ISOLATION, PURIFICATION AND CHRACTERIZATION OF LIPOXYGENASE ISOZYMES FROM CANOLA (*Brassica napus* cv, Westar) SEED

By

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ABSTRACT

Lipoxygenase was extracted from Canola seeds *(Brassica napus* cv, Westar) and partially purified by precipitated with ammonium sulfate at 20-50% of saturation. The optimum pH for the enzyme activity was 7.5 and its K_m value was 2.0×10^{-4} M. The activity of the enzyme extract was considerably greater on linoleic acid than on its ester or on linolenic acid. The effect of cyanide on the enzyme activity was also investigated.

Further purification of the enzyme extract was performed by successive chromatography on ion-exchange and gel filtration, using FPLC system. Four lipoxygenase isozymes (I, IIA, IIB and III) were separated. The homogeneity of each isozyme was demonstrated by the presence of a single protein band on SDS-PAGE gel electophoresis. The molecular weights of isozymes I, IIA, IIB and III were, respectively, 72 000, 106 000, 78 000 and 62 000. The optimum pH for lipoxygenase activity was 6.5 for isozyme I and 6.0 for isozymes IIA, IIB and III. The apparent K_m values for isozymes I, IIA, IIB and III were, respectively, 5.5×10^{-4} M, 3.4×10^{-4} M, 4.0×10^{-4} M and 3.8×10^{-4} M. The maximum absorbance of linoleate hydroperoxides end products was 234 nm for isozymes I, IIB and III and 238 nm for isozyme IIA. The substrate specificity showed that isozyme I displayed a preferential activity for mono- and dilinoleate whereas isozyme IIA showed a preferential activity for dilinoleate followed by a tendency for mono- and trilinoleate. However, isozymes I and IIA did not show any activity toward free linoleic acid. Isozyme IIB showed a common activity towards free linoleate as well as mono-, di- and trilinoleate. Isozyme III showed a preferential activity towards free linoleate. The effect of cyanide on lipoxygenase activity showed that isozymes I and IIA were inhibited completely. However, the addition of 3 mM and 10 mM cyanide to isozymes IIB and III, respectively, increased the activity before being decreased at higher concentration of cyanide.

Resumé

La lipoxygénase du grain de "Canola" (*Brassica napus* cv, Westar) a été extraite et partiellement purifiée par une précipitation au sulfate d'ammonium à 20-50% de saturation. Le pH optimal de l'activité enzymatique est de 7,5 et la valeur du K_m est de 2×1^{-4} M. L'activité enzymatique a été plus grande pour l'acide linoléique libre que celle obtenue pour ses esters.

L'enzyme partiellement purifié a été d'avantage purifier par chromatographie sur échangeur d'ion et sur gel de filtration en utilisant le système "Fast Protein Liquid Chromatography, FPLC". Quatre isozymes de la lipoxygénase (I, IIA, IIB et III) ont été séparées. L'homogénéité de chaque isozyme a été confirmée par la présence d'une seule fraction sur électrophorèse en gel (SDS-PAGE). Le poids moléculaire des isozymes I, IIA, IIB et III sont respectivement de 72000, 106000, 78000 et de 62000. Le pH optimal pour l'activité lipoxygénasique a été de 6,5 pour l'isozyme I et de 6,0 pour les isozymes IIA, IIB et III. Les valeurs de K_m pour les isozymes I, IIA, IIB et III sont respectivement de $5,5 \times 10^{-4}$ M, 3.4×10^{-4} M, $4,0 \times 10^{-4}$ M et de $3,8 \times 10^{-4}$ M. L'absorbance maximale des hydroperoxides de l'acide linoléique a été de 234 nm pour les isozymes I, IIB et III et de 238 nm pour l'isozyme IIA. La spécificité du substrat a montré que l'isozyme I a une activité préférentielle pour le monolinoléate et le dilinoléate tandis que l'isozyme IIA a montré une activité préférentielle pour le monolinoléate, le dilinoléate et le trilinoléate. Cependant, les isozymes I et IIA n'ont montré aucune activité vis-à-vis l'acide linoléique libre. L'isozyme IIB a montré une activité commune pour l'acide linoléique libre ainsi que pour ces esters. Par contre, l'isozyme III a montré une activité préférentielle seulement pour l'acide linoléique libre. L'effet du cyanure sur l'activité lipoxygénasique a montré que les isozymes I et IIA sont complètement inhibés. Les résultats obtenus montrent aussi que la présence de 3 mM et de 10 mM du cyanure va respectivement augmenter l'activité lipoxygénasique des isozymes IIB et III, cependant, une concentration plus grande a pour effet d'inhiber cette activité.

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Dedicated to:

My parents, family and particularly

my aunt and my fiancée.

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CHAPTER I

INTRODUCTION

Lipoxygenase (E.C. 1.13.11.12) is a dioxygenase which catalyzes, as its primary activity, the hydroperoxidation by molecular oxygen of linoleic acid and other polyunsaturated lipids containing a *cis*, *cis*-1,4-pentadiene moiety (Axelrod *et al.*, 1981). Lipoxygenase activity has been demonstrated in a wide range of plant tissues (Pinsky *et al.*, 1971). The enzyme has been well studied in soybean, peanut and pea (Christopher *et al.*, 1972; Sanders *et al.*, 1975; Yoon and Klein, 1979). Soybean lipoxygenase has received the most attention because soybean seeds are the richest known source of lipoxygenase (Axelrod *et al.*, 1981).

Isozymes of lipoxygenase have been isolated from soybean (Christopher *et al.*, 1970, 1972), pea (Anstis and Friend, 1974; Eriksson and Svensson, 1970) and alfalfa (Ben-Aziz *et al.*, 1971). Crude and purified lipoxygenase from various sources differ in their pH optima, substrate specificity, number of isozymes and other characteristic (Christopher *et al.*, 1972; Dillard *et al.*, 1961; Haydar and Haziyev 1973; Siddiqi and Tapple 1957;). A number of isozymes also differ in physicochemical properties as well as mode of action. The fragility of the enzyme, the complexity of its isolation and purification procedures and its mode of action are some of the factors which contribute to the variation in the results reported in the literature (Galliard and Chan, 1980).

Interest in lipoxygenase is related to its action on endogenous unsaturated fatty acids which results in the production of hydroperoxides. These hydroperoxides contribute to the formation of many aldehydes and alcohols which are responsible either for the desirable, fresh-vegetable flavors associated with normal metabolism of growing plants or undesirable off-flavors which occur after harvesting and during storage or processing of many foods (Sessa, 1979).

The lipoxygenase activity in beans is characterized by a wide range of optimum pH varying from 6.0 to 9.0 and becoming inactive at pH greater than 11.0 (Galliard and Phillips, 1971). Tapple (1961) reported that soybean lipoxygenase activity was affected significantly by modification of the pH of the medium. The substrate selectivity for soybean lipoxygenase (Verhue and Franke, 1972), French bean lipoxygenase (Kermasha and Metche, 1986) and asparagus lipoxygenase (Ganthavorn and Powers, 1989) has been studied. Cyanide ion is a well known inhibitor of heme-proteins such as peroxidase and catalase (Siddiqi and Tapple, 1956), however, the addition of cyanide at certain concentration to the partially purified lipoxygenase resulted in an apparent increase in lipoxygenase activity (Kermasha and Metche, 1986). This apparent increase in activity could be explained by the fact that the partially purified extract may contain other competing enzymes such as catalase and peroxidases, which are also known to be inhibited by cyanide (Siddiqi and Tapple, 1956). Cyanide has been used to distinguish between heme-protein and lipoxygenase-catalyzed oxidation (Ganthavorn and Powers, 1989).

Canola, an improved cultivar of rapeseed (*Brassica sp.*) is one of the principal commercial oilseeds in the world. Canada has become the largest exporter of this crop in the world over the past sixteen years. Franke and Freshe (1954) reported the absence of lipoxygenase activity in rapeseed while Bronisz *et al.* (1958) found a small degree of lipoxygenase activity in rapeseed extracts. St. Angelo *et al.* (1979) did not confirm the presence of lipoxygenase in rapeseed. Appleqvist (1972) suggested that the enzyme was probably present in rapeseed but could not be detected because of the presence of an inhibitor. Christophersen and Bremer (1972) reported that erucic acid inhibited fatty acid oxidation in mitochondria from rat heart and liver. Since erucic acid is the most abundant fatty acid (40% of total fatty acid) in rapeseed, it was suggested that this acid could have an inhibitory effect on lipoxygenase activity. In addition, St. Angelo *et al.*

lipoxygenase enzymes from soybean and peanut. Phenolic compounds are also known to form several types of bonds with proteins, e.g., hydrogen, covalent, ionic and hydrophobic (Loomis, 1974). The mechanism of inhibition of lipoxygenase and the type of binding to lipoxygenase by erucic acid and by phenolic compounds have not yet been determined (Sessa, 1979). The fact that Canola seeds contain less than 5 % erucic acid (of total fatty acid) while rapeseed contains approximately 40 % erucic acid, suggests that the profile of lipoxygenase activity of the two types of *Brassica* could be entirely different.

