjusted from 3.60 to 9.88 by using 0.1 N HCl or 0.1 N NaOH. The results showed that isoenzyme-1 was quite stable at 25 °C in the pH range from 4.5 to 8.0 (Figure 5).

Heat Stability of Isoenzyme-1. The effect of temperature on enzyme stability was investigated by measuring the residual activity after holding isoenzyme-1 at pH 7.0 for various times at temperatures ranging from 35 to

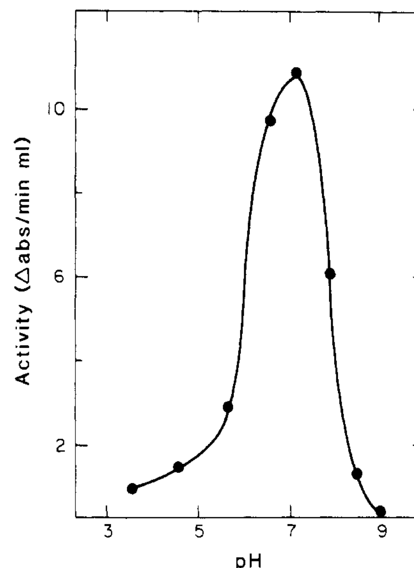

Figure 4. Optimum pH for isolated pea lipoxygenase isoenzyme-1. Activity was assayed with 2.5 mM linoleic acid containing 0.078% Tween-20 at various pHs at 25 °C. The buffer system was 0.05 M piperazine–0.05 M glycylglycine, containing 0.2 M NaCl, and the pH was adjusted by using 0.1 N HCl or 0.1 N NaOH. The enzyme concentration was 16 nM.

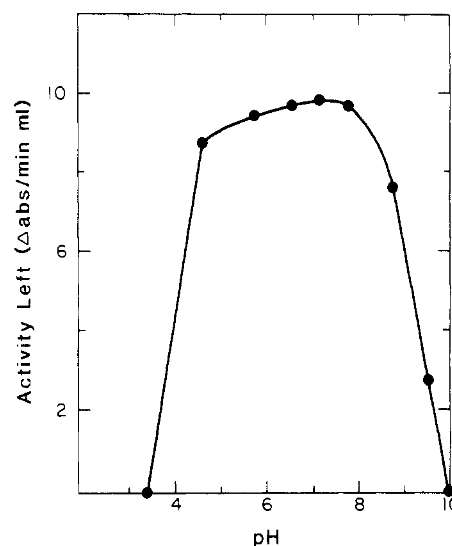

Figure 5. pH stability curve for isolated pea lipoxygenase isoenzyme-1. The enzyme was incubated at various pHs for 1 h at 25 °C followed by assay of remaining activity at pH 7.0 and 25 °C using 2.5 mM linoleic acid containing 0.078% Tween-20 in 0.2 M sodium phosphate buffer. The buffer system used was same as described in Figure 4.

80 °C. Samples were removed at various time intervals, cooled in ice water (0 °C) immediately, and then assayed. The results are shown in Figure 6. The enzyme was completely inactivated after heating 30 s at 80 °C and after 4 min at 65 °C. About 60% activity remained after heating for 25 min at 50 °C.

Activity–Temperature Profile for Isoenzyme-1. Figure 7 shows an Arrhenius plot of log activity vs. $1/T$ (K). The activation energy (E_a) for denaturation was 24.6 kcal/mol as calculated from the left-side slope. The E_a for the conversion of substrate to product at substrate concentration about equal to K_m gave a value of 4.53 kcal/mol. In general, activation energies for transformation of reactants to products in enzyme-catalyzed reactions are in the range of 6–15 kcal/mol while activation energies for denaturation of enzymes are in the range of 50–150

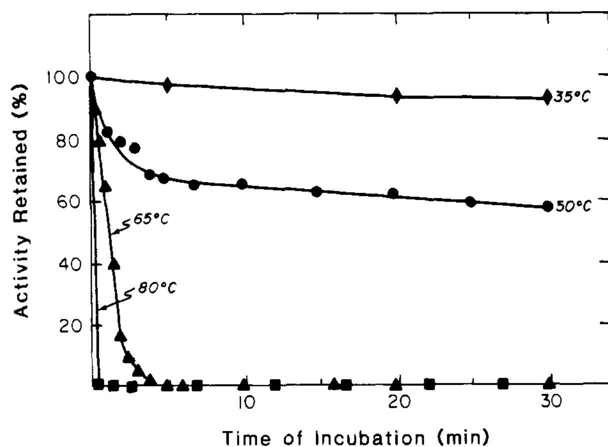

Figure 6. Heat stability of isolated pea lipoxygenase isoenzyme-1. The enzyme was incubated at various temperatures for up to 30 min followed by rapid cooling in an ice bath and assay at 25 °C for remaining activity. Enzyme solutions were incubated at 4 μM in 0.2 M sodium phosphate buffer, pH 7.0. Activity left was determined as described in Figure 5.