The lipoxygenase of Canola has not been studied either in terms of isolation or in terms of its physicochemical characteristics. The aim of this work was to prepare an active lipoxygenase extract, to purify this extract, to separate and characterize its isozymes.

CHAPTER II

LITERATURE REVIEW

2.1. Definition of Lipoxygenase

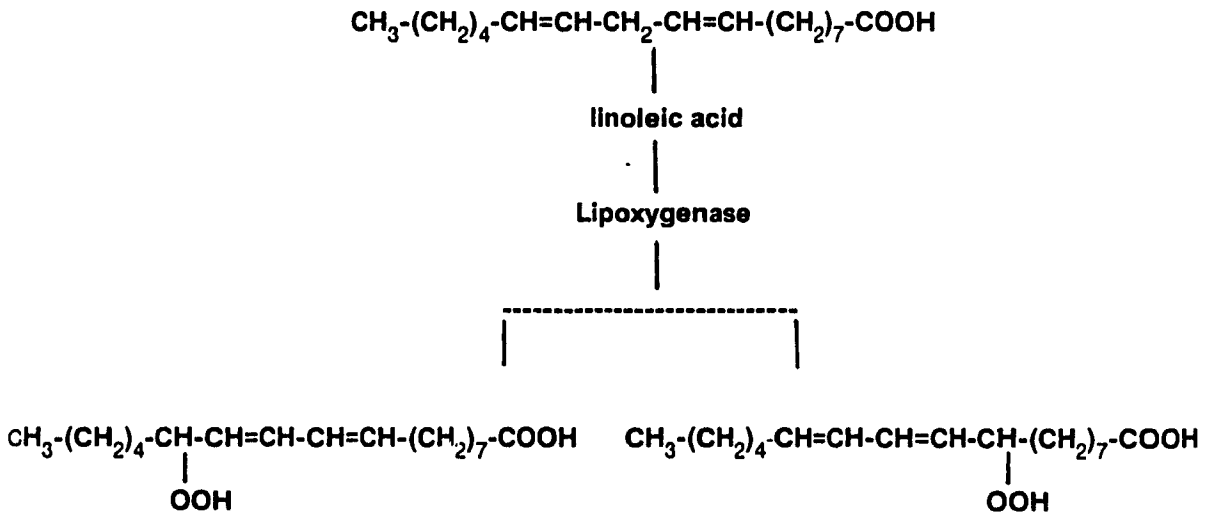
Lipoxygenase (linoleate: oxygen oxidoreductase; EC 1.13.1. 13) is considered to be a key enzyme in the oxidative degradation of lipids. It is a dioxygenase which catalyzes as an initial reaction, the hydroperoxidation of linoleic acid and other polyunsaturated fatty acids (PUFA) and their esters containing a *cis*, *cis*-1,4-pentadiene moiety (Axelrod *et al.*, 1981).

2.2. Mechansim of Enzymatic Action

The enzymatic oxidation of endogenous linoleic acid substrate in plants results in the formation of optically active derivatives of 9-D- and -13-L-hydroperoxydienes. A proposed mechanism for lipoxygenase action (Galliard, 1975) is given in Figure 1. The formation of hydroperoxide, mediated by lipoxygenase activity, probably begins at the site of activated methylene (-CH_2-) and then oxidative reactions proceed by a free radical mechanism. Although the mechanisms for lipoxygenase-induced oxidation of fatty acids are not identical to autoxidative mechanisms, hydroperoxides can ultimately be formed (Zapsalis and Beck, 1985). The heterogeneity and the source of the enzyme are some of the factors which contribute to the variation in the proportion of 9-*D* and 13-*L*-hydroperoxy derivatives of linoleic acid (Kermasha and Metche, 1986).

The resulting *cis*, *trans* conjugated diene hydroperoxides resulting from lipoxygenase activity, contribute to the formation of many aldehydes and alcohols which are responsible either for the desirable, fresh-vegetable flavors associated with normal metabolism of the growing plant or undesirable off-flavors that occur after harvesting and

Fig 1. Mechansim of action of lipoxygenase



13-hydroperoxyoctadeca-9,11-dienoic acid

9-hydroperoxyoctadeca-10,12-dienoic acid

during storage or processing (Sessa, 1979). However, the mechanism of lipoxygenase for further bio-degradation of hydroperoxides of linoleic acid to ketols and carbonyl compounds does not appear consistent from a biological system to another. The understanding of the "oxidation mechanism" could have considerable implications in the final quality of oilseeds and edible oil products.

Lipoxygenase is not even the only catalyst capable of forming carbonyl compounds from polyunsaturated fatty acids (Gardner, 1979). There are some other hemoproteins of higher plants, such as peroxidase, catalase, and cytochrome *c*, that are powerful lipid peroxidizers (Eriksson and Svensson, 1970). However, lipoxygenase enzyme is the biological catalyst considered to be the principal cause of peroxidation of polyunsaturated fatty acids in many raw plants products (Ste-Angelo *et al.*, 1977). The hemoproteins but not lipoxygenase are inhibited by cyanide, therefore one can distinguish between hemoprotein and lipoxygenase catalysts (Tapple, 1953).

Lipoxygenase is particularly relevant to food of plants origin because its oxidizes the essential PUFA such as linoleic acid, linolenic acid and arachidonic acid producing hydroperoxides that can decompose to form derivatives with characteristic tastes and odors both desirable and undesirable (Galliard and Chan, 1980; Whitaker, 1972).

2.3. Sources of Lipoxygenase

Lipoxygenase was discovered in 1928 when Bohn and Haas reported the presence of a carotene-destroying enzyme "*carotene oxidase*" in soybean. Andre and Hou (1932) reported that soybean contains an enzyme which oxidizes PUFA and subsequently named "Lipoxidase". Summer and Summer (1940) success in showing that partially purified lipoxygenase has a much lower activity in oxidizing carotene than the crude enzyme, have raised the possibility that there is a second enzyme which oxidizes

carotene (Kies *et al.*, 1969). Since the lipoxygenase enzyme has been known for many years, it was one of the first enzymes obtained in a crystalline form (Theorell *et al.*, 1947). The occurrence of the enzyme was originally thought to be confined to seeds of leguminous plants and some cereals (Tapple, 1963), but it is now known to be more widely distributed among higher plants (Pinsky *et al.*, 1971; Grosch, 1972). Lipoxygenase is present in a wide variety of plants especially legumes and is believed to be one of the main oxidative catalysts in vegetables (Reynolds and Klein, 1982). Particularly rich sources of lipoxygenase are soybean (Axelrod *et al.*, 1981; Wagenstecht and Lee, 1956), potato tubers (Galliard and Phillips., 1971) and eggplant (Grossman *et al.*, 1972a). Lipoxygenase also occurs in leaves (Holden, 1970; Grossman *et al.*, 1972b; Anstis and Friend, 1972b) and green alage (Zimmerman and Vick 1973). Plant sources of lipoxygenase are given in Table 1. The occurrence of lipoxygenase in animal tissues was first questionable, since lipid peroxidation was believed to be exclusively due to ubiquitous heme compounds (Boyd and Adams, 1955; Tapple, 1963). The unfolding prostaglandin research in the mid 1970s led to the discovery of lipoxygenase activity in platelets (Veldink *et al.*, 1977) and leukocytes (Borgeat *et al.*, 1976). Recently, lipoxygenase has been found in chicken muscle (Grossman *et al.*, 1988). Hawkins and Brash (1989) studied the lipoxygenase metabolism of PUFA in oocytes of the frog *Xenopus laevis*. Tsukuda and Amano (1968) found that lipoxygenase is responsible for the lipid oxidation in fish. Furthermore, the presence of lipoxygenase in fish tissues was found to initiate the oxidation of PUFA to produce acyl hydroperoxides (Hsieh and Kinsella, 1989).

2.4. Off-Flavor Production

In soybeans, where the lipid content is very high, the production of undesirable volatiles from unsaturated fatty acid by lipoxygenase is well known (Grosch and

Table 1. Plant sources of lipoxygenase.