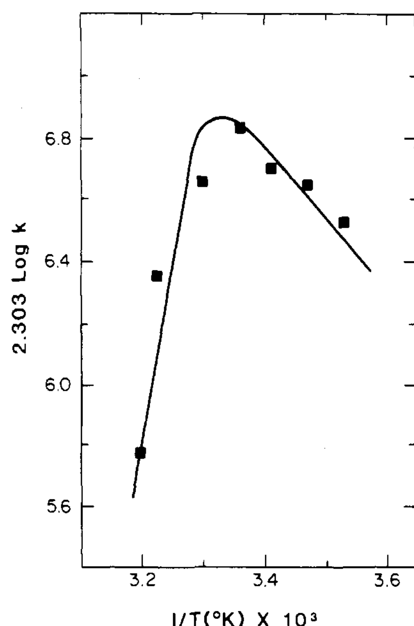

Figure 7. Activity-temperature profile of isolated pea lipoxygenase isoenzyme-1 plotted according to the Arrhenius equation. The activity was assayed with 2.5 mM linoleic acid containing 0.078% Tween-20 in 0.2 M sodium phosphate buffer, pH 7.0, at various temperatures. The enzyme concentration used was 1.6 nM.

kcal/mol (Whitaker, 1972). E_a for conversion of substrate to product is similar to that reported for other lipoxygenases. Tappel et al. (1953) reported a value of 4.3 kcal/mol for the activation energy of the oxidation of linoleic acid catalyzed by soybean lipoxygenase. Irvine and Anderson (1953) reported a value of 6.5 kcal/mol for wheat lipoxygenase. Al-Obaidy and Siddiqi (1981) reported a value of 7.1 kcal/mol for broad bean lipoxygenase.

Kinetics of Hydroperoxidation of Linoleic Acid by Isoenzyme-1. The Lineweaver–Burk method (1934) was used to analyze the effect of substrate concentration on initial velocities of hydroperoxidation of linoleic acid catalyzed by isoenzyme-1 in the presence of O_2. Figure 8A shows the double-reciprocal plots of initial velocities vs. linoleic acid concentrations ranging from 0.44 to 2.5 mM in Tween-20 at five different fixed O_2 concentrations ranging from 0.066 to 0.264 mM. The results indicate that

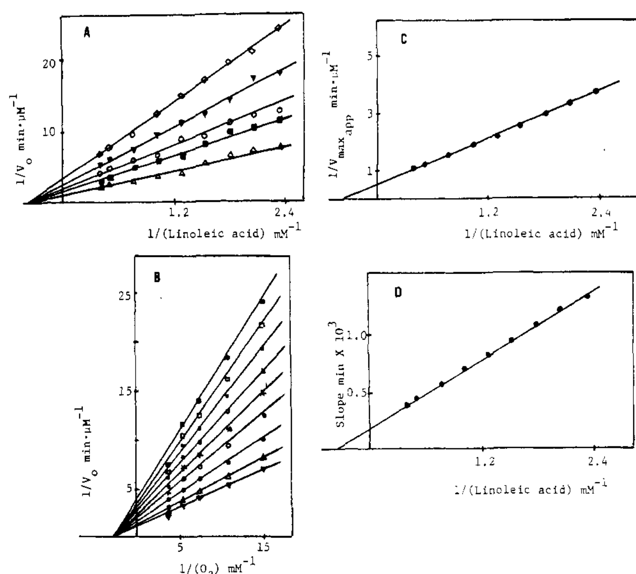

Figure 8. (A) Effect of concentration of linoleic acid and of oxygen on initial velocities of isolated pea lipoxygenase isoenzyme-1. The stock substrate solution was made by mixing 157.2 μL of linoleic acid, 157.2 μL of Tween-20, and 8 mL of deionized distilled water, clarified by adding 1.0 mL of 1 M NaOH and made to 50 mL with deionized distilled water. For each linoleic acid/Tween-20 assay solution, the stock solution was then diluted with 0.2 M phosphate buffer, pH 7.0. The specially designed cuvettes containing linoleic acid/Tween-20 solution were flushed with a gas mixture of known O_2 concentration; isolated pea lipoxygenase isoenzyme-1 (0.9 nM) was added by using a syringe, and the absorbance at 234 nm was measured with a Cary-219 spectrophotometer at 24 °C. The O_2 concentration in each reaction was determined with an O_2 electrode: ◇, 0.066 mM O_2; ▼, 0.0925 mM O_2; ○, 0.132 mM O_2; ■, 0.185 mM O_2; △, 0.264 mM O_2. (B) Effect of concentration of linoleic acid and oxygen on initial velocities of isolated pea lipoxygenase isoenzyme-1. Data replotted from Figure 8A: ■, 0.43 mM; □, 0.48 mM; ▪, 0.55 mM; □, 0.65 mM; ★, 0.77 mM; ○, 0.96 mM; ●, 1.29 mM; △, 1.93 mM; ▼, 2.42 mM linoleic acid. (C) Replot of the y intercepts of the data of Figure 8B vs. 1/[linoleic acid]. (D) Replot of the slopes of the data of Figure 8B vs. 1/[linoleic acid].

the mechanism of this reaction is sequential.

The data of Figure 8A were replotted by the double-reciprocal method as reciprocal initial velocities vs. reciprocal O_2 concentrations (Figure 8B). The K_m for O_2 was 0.36 mM. The intercept replot (data from Figure 8B) shown in Figure 8C gave V_{max} 2 μM/min and K_m 2.67 mM for linoleic acid. The slope replot (data of Figure 8B) shown in Figure 8D gave K_m 0.36 mM for O_2, and the k_{cat} calculated for these reactions was 2.2×10^3 min^{-1}.

For comparison, commercially purchased Sigma soybean lipoxygenase-1 was studied under the same conditions as used for isoenzyme-1. The double-reciprocal plots of initial velocities vs. linoleic acid concentrations are shown in Figure 9A. The data indicate a sequential mechanism. The K_m for linoleic acid was 1.63 mM. The data from Figure 9A were replotted as reciprocal initial velocities vs. reciprocal O_2 concentration (Figure 9B). This replot gave K_m 0.18 mM for O_2. The intercept replot (data from Figure 9B) shown in Figure 9C gave V_{max} 16 μM/min and K_m 1.63 mM for linoleic acid. The slope replot (data from Figure 9B) shown in Figure 9D gave K_m 0.18 mM for O_2, and the k_{cat} calculated was 2.3×10^3 min^{-1}, which is essentially identical with that of the isoenzyme-1 (above).

Egmond et al. (1976) studied the steady-state kinetics of the peroxidation of linoleic acid catalyzed by soybean lipoxygenase. The linoleic acid concentrations ranged from 5.4 to 140 μM with no detergents added, and the O_2 con-

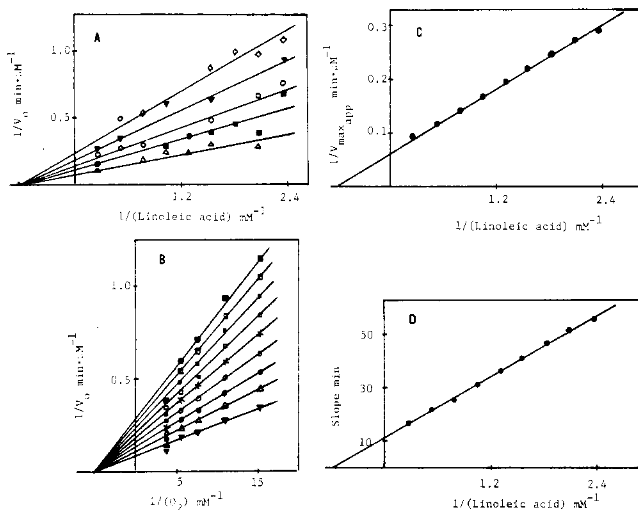

Figure 9. (A) Effect of concentration of linoleic acid and oxygen on (initial velocities of soybean lipoxygenase)$^{-1}$. The assay conditions were as described in Figure 8A. The concentrations of O_2 and symbols are as in Figure 8A. (B) Effect of concentrations of linoleic acid and oxygen on initial velocities of soybean lipoxygenase-1 catalyzed reaction. Data were replotted from Figure 9A. The concentrations of linoleic acid and symbols are as in Figure 8B. (C) Replot of the y intercepts of the data of Figure 9B vs. 1/[linoleic acid]. (D) Replot of the slopes of the data of Figure 9B vs. 1/[linoleic acid].