Alfalfa	Peanut
Apple	Peppares
Asparagus	Potaties
Barley	Pumkin
Caulifower	Rapeseed
Eggplant	Red beans
Flaxseed	Rice
Green beans	Snap beans
Lentil	Soybean seed
Lima beans	Squash
Lupin	Sunflower seed
Maize	Tomatoes
Mustard	Watermelon
Navy bean	Wheat
Peas	Winged bean

Laskawy, 1975; Wolf, 1975). The development of rancid off-flavors in unblanched vegetables during frozen storage was associated with an increase in hydroperoxides and carbonyl compounds which were thought to be the result of lipoxygenase action on lipids in vegetables (Siddiqi and Tapple, 1956; Wagenknecht and Lee, 1958). Development of off-flavors can be controlled by inactivation of lipoxygenase with heat, acid, alcohol or antioxidants (Sessa, 1979). Wagenkencht and Lee (1956) showed that the endogenous lipase acting on unblanched frozen peas released fatty acids which served as substrates for lipoxygenase. Eriksson (1967) substantiated the role of lipoxygenase in the development of off-odor compounds and showed that the enzymes was in the inner part of the cotyledon. Wagenkencht and Lee (1959) attributed off-flavors in unbalanced sweet corn to lipoxygenase.

The high level of polyunsaturated fatty acids (78%) for lupin and (80%) for peanuts is susceptible to oxidative rancidity and creates a storage problem (Ollas and Valle, 1988; St-Angelo *et al.*, 1979). The causes of lipid oxidation in vegetable oils are numerous and involve such factors as enzymes, particularly lipoxygenase which act on PUFA that could be decomposed into acids, ketones, aldehydes and alcohols or other substance during storage or processing. These degradative products can then react with amino acids and proteins, impairing flavor and lowering the nutritive value of the lupin themselves or the food products into which they are incorporated (Gardner 1979; St.Angelo *et al.* 1979). Protein and amino acids are only one class of biochemicals in foods that is susceptible to the damaging effect of lipid hydroperoxides, (Gardner, 1979). The level of linoleic acid (> 50%) in faba bean is particularly susceptible to oxidation and is responsible for the development of off-flavors; the rancidity of processed faba beans was observed soon after grinding, representing a major storage problem (Eskin and Henderson, 1974).

A number of researchers reported the poor quality of oil as well as the reduced oil yield from field and storage damaged soybeans (Evans *et al.*, 1974; List *et al.*, 1977; Robertson *et al.*, 1973). This may be due to higher lipoxygenase activity in damaged beans. This finding was supported by those of Rice *et al.*, (1981) who found that crude oils extracted from enzyme inactivated whole soybeans were of higher quality than oils from beans with normal enzymatic activity.

Lipoxygenase is an important factor in the generation of the flavor compounds from lipids when soybean are processed under high moisture condition such as in the preparation of soy milk by the traditional process. Less certain is the significance of lipoxygenase action when soybean are processed under low moisture conditions, as in the commercial extraction of oil. However, the potency of the flavor compounds that can arise by the decomposition of hydroperoxides generated by lipoxygenase suggested that very little oxidation may be needed to give rise to objectionable levels of flavor (Wolf, 1975). Much of the literature on lipoxygenase from soybean has been concerned with the fact that the enzyme is known to be involved in the formation of beany off-flavors in processed products (Sessa, 1979). The hydroperoxides may also be involved in loss of the characteristic color of some products as a result of the co-oxidation of pigments (Klein *et al.*, 1984).

2.5. Application of Lipoxygenase to Food and Agricultural Science

The presence of lipoxygenase in plants may affect their storage and processing since lipoxygenase promotes the peroxidation of the PUFA (which are nutritionally essential) and can affect taste, odor, and color. Moreover, lipoxygenase may influence ripening and abscission. In fact, lipoxygenase has been used to modify fatty acids to bleach wheat flour and to improve the rheological properties of wheat dough (Axelrod, 1974).

2.6. Enzyme Extraction

Endogeneous lipids have been extracted from peas with acetone and diethyl ether (-30^{O}C) and the crude enzyme was then extracted using Tris-HCl buffer (Eriksson and Svensson, 1970). Ganthavorn and Powers (1989) reported that lipids were extracted from asparagus using an acetone wash (-30^{O}C) and the enzyme was then extracted from the defatted powder using potassium phosphate buffer at 4^{O}C. Kermasha and Metche (1986) reported that lipoxygenase from *Phaseolus vulgaris*, defatted by successive washing with acetone and diethyl ether, resulted in a two-fold increase in enzyme activity when compared to analogous washes using hexane. This effect may be related to the polarity of the solvents, since both diethyl ether and acetone have a higher polarity index (2.8 and 5.1, respectively) than that of hexane (0.1). The effect of organic solvents on lipoxygenase activity was investigated by Allen (1968). Galpin and Allen (1977) have reported that the effect of *n*-alcohols and *n*-carboxylic acid on lipoxygenase activity resulted from the physicochemical interactions between these organic solutions and the substrate rather than their direct effects on the enzyme configuration. However, some workers reported that lipoxygenase activity was determined in the crude extract without using solvents. The lipoxygenase activity of crude extracts of fourteen legumes was reported by Chang and McCurdy (1985). Pinsky *et al.* (1971) reported also that crude lipoxygenase was extracted from various plants using phosphate buffer at 4^{O}C.

2.7. Enzyme Purification

Lipoxygenase enzyme was purified by conventional techniques of protein isolation, such as ammonium sulfate fractionations, gel filtration and ion-exchange chromatography. These techniques are reliable for the isolation and the separation of the enzyme from other proteins and are based on its physical and chemical properties (Eskin *et al.*, 1977).

2.7.1. Ammonium Sulfate Fractionation

One of the earliest successes achieved in enzyme purification was using salt for protein separation (Northrop *et al.*, 1948) and a great number of enzymes have been subsequently isolated in this way. The most frequently used salt is the ammonium sulfate and its principal advantage is having a solubility (*ca.* 700 g/liter) which permit the precipitation of a wide scale of protein molecules.

The first soybean lipoxygenase extract was partially purified by Theorell *et al.* (1947) using ammonium sulfate fractionation and the final product was crystallized. Since then, many partially purified preparations of lipoxygenase have been reported in the literature (Change *et al.*, 1971; Stevens *et al.*, 1970; Tapple, 1961; Verhue and Franke, 1972; Yamamoto *et al.*, 1970). Lipoxygenase active proteins isolated from various sources were precipitated with ammonium sulfate using different levels of saturation. Olias and Valle (1988) reported that lipoxygenase was partially purified from lupin (38.4-fold) by ammonium sulfate at 25% saturation. Chen and Whitaker (1986) as well as Eriksson and Svensson (1970) reported that lipoxygenase, respectively from immature English pea and pea were precipitated by ammonium sulfate at 25-50% of saturation. Ammonium sulfate at 20 to 50% of saturation was also used to precipitate the partially purified lipoxygenase of *Phaseolus vulgaris* (Kermasha and Metche, 1986) and of pea seed (Haydar and Hadziyev, 1973). Kermasha and Metche (1986) reported that 98% of French bean lipoxygenase activity was located in the fraction precipitated by ammonium sulfate at 20-50% of saturation. Klein (1976) indicated that lipoxygenase was isolated from dried split pea seeds, frozen raw peas and snap beans by ammonium sulfate fractionation (30-50%). This range was also used to isolate and purify lipoxygenase from dried winged beans seeds (Van Den *et al.*, 1982a). Arens *et al.* (1973) have used 30-60% saturation to isolate lipoxygenase from peas. Partially purified lipoxygenase from asparagus (Ganthavorn and Powers, 1989) and from potato tubers (Galliard and Phillips,

1971) were obtained by precipitation with ammonium sulfate at 0-50% saturation. Reynolds and Klein (1982) indicated that lipoxygenase-1 was purified from pea seeds by 25-60% whereas commercial soybean lipoxygenase (Stevenes *et al.*, 1970) and green bean lipoxygenase (Adams *et al.*, 1989) were purified at 40-60% of saturation. Sanders *et al.* (1975) reported that lipoxygenase was isolated and partially purified from peanut seeds with ammonium sulfate at 40-65% of saturation.