centrations ranged from 5 to 100 μM. On the basis of the results of double-reciprocal plots of the rates as a function of O_2 concentrations at several fixed levels of linoleic acid and added 13-hydroperoxide, the K_m was 20 μM for linoleic acid and 3.5 μM for O_2. In the presence of the product of the reaction, 13-L_s-hydroperoxylinoleic acid, the apparent substrate inhibition only affected the apparent affinity for O_2 and was of a hyperbolic type. Tappel et al. (1952) reported the apparent K_m for O_2 at two different concentrations of linoleic acid to be 30 and 290 μM at 0.36 and 7.2 mM linoleic acid, respectively, for soybean lipoxygenase. Haydar and Hadziyev (1973) reported the K_m for linoleate to be 2.3 mM for lipoxygenases partially purified from dry English pea seeds (var. Homesteader, Laxton and Strategem). Reynolds and Klein (1982b) reported the K_m values for isoenzymes LP-2 and LP-3 purified from dry English pea seeds (var. Little Marvel) to be 2.66 mM (by spectrophotometry; 3.08 mM by polarography) and 2.22 mM (by spectrophotometry; 2.99 mM by polarography), respectively.

Therefore, the K_m values for linoleic acid and O_2 by isoenzyme-1 reported here for immature English peas are in agreement with the K_m values for linoleic acid and O_2 of lipoxygenase from dry English peas determined by Haydar and Hadziyev (1973) and Reynolds and Klein (1982b).

Substrate and Product Specificities. The substrate specificity of isoenzyme-1 of English pea and soybean lipoxygenase-1 was studied under identical conditions. The results are summarized in Table III. The results clearly show that soybean lipoxygenase-1 preferred the substrates with a free carboxyl group and had almost no activity on substrates with a modified carboxyl group while isoenzyme-1 of English pea still had a considerable amount of activity on substrates with a modified carboxyl group.

Reynolds and Klein (1982b) reported that type-2 lipoxygenase isoenzyme LP-2 purified from dry English pea seeds (var. Little Marvel) had similar activities on free linoleic and linolenic acids, methyl linoleate, and trilinolein, while type-2 lipoxygenase isoenzyme LP-3 and type-1 isoenzyme LP-1 (Reynolds and Klein, 1982a) preferred the free fatty acids.

Table III. Substrate Specificity of Isolated Isoenzyme-1 from English Pea and Soybean Lipoxygenase-1[a]

		pea isoenzyme-1		soybean lipoxygenase-1	
substrate[b]	detergent or solvent	act., mM min^{-1}	%[c]	act., mM min^{-1}	%[c]
linoleic acid	Tween-20	0.197	188	0.467	45.8
linoleic acid	ethanol	0.105	100	1.02	100
linoleic acid	ethanol + 0.05% BSA	0.048	46	0.277	27.2
linoleyl alcohol	ethanol	0.073	70	0.0095	0.93
linoleyl acetate	ethanol	0.030	29	0.0047	0.46
methyl linoleate	Tween-20	0.054	51	0.0026	0.25
methyl linoleate	ethanol	0.036	34	0.0066	0.65
ethyl linoleate	ethanol	0.019	18	0.0036	0.35
propyl linoleate	ethanol	0.023	22	0.0098	0.96
trilinolein	ethanol	0.081	77	0.013	1.3

[a] 0.2 M phosphate buffer, pH 7.0, at 24 °C. [b] Substrate concentration was 0.2 mM in all cases. [c] Relative to linoleic acid in ethanol as 100%.

The product specificity of linoleate hydroperoxidation catalyzed by isolated isoenzyme-1 of immature English peas and soybean lipoxygenase-1 was studied by using radioactive [1-^{14}C]linoleic acid. The results gave 87.7% 13-LOOH and 12.3% 9-LOOH, respectively, for soybean lipoxygenase-1 at pH 9.0, while isoenzyme-1 of pea produced 50.3% of 13-LOOH and 49.7% of 9-LOOH at pH 7.0. Hamberg (1971) and Roza and Francke (1973) reported that dry English pea seed lipoxygenase-catalyzed linoleate hydroperoxidation gave a ratio of 13-LOOH to 9-LOOH of 50:50. Dolev et al. (1967) and Leu (1974) reported that the ratio of 13-LOOH to 9-LOOH for soybean lipoxygenase-1 was 90:10.

It has been reported that pH is important in determining the ratio of product isomers formed by oxidation of linoleic acid by the same enzyme. Roza and Francke (1973) reported that oxidation of linoleic acid by soybean lipoxygenase-1 at pH 9 gave predominantly the 13-isomer, while at pH 7 the same enzyme gave almost equal amounts of the 9- and 13-isomers. Galliard and Phillips (1971) reported that oxidation of linoleic acid by soybean lipoxygenase at pH 5.5 resulted in a ratio of 54:46 of 9- to 13-isomers. However, Christopher and Axelrod (1971) suggested that the enzyme preparations used by the above workers may have contained more than one isoenzyme and that the pH effects observed were due to the isoenzymes being more active at different pHs. Soybeans contain a type-1 lipoxygenase with an optimum pH of 9.0 and type-2 lipoxygenases with an optimum pH at 6.5. Veldink et al. (1968) reported that, at pH 7.4, oxidation of linoleic acid by soybean lipoxygenase-1 resulted in a ratio of 1:10 of 9- to 13-isomers.

These studies on substrate and product specificity indicate that the structure of the active site must be different for the immature pea isoenzyme-1 and soybean lipoxygenase-1. There appears to be no recognition site in pea isoenzyme-1 for the carboxyl group, unlike the soybean lipoxygenase-1 active site.

DISCUSSION IN SUMMARY

We have isolated the major lipoxygenase isoenzyme (isoenzyme-1) of immature English peas (var. Dark Skinned Perfection), using English peas at the stage of maturity selected by the frozen food industry for processing. Pre-

vious attempts (Siddiqi and Tappel, 1956; Wagenknecht and Lee, 1956; Klein, 1976) to isolate lipoxygenase from the immature English pea had not been very successful due to instability of the enzyme. The definitive published work on lipoxygenase of English peas has started with the ripe dry pea seeds. Therefore, it is essential to determine the similarities among the lipoxygenases at the different stages of maturity.

The isoenzyme isolated here, designated isoenzyme-1, is the major one in immature English green peas and appears to be similar, if not identical, with the major isoenzyme in ripe dry English pea seeds. The chromatographic behavior on DEAE–cellulose (or DEAE–Sephadex) is similar (Eriksson and Svensson, 1970; Yoon and Klein, 1979), as are the optimum pH near 7.0 (Eriksson and Svensson, 1970; Arens et al., 1973; Yoon and Klein, 1979; Reynolds and Klein, 1982a), the p*I* of 5.8 (Eriksson and Svensson, 1970; Reynolds, 1982), and substrate specificity (Reynolds and Klein, 1982b). The molecular weight of 100 000 of the isoenzyme-1 from immature English peas is similar to that reported for the major isoenzyme from ripe dry English pea seeds by some workers (Haydar et al., 1975; Spaapen et al., 1977; Reynolds, 1982) but quite different from the molecular weights reported by Eriksson and Svensson (1970) and Arens et al. (1973) of 72 000 and 78 000, respectively. The explanation for this difference is not known. The amino acid composition of isoenzyme-1 is similar to the dry pea seed enzyme isolated by Arens et al. (1973) except for methionine and half-cystine and differs somewhat from the values reported by Eriksson and Svensson (1970).

A major difference between lipoxygenases isolated from the immature and ripe dry English pea seeds is in terms of stability. The major isoenzyme is relatively easy to isolate in high purity (sp act. 287 200 units/mg of protein; Klein, 1976) from the ripe dry seed, while it has been reported to be very unstable from the immature pea (17 100 units/mg of protein; Klein, 1976). While we lost some activity on purification of the isoenzyme-1, the recovery of activity is no worse than in many enzyme purifications requiring multiple steps. Apparently, we were able to minimize the factor(s) accounting for this instability.

For the first time, one can feel reasonably comfortable that the data obtained for the major lipoxygenase isoenzyme of ripe, dry English pea seeds have relevance to lipoxygenase in the immature pea used in frozen food processing.

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