2.7.2. Liquid Chromatography

Liquid chromatography is an instrumental technique which separates complex mixtures into their component parts and provides qualitative and quantitative information on each component. Ion-exchange chromatography is a technique which separates biomolecules according to their difference in charges. Proteins bind to the ion exchanger by electrostatic forces mainly between the proteins surface charges and the dense clusters of charged groups on the exchangers (Scopes, 1987). There are several types of resins, the most commonly used being DEAE-cellulose, which were introduced by Peterson and Sober (1956). Pea lipoxygenase has been purified using DEAE-cellulose (Arens *et al.*; Chen and Whitaker, 1986; Eriksson and Svensson, 1970 and Haydar and Hadziyev 1973). However, there are other ion-exchange resins that have also been used in the purification of lipoxygenase such as DEAE-sephacel (Ida *et al.*, 1983) and DEAE-Sephadex (Reynolds and Klein, 1982; Sanders *et al.*, 1975; Sekiya *et al.*, 1977; Van Den *et al.*, 1982a). Wallace and Wheeler (1979) indicated that two isozymes of wheat germ lipoxygenase were separated and purified using DEAE-cellulose. These authors demonstrated that peroxidase was separated from lipoxygenase-1 at pH 7.8, since peroxidase did not bind to DEAE-cellulose. Peroxidase and lipoxygenase-2 were also separated by a combined pH and concentration gradient through CM-cellulose.

Ganthavorn and Powers (1989) purified lipoxygenase from asparagus by carboxymethylcellulose (CMC) chromatography.

Gel filtration chromatography is a simple and reliable technique used for the separation of biomolecules according to their size. It is suitable for the purification and size estimation of a wide range of biomolecules, with Sephadex being the most commonly used. Lipoxygenase from pea, peanut, commercial soybean and winged beans lipoxygenase was purified by Sephadex G-150, (Eriksson and Svensson, 1970; Klein, 1976; Sanders *et al.*, 1975; Stevens *et al.*, 1970; Van Den *et al.*, 1982a). Lipoxygenase from pea seeds was purified by Sephadex G-200 (Yoon and Klein, 1979). Lipoxygenase from lupin was purified by Phenyl Sepharose CL-4B was used by (Olias and Valle, 1988). Combination of Sepharose CL-2B and differential centrifugation was used to separated and purify lipoxygenase from apple (Kim and Grosch ,1979). Grossman *et al.* (1972) isolated and characterized eggplant lipoxygenase on Ecteola cellulose into two active fractions, with one of the two fractions being more active than the other. The more active fraction was further purified on Sephadex G-200 and was 20 times more active compared to the crude extract. Andrawis *et al.* (1982) reported that isozyme lipoxygenase-2 from soybean was isolated by affinity chromatography.

2.8. Lipoxygenase Assay

The primary products of lipoxygenase enzymatic reaction are *cis,trans*-conjugated diene hydroperoxides. There are several methods that have been developed for determining the rate of the enzymatic reaction. The most commonly used methods are the manometric technique, polarographic measurement of oxygen uptake by the substrate and the spectrophotometric assay based on the increase in extinction brought about by the formation of the conjugated diene.

The manometric procedure has a wide applicability, since it may be employed with crude preparations as well as with purified extracts. However, it has some disadvantages including complicated applications which require an extended assay period (up to 30 min) in which initial consumption cannot be measured. This time period can allow secondary reactions to become more significant. In addition, the shaking which is essential in this method tends to inactivate the purified enzyme (Eskin *et al.*, 1977).

The polarographic or oxygen electrode method measures the reaction continually from the start and the whole reaction may be completed in a few mins. This method is especially suitable for kinetic studies both with the pure enzyme and the crude extract (Mitsuda *et al.*, 1967; Grossman *et al.*, 1969).

Spectrophotometric determination of lipoxygenase activity based on chromogenic reaction first was reported by Holman (1947). However direct spectrophotometric determination of enzyme activity based on the absorbance at 234 nm of the conjugated diene hydroperoxide formed was also reported (Theorell *et al.*, 1947; Surrey, 1964 and Ben-Aziz *et al.*, 1970). The advantage of this method that it is simple and rapid, however, it is difficult to determine lipoxygenase activity of crude extracts because of the turbidity of the preparation (Eskin *et al.*, 1977).

Linoleic acid is probably the best substrate used for the determination of lipoxygenase activity (Eskin, 1977), however its pH-dependent solubility in the aqueous reaction medium causes many difficulties. The poor water solubility of linoleic acid can be improved by the addition of emulsifier such as Tween-20 (polyoxyethelensorbitan monolaurate) to the reaction mixture (Ben Aziz *et al.*, 1970; Grossman *et al.*, 1969; Surrey 1964).

Demonstration of lipoxygenase activity could also be performed by an electrophoretic preparation. This method is based on the reaction of the hydroperoxide product with acidic potassium iodide to oxidize the iodide to the elementary form. The oxidized iodide then reacts with starch at the active enzymatic fractions which is resulting by a chromogenic reaction (Guss *et al.*, 1968). Another chromogenic reaction based on the reaction between the linoleic acid hydroperoxide and a mixture of ferrous ammonium sulfate and ammonium thiocyanate was also reported (Grossman *et al.*, 1971)

2.9. Physico-chemical Properties

The lipoxygenase activity in beans is characterized by a wide range of optimum pH varying from 6.0 to 9.0 and become inactive at pH greater than 11.0 (Galliard and Phillips, 1971). Tapple (1961) reported that soybean lipoxygenase activity was affected significantly by modification of the pH of the medium.

Whitaker (1972) indicated that cyanide inhibited many enzymes which contain essential iron or copper; cyanide are poisons that combine with particular enzymes (usually those containing copper or iron) associated with cellular oxidation in the living organisms. This reaction occurs because the cyanide can form very stable complexes with the heavy metals. Pistorius and Axelrod (194) investigated that all isozymes of soybean lipoxygenase contains one atom of iron per molecule. The nature of the binding of iron to the enzyme is not known (Eskin *et al.*, 1977). Cyanide has been used to distinguish between-protein and lipoxygenase-catalyzed (Ganthavorn and Powers, 1989). Siddiqi and Tapple (1957) reported that cyanide ion inhibited the partially purified lipoxygenase from peanut at concentrations greater than 1 mM. These results have also been confirmed by St. Angelo and Ory (1972) and Kermasha and Metche (1986). Galliard and Phillips (1971) noted that the partially purified lipoxygenase from potato tubers was not inhibited by cyanide (1 mM). Grossman *et al.* (1972) reported that

cyanide has no inhibitory effect on eggplant lipoxygenase. It was also reported that cyanide caused an increase in pH, which decreased the activity of the acid isozyme (St. Angelo and Koch, 1977).

Gibian and Galaway (1976) reported that the oxygenation of linoleat and arachidonate catalyzed by soybean lipoxygenase was subjected to competitive product inhibition. For normal conditions there was an additional inhibition due to product that causes the reaction to cease before completion. This process was reversible upon addition of further substrate and was proposed to be a chemical (reversible) change of the enyzme.

The soybean lipoxygenase was first crystallized by Theorell *et al.* (1947). These workers reported that the enzyme globulin has a molecular weight of 100,000 and possess no components other than amino acids. These results suggested that there was no evidence for the presence of a prosthetic group or metal in the enzyme structure. However, Stevens *et al.* (1970) reported that soybean lipoxygenase can be dissociated into two subunits of equal size and have a molecular weight of 100,000 to 108,000. Olias and Valle (1988) indicated that the molecular weight of the partially purified lipoxygenase from lupin was 92,000, from avocado was 74,000 (Marcus *et al.*, 1988), from potato tubers was 100,000 (Galliard and Phillips, 1971) and from tomato fruit was 87,000 (Bonnet and Crouzet, 1977). Germinating sunflower seed lipoxygenase separated on SDS-PAGE and gel filtration sephadex G-200 has a molecular weight of 250,000 (Leoni *et al.*, 1985).

2.10. Isozymes

Kies (1947) who questioned the identity of lipoxygenase and carotene oxidase, thought that lipoxygenase activity of soybeans was caused by more than one enzyme.

Kies *et al.* (1969) raised a question about whether soybean lipoxygenase or some other enzymes in the extracts cause ß-carotene oxidation. That question was answered with the discovery of isozymes of soybean lipoxygenase (Guss *et al.*, 1967; Christopher *et al.*, 1970 and 1972; Verhue and Francke, 1972).

Koch *et al.* (1958) had claimed earlier, on the basis of measurement of peroxide-forming ability, that soybean contained two lipoxygenases, one whose preference was for the triglyceride and the other was for free acids. Christopher *et al.* (1970) purified a second isozyme from soybean which differed from the first in pH optimum and ester-acid preference. The original enzyme (Theorell *et al.*, 1947) was designated as lipoxygenase-1 and the new isozyme as lipoxygenase-2. Yamamto *et al.* (1970) also isolated an enzyme that designated as "b" and which may be identical to lipoxygenase-2. The guaiacol-linoleic hydroperoxide peroxidase of Grosch *et al.* (1972) may also be identical to lipoxygenase-2. Christopher *et al.* (1972) isolated a third isozyme, lipoxygenase-3, from soybean. It was distinct from the other two isozymes in its elution profile from DEAE-Sephadex column, isolectric point, pH-profile and effect of calcium ion on its' activity. The more rapidly eluting component is lipoxygenase-3 and the slower component is lipoxygenase-4 (Whitaker, 1973). Hurt and Axelrod (1977) indicated the presence of four isozymes L-1, L-2, L-3 and L-4 in soybean. Calcium stimulates the activity of lipoxygenase-2 but inhibits the activity of lipoxygenase-3 under similar conditions (Christopher *et al.*, 1972). The pH activity profiles of each isozyme was significantly different under similar assay condition; L-1 and L-2 have optimum activity at pH 9.5 and 6.5, respectively, whereas L-3 ranged from pH 4.5-9.0 (Christopher *et al.*, 1972). The role of the calcium in activitating soybean L-2 was due to its interaction with linoleate substrate, to make it more accessible to the enzyme rather than to a direct interaction with the enzyme (Zimmerman and Snyder, 1974). Increasing the activity of L-

2 by calcium occurred because this ion changed the physical state of the substrate by eliminating the floating micelle fraction (Reynolds and Klein, 1982).

Four active fractions have been isolated from soybean lipoxygenase and designated as L-1, L-2, L-3a, L-3b, respectively (Axelrod *et al.*, 1981). These authors demonstrated that L-3a and L-3b were very similar in their properties and suggested that the two isozyme were identical and referred to as a single isozyme (L-3). These three isozymes (L-1, L-2 and L-3) were exhibiting different kinetic behavior (Axelrod *et al.*, 1981; Galliard and Chan, 1981). L-2 is considered as a neutral plant enzyme because it has optimum pH at 6.5-7.0. Therefore, each isozyme might be expected to take part in the development of grassy, beany and green flavors through a different mode of action (Matoba *et al.*, (1985).

The presence of lipoxygenase isozymes is not limited to soybean. There are many reports in the literature describing the existence of isozymes of lipoxygenase from different plant sources (Anstis and Friend, 1974; Hale *et al.*, 1969 and Wallace and Wheeler, 1979). Anstis and Friend (1974) separated four isozymes from pea seed and five active fractions for pea seedlings. However, Arens *et al.* (1974) reported that only two to three isozymes were separated from fresh green beas, whereas Hale *et al.* (1969) showed the presence of one to two isozyme from green beas seeds. Two isozymes were isolated from faba beans (Beaux *et al.*, 1973; Eskin and Henderson, 1974), winged bean (Van Den *et al.* 1982) and wheat flour extract (Graveland, 1970; Wallace and Wheeler, 1979).

2.11. Characterization

Characterization of the enzymes through their enzymatic properties shows that the lipoxygenases comprise a large group of diverse enzymes (Axelrod, 1974).

2.11.1. Effect pH on Lipoxygenase Isozymes Activity

Almost all plant lipoxygenase, except soybean lipoxygenase-1, have optimum activities at pH 6.5-7.0 (Siddiqi and Tapple, 1956; Eriksson and Svensson, 1970; Galliard and Phillips, 1970; Arens *et al.*, 1977; Yoon and Klein, 1979; Reynolds and Klein 1982a; Galliard and Chan, 1980 and Chen and Whitaker, 1986).

Type-1 lipoxygenase purified from pea seeds was very unstable, especially at pH values below 6 (Reynolds and Klein, 1982). Ida *et al.* (1983) reported that the optimum pH for rice bran isozymes L-1, L-2 and L-3 were, respectively 4.5, 5.5 and 7.0. Galliard and Phillips (1970) also reported that it seems likely that two major types of lipoxygenase activity exist in plants, one active at acidic pH and one active at alkaline pH. Some plants presumably contain both types of enzyme, e.g. soybean whereas potato contain mainly one type.

2.11.2. Specificity

Substrate specificity for lipoxygenase may be straight chain fatty acids, esters, alcohols and hydroxyamates (Haining, 1974) and halides (Balin and Shearer, 1965) which contain *cis,cis*-1,4-pentadiene structure. The most common substrate used are essential fatty acids such as linoleic, linolenic and arachidonic acids (Hamberg and Samuelsson, 1965 and 1967). Linoleic acid is probably the best substrate for lipoxygenase activity (Axelrod, 1974; Eskin, 1977 and Takagi and Mijashita, 1987). Holman *et al.* (1969) studied the specificity of soybean lipoxygenase-1 using linoleic acid. Beare-Rogers *et al.* (1969) have used PUFA from marine oil as substrate and they indicated that only the 1,4-pentadiene structure was oxidized and which other structures in the molecule were not attacked. Several reports have investigated the positional specificity of lipoxygenase (Galliard and Phillips, 1971 and Zimmerman and Vick, 1970).

The ratio of 13-L to 9-D hydroperoxy derivatives formed from linoleic acid with potato lipoxygenase was almost 95% to 5% (Galliard and Phillips, 1971), for soybean lipoxygenase was 70% to 30% (Hambarg and Stamuelsson, 1965), for flax seed lipoxygenase was 40% to 10% (Zimmerman and Vick, 1970) and for dry immature English pea seed lipoxygenase was 50 % to 50% (Rosa and Francke, 1973), for immature English pea lipoxygenase was 87.7% to 12.3% (Chen and Whitaker, 1986). Specificity for the site of O_2 insertion into substrate during oxidation is affected by many factors including, source of lipoxygenase, type of isozymes, substrate and experimental conditions such as pH, temperature and O_2 tension (Eskin *et al.*, 1977).

2.11.3. Electrophoresis

Electrophoresis is essentially based on the migration of molecules by the charge and the size (Andrews, 1986). The migration of charged molecules in solution is due to the influence of an externally applied electric field. In polyacrylamide gel electrophoresis, the molecules being analyzed migrate in an electric field that is imposed on the aqueous solution trapped in the matrix of the gel. Guss *et al.* (1967) reported, on the basis of a chromogenic reaction disc electrophoresis that there are three to four lipoxygenases existing in soybean extract. Klein (1976) reported, based on electrophortic analysis that lipoxygenase from dried split pea seeds separated into one major and two trace bands in the raw. However, three to four pea seed lipoxygenase bands were observed by Haydar and Hadziyev (1973). Guss *et al.* (1968) indicated that the electrophoresis of soybeans extracts showed the presence of three to four isozyme bands whereas the electrophoresis separation of wheat-milling fractions resulted by the appearance of two to four bands. Hale *et al.* (1969) reported that the gel patterns indicated the presence of two to three bands in the pea seeds and four bands in the wheat extract.

Canola, an improved cultivar of rapeseed (*Brassica sp.*), is one of the principal commercial oilseeds in the world. Franke and Freshe (1954) reported the absence of lipoxygenase activity in rapeseed while Bronisz *et al.* (1958) found a small degree of lipoxygenase activity in rapeseed extracts. Appleqvist (1972) has suggested that the lipoxygenase was probably present in rapeseed, but could not be detected because of the presence of an inhibitor. Christopher and Bremer (1972) reported that erucic acid inhibited fatty acid oxidation in mitochondria from rate heart and liver; since erucic acid is the most abundant fatty acid (40% of total fatty acid) in rapeseed. It was suggested that this acid could have an inhibitory effect on lipoxygenase activity. St. Angelo *et al.* (1979) reported that by examining rapeseed by the same procedures and techniques used for measuring lipoxygenase activity in soybean and peanut, they were unable to confirm the presence of lipoxygenase in rapeseed. Phenolic compounds are also known to form several types of bonds with proteins e.g., hydrogen covalent, ionic and hydrophobic (Loomis, 1974). The mechanism of inhibition of lipoxygenase and the type of binding to lipoxygenase by erucic acid and the phenolic compounds have not yet been determined (Sessa, 1979). The fact that Canola seeds contain less the 5% erucic acid (of total fatty acid) while rapeseed contains approximately 40% erucic acid, suggested that the lipoxygenase activity of two types of *Brassica* could be entirely different.

OBJECTIVE OF THIS STUDY

Canola seed (*Brassica sp.*), is one of the principal commercial oilseeds in the world and Canada has become the largest exporter of this crop in the world. Interest in lipoxygenase is related to its action on endogenous polyunsaturated fatty acids resulting in the production of hydroperoxides. These hydroperoxides contribute to the formation of many aldehydes and alcohols which are responsible either for the desirable, fresh-vegetable flavors associated with normal metabolism of growing plants or undesirable off-flavors which occur after harvesting and during storage. However, there are conflicting reports regarding the presence of this enzyme in the rapeseed. These reports have suggested that lipoxygenase was probably present in rapeseed but could not be detected. Lipoxygenase of Canola has not been studied either in terms of isolation or in terms of its physico-chemical characteristics.

The aim of this work is:

1. To demonstrate the presence of an active lipoxygenase extract in the Canola seed.
2. To investigate the extraction procedure of the active fraction from Canola seed .
3. To separate, purify and characterize lipoxygenase isozymes of Canola seed.
4. To develop for the first time, the methodology for purification of lipoxygenase by the use of Fast Protein Liquid Chromatography (FPLC) .

CHAPTER III

MATERIALS AND METHODS

Certified seeds of Canola (*Brascica napus* cv Westar), obtained from Agriculture Canada, Saskatoon, Saskatchewan, Canada were used in this study. The seeds were homogenized to a fine powder and defatted with organic solvents. The partially purified enzyme extracts were obtained from the defatted powder by centrifugation and precipitation with ammonium sulfate. The purification and separation of lipoxygenase isozymes were performed by ion-exchange and gel filtration chromatographies. The physicochemical and kinetic charateristices of enzyme fractions were performed.

3.1. Defatting of Canola Seed Powder Extract

Lipoxygenase was extracted from the ground Canola seed using a modification of the procedure described by Kermasha and Metche (1986). The seed (750 g) were frozen in a dry ice bath (-30°C), ground using a mortar and pestle and further reduced to a fine powder using a Vitris-23 homogenizer at high speed for 2-3 min with dry ice. The fine powder was defatted successively with acetone and diethyl ether (Anachemia Co.) at -30°C, in a dry ice bath. The defatting procedure was repeated five times. The Canola powder was transferred to a vacuum erlenmeyer flask at 4°C and subjected to a vacuum for 8 h to remove traces of solvents. Lipid extract was concentrated, by removing solvents, using rotary evaporator (Tokyo Rikakikai Co., Ltd model N) and transferred into small vials and flashed with nitrogen and kept at -80°C. The lipid extract was used as endogenous substrate for the determination of lipoxygenase activity.

3.2. Preparation of Crude Extract

The defatted powder was suspended in Tris-HCl buffer 0.1 M at pH 7.3 (1/3 W/V) containing 0.1% Trition X-100 (polyethyleneglycol) obtained from Schwarz Mann Co. All the procedures were performed at 4°C unless otherwise indicated. The suspension was subjected to mechanical stirring for 16 h and the mixture was centrifuged (Beckman J2-21) twice at 48,000xg for 30 minutes. The precipitate was discarded and the supernatant was subjected to ultracentrifugation (Beckman L5-65) for 3 h at 200,000xg. The precipitate was discarded and the supernatant was further subjected two times to ultracentrifugation and the clear supernatant was designated as fraction 1. A small portion of fraction 1 was subjected to protein, polyphenol and lipoxygenase activity determinations.

3.3. Ammonium Sulfate Fractionation

The supernatant (fraction 1) was treated with solid ammonium sulfate (Schwarz Mann Co.) at 20% of saturation and mechanically stirred for 5 min and allowed to stand for 30 min. The precipitate (fraction 2) was obtained by centrifugation at 48,000 xg for 1 h and the resulting supernatant was treated further with ammonium sulfate at 50% of saturation. The precipitate (fraction 3) was obtained by centrifugation at 48,000 xg for 1 h and the supernatant was further treated with ammonium sulfate at 100%. The precipitate (fraction 4) was obtained by centrifugation at 48,000xg for 1 h. Fractions 2, 3 and 4 were re-suspended in a minimal amount of Tris-HCl buffer solution (0.1 M, pH 7.3), dialyzed against the same buffer and any resulting insoluble materials are removed by further centrifugation (1 h, 48,000xg).

3.4. Protein Determination

The different fractions (1, 2, 3 and 4) obtained during the purification procedures by ammonium sulfate precipitate were assayed for protein content according to the method of Hartree (1972), a modification of Lowry *et al.* (1951), using crystalline bovine serum albumin solution (BSA) as a standard.

Protein samples were solubilized in sodium chloride (8.5%) and 1 ml of this protein solution was placed into each of a set test tubes. The compositions of solutions used are given in Table 2. By adding 0.9 ml of solution A to protein and samples, this set of tubes was set-up in a water-bath for 10 min at 50°C and let then cool to room temperature. Following the addition of 0.1 ml of solution B, the set of tubes was kept at room temperature for 10 min before adding solution C and rapidly mixed. The tubes were then incubated again in the water-bath for 10 min at 50°C, cooled to room temperature, after which 3 ml was taken from each tube and measured spectrophometrically at 650 nm, using a Beckman DU-65 Spectrophotometer.

A protein calibration curve was prepared by dissolving 12.5 mg BSA in NaCl solution (8.5%) in a volumetric flask of 50 ml giving a final concentration of 0.25 mg BSA/ml. A set of protein standards prepared to cover the range to be used of this assay is shown in Figure 2. The protein standard determination was carried out under the same condition as for the protein samples.

3.5. Kinetics Studies of Partially Purified Lipoxygenase

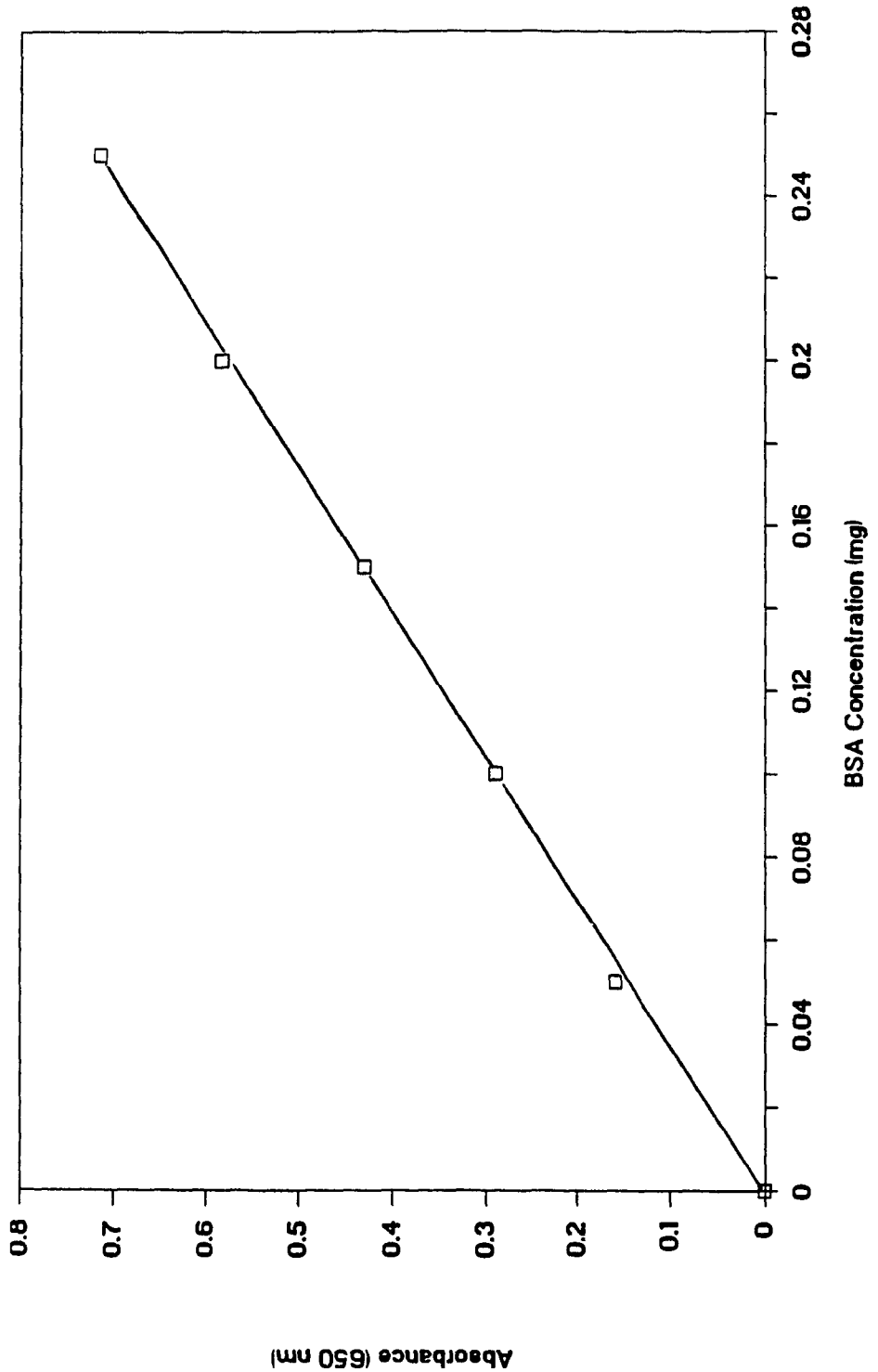
3.5.1. Substrate Preparation

Linoleic acid [*cis*-9,*cis*-12-octadectdienic acid], linolenic acid (*cis*,*cis*,*cis*-9,12,15-octadecatrienoic acid), monolinolein (1-mon-*cis*-*cis*-9,12-octadecadienoly) rac-

Table 2. Solutions required for protein determination.

Solution A:	Potassium sodium tartrate 2 g, and 100 g of sodium carbonate are dissolved in 500 ml 1N, diluted to 1 liter with deionized water.
Solution B:	Potassium sodium tartrate 2 g and 1 g cuperic sulfate are dissolved in 90 ml water and 10 ml NaOH was added.
Solution C:	Folin-Ciocalten reagent 1 volume diluted with 15 volume water, this solution was prepared dialy.

Fig 2. Standard curve for protein determination using BSA.


Absorbance (650 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.04
0.08
0.12
0.16
0.2
0.24
0.28
BSA Concentration (mg)

glycerol, dilinolein (1,3-*di*-cis,*cis*-9,12-octadecenyl) rac-glycerol and trilinolein (1,2,3-*tri*-*cis*-9,12,15-octadecadienoly) rac-glycerol purchased from Sigma Chemical Co. as well as Canola lipid extract were used as substrates for the measurement of lipoxygenase activity. The substrates were prepared in Tris-HCl buffer solution (0.1 M, pH 7.3) according to the method described previously (Kermasha and Metche, 1986). The suspension was neutralized by dropwise addition of 1% sodium carbonate solution. The buffer-substrate was homogenized by adding 0.5% (V/V) of Tween-20 (Polyoxyethenesorbitan monolaurate, Sigma Chemical Co.). The final concentration of substrate in the solution was 4×10^{-3} M.

3.5.2. Enzyme Assay

Lipoxygenase activity was determined spectrophotometrically (Beckman DU-65 spectrophotometer) according to the procedure described by Surrey (1964). Lipoxygenase activity was measured by the increase in absorbance at 234 nm due to the formation of linoleic acid hydroperoxide. The reaction medium was pre-incubated for 10 min at 25^{o}C in a 3 ml or a 0.8 ml spectrophotometer cell, the mixture of enzyme: substrate: buffer (1: 2: 27 V/V/V). A control assay, containing all the components except the enzyme preparation, was run in tandem with these trails.

For the substrate specificity, the reaction medium consisted of substrate (2 ml) and sufficient Tris-HCl buffer (0.1 M, pH 7.3) to adjust the final volume of the mixture to 30 ml. The reaction medium was maintained under a gentle air stream in 500 ml double layered flask at 25^{o}C. The reaction was initiated by adding pre-incubated enzyme (1 ml) with constant mechanical stirring for 3 h and the reaction medium was taken every 30 min. A control solution containing all the components minus the enzyme preparation was run in tandem with these trials. The formation of hydroperoxides was measured by an increase in the absorbance at 234 nm. The preparation of the

was expressed as the increase of A/mg protein/min, where A is equal to 0.001 absorbance at 234 nm (Ali Asbi *et al.*, 1989; Ganthavon and Powers, 1989; Kermasha and Metche, 1986; Klein, 1976; McCurdy *et al.*, 1983 and Shastry and Raghavendra, 1975).

3.5.3. Effect of Potassium Cyanide

Potassium cyanide (KCN) was used as inhibitor for lipoxygenase activity. Cyanide (Anachemia) was prepared to a final concentration of 50 mM with deionized water. The reaction mixtures containing enzyme extract (100 mg protein), substrate (20-200 µl) linoleic acid and various concentrations of (0 to 50 mM) KCN were used with constant volume and sufficient amount of Tris-HCl buffer (0.1 M, pH 7.3) in a 3 ml or 0.8 ml spectrophotometer cell. The hydroperoxides resulting from the formation of linoleic acid were measured spectrophotometrically at 234 nm.

3.6. Gas Chromatography

Fatty acids of Canola lipid extract were methylated and prepared for gas chromatography (GC) analysis according to the method describes by Badings and Jong (1983). The Canola lipid extract (100 mg) was weighed into a 8 ml Sovirel culture tube with a screw-cap. Heptane (6 ml) was used to dissolve the lipid and 0.06 ml of 2 M sodium methoxide solution was added and the contents of the capped tube were stirred vigorously for 1 min at room temperature using Vortex mixer. The sediment of sodium glycerolate was separated by centrifugation at 1000xg for 3 min. A sample from the clear supernatant (1 µl) was then subjected for GC analysis of the fatty acid methyl ester (FAMEs). GC analysis of the methylated fatty acids was carried out using a FID equipped Varian 3700 gas-liquid chromatograph fitted with a capillary DB 17 (0.53 mm x 30m; Chromatographic Specialities Inc.). The initial column temperature was 120°C, held for 2

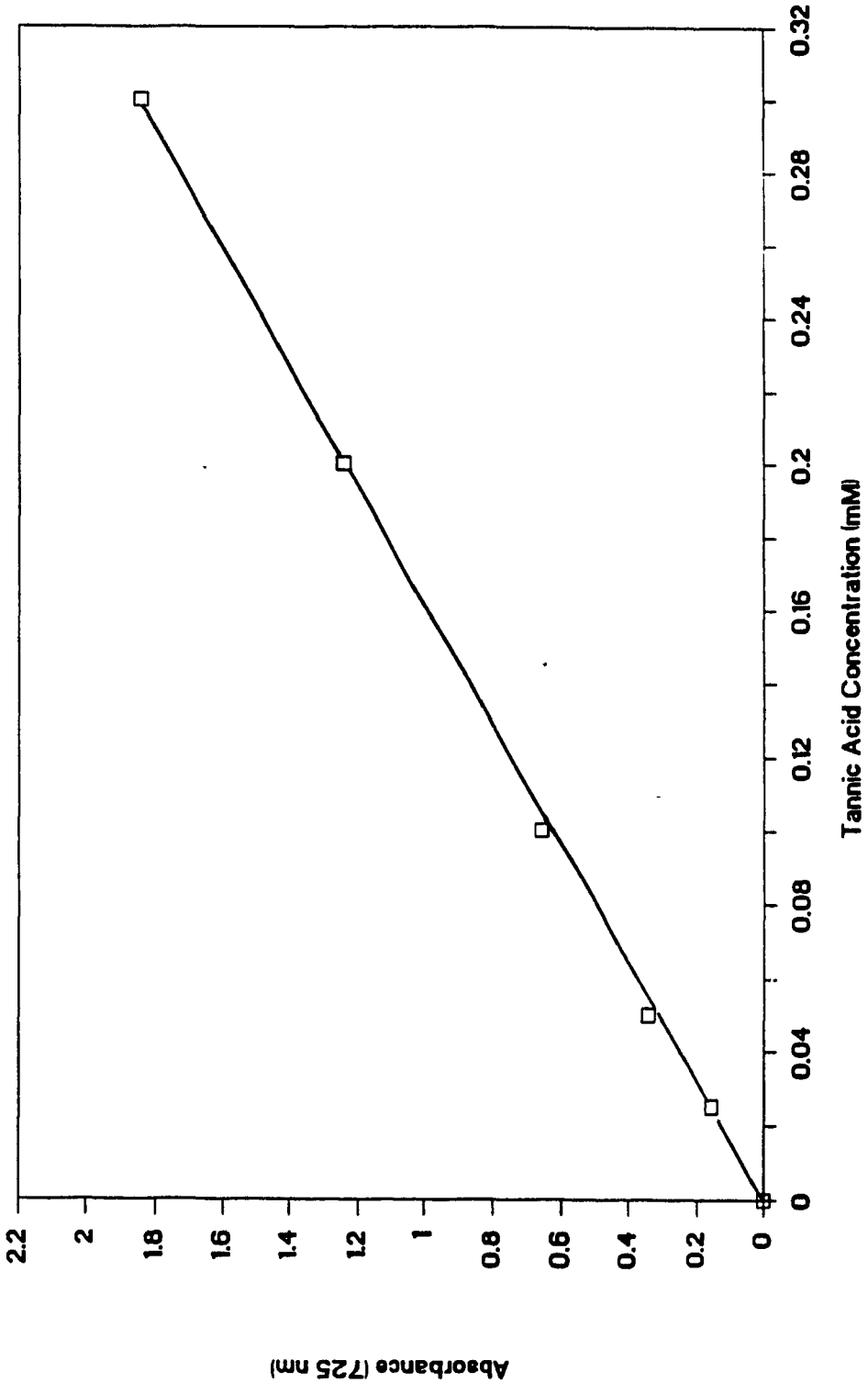
DB 17 (0.53 mm x 30m; Chromatographic Specialities Inc.). The initial column temperature was 120°C, held for 2 min before being increased at 3°C/min to a maximum of 260°C, a then held for 15 min. Flow rates were set at 20 and 300ml/min for hydrogen and air, respectively, while the carrier gas (N_2) flow rate was 0.6 ml/min. Injector and detector temperatures were 200°C and 250°C, respectively. Identification of individual fatty acids was based on retention times of purchased standards of rapeseed oil (Chromatographic Specialities Inc.). Quantitation was based on detector response relative to the concentration of each individual standard initially and also by use of an internal standard.

3.7. Determination of Phenolic Compounds

Phenolic compounds in the defatted Canola seed, the crude enzymatic extract (fraction 1) and the partially purified lipoxygenase (fraction 3) were extracted according to the procedure described by Otsuka *et al.* (1989). The powder samples (2 g) were washed successively with 10 ml hexane, acetone and a mixture of methanol: chloroform (2:1 V/V) and the suspension was stirred and filtered through glass wool. The washed powder was suspended in 5 ml deionized water and the phenolic compounds were extracted with 10 ml of n-butanol (Aldrich Chemical Co.).

The total phenolic compounds was measured spectrophotometrically at 725 nm according to a modification of the method of Goldstein and Swain (1963). The organic phase (0.5 ml) containing phenolic compounds was introduced in a test tube with 7 ml of deionized water and 0.5 ml of 2 N Folin Ciocalteau solution (Fisher Scientific) was added. The mixture was shaked vigorously and allowed to stand for 3 min before the addition of 1 ml sodium carbonate solution (35%). The mixture was then allowed to stand in the dark for 30 min and the absorbance was measured spectrophotometrically at 725 nm using Beckman DU-65 spectrophometer. A calibration curve was prepared

Fig 3. The standard curve for phenolic compounds determination using tannic acid.


Absorbance (725 nm)
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
0.04
0.08
0.12
0.16
0.2
0.24
0.28
0.32
Tannic Acid Concentration (mM)

using tannic acid (Fisher Scientific) with various concentrations ranging from 0.025-0.30 mg/ml (Fig. 3). A control solution, containing all the components minus the samples was run in tandem with these trials.

3.8. Electrophoresis on Partially Purified

3.8.1. Polyacrylamide Gel Electrophoresis

A polyacrylamide gel disc electrophoresis was performed, with some modification according to the method of Davis (1964). Duplicate gels for each sample were run, one was stained for protein with Coomassie Brilliant Blue R-250 and the other for a chromogenic reaction for detecting lipoxygenase activity. The gel system used containing 1% soluble starch was stacked at pH 8.9 and run at pH 8.3 according to the procedure described by Guss *et al.* (1967). Disc electrophoresis tubes (length = 90 mm; internal diameter = 5 mm) were used and one end of each tube was sealed with parafilm. The composition of polyacrylamide gel is given in Table 3. The separation gel solution (1.2 ml) was introduced in the disc electrophoresis tubes. Deionized water was placed on the top of the separation gel, to prevent dehydration of the gel during the polymerization to give a layer of approximately 2 mm. The gels were left to polymerize for one hour and the water layer was then removed. Spacer solution (0.2 ml) was placed on top of the separation gel. The tubes were then placed under a fluorescent lamp for twenty minutes to allow photo-polymerization of the spacer gel. Protein samples (50 µl) containing 30 to 60 µg protein were placed on top of the spacer gel. The tubes were placed again under the fluorescent lamp for twenty minutes for photo-polymerisation of sample gel.

The parafilm was carefully removed, and the tubes were inserted into the upper buffer reservoir. Buffer solution was added to the upper and the lower reservoirs, so it completely covers the electrodes.The composition of electrode buffer is given in Table

Table 3. Solution required for the preparation of polyacrylamide gel.

Solution Gel	Composition	Mixing Ratios of Solution (ml)
Separation Gel		
(1)	48.0 ml HCl 1N 36.6 g Tris[a] 0.23 ml TEMED[b] Diluted to 100ml water (pH 8.9)	1
(2)	30.0g Acylamide 0.8 g Bisacryalmide[c] Water to 100 ml	2
(3)	0.14 g Per[d] water to 100 ml	4
(4)	Soluble starch 1 g to 100 ml boiling Water	1
(5)	Deionized water	1
Spacer Gel		
(5)	48.0 ml HCl 1N 5.98 g Tis[a] 0.46 ml TEMED[b] Diluted to 100 ml Water (pH 6.7)	1
(6)	12.0 g Acryalmide 3.0 g Bisacryalmide Water to 100 ml	2
(7)	4.0 mg Riboflavin Water to 100 ml	1
(8)	40 g Sucrose Water to 100 ml	4

Tris[a]: Tris (hydroxymethyl) aminomethane.
TEMED[b]: N, N, N, N- tertramethylethylenediamine.
Bis[d]: N, N,-methylenelbisacryalmide.
Per[d]: Ammonium persulphate prepared immediately prior to use.

4. Three drops of bromophenol blue solution (0.25 %) were added to the upper reservoir. The current was adjusted to 3-6 mA per tube. Electrophoresis was stopped when the bromophenol blue indicator reached the bottom of the tubes (3-6.4 h). The gels were removed from each tube and introduced to individual test tubes (10 mm x 100 mm).

3.8.1.1. Protein Staining

For protein staining, the solutions used are given in Table 4. The gels were first fixed for 1 h with 11.4% trichloroacetic acid (TCA) prepared in 30% methanol containing 3.4% sulfosallicylic acid. The gels were then immersed for 3.5 h in 0.25% Coomassie Brilliant Blue R-250 prepared in 50% methanol containing 9.2% acetic acid. The gels were destained with 10% of TCA and kept in the destaining solution for several days with frequent changes of the destaining solution. The gels were then stored in 7% acetic acid solution.

3.8.1.2. Chromogenic Reaction of Enzyme Activity

When electrophoresis was completed, the gels were removed from the tubes and placed in 10x100 mm test tubes. The gels were incubated with linoleic acid as substrate for 30 minutes at 25°C. The working solution of enzyme reaction was prepared as described previously for the enzyme assay (Materials and Methods). At the end of the enzyme reaction, the gels were washed with deionized water and treated with 5% KI prepared in 15% acetic acid for 30 min and the appearance of the brown-to-blue bands indicated the presence of a lipoxygenase activity. The brown bands changed to blue color for easy visualization within 20 min. A control was run containing (a): all the compounds minus the enzyme extract (b): all the compounds minus the substrate.

Table 4. Solution prepared for electrophoresis.

Solution	Composition
Electrode buffer	6.0 g Tris, (28.8 g) Glycine, (pH 8.3, 5 mM) deionized water to one liter, then diluted ten times before use.
Indicator solution	Bromophenol Blue (0.25 %) deionized water to 100 ml
Fixative solution	11.4 g (TCA)[a], 3.4 g sulfosallicylic acid in 30% methanol to 100 ml.
Staining solution	0.25 g Coomassie Brilliant Blue R-250, 50 ml methanol, 9.2 ml acetic acid, deionized water to 100 ml.
Activity staining	5 g KI[b], 15 ml aectic acid, deionized water to 100 ml.
Destaining solution	TCA (10 g) deionized water to 100 ml.
Storage solution	Glacial acetic acid (7 ml), deionized water to 100 ml.

[a]TCA: Trichloroacetic Acid.

[b]KI: Potassium Iodide.

To prevent background staining due to autoxidation of the KI, the acetic acid solution is degased by the application of vacuum prior to the addition of KI. The saturated KI solution was prepared daily and was placed under nitrogen just prior to use.

3.8.2. SDS-PAGE

Electrophoresis was performed in the presence of sodium docdecyl sulfate (SDS) according to the method of Weber *et al.* (1972) with some modification.

3.8.2.1. Preparation of Gels:

The composition of gel electrophoresis preparations are given in Table 5 and Table 6. The gels were mixed thoroughly and the resultant solution was placed at 4°C for for 5 min. Ammonium persulphate (1.5 ml) was prepared just before use and mixed with the cold gel solution. A portion (1.2 ml) of the resultant solution was placed in an electrophoresis tube (5X90 mm). Before the gel polymerized, two drops of deionized water were layered on the top of the gel solution to ensure a flat gel surface. The solution was allowed to stand (30 minutes, at 25°C) to polymerize the gels.

3.8.2.3. Preparation of sample.

Proteins samples (2-3 mg) were placed in a screw-cap test tube (0.5 ml) with SDS/2-mercaptoethanol solution (2%) and 0.5 ml of sodium phosphate buffer solution (0.01 M, pH 7.2). The tubes were first heated in a boiling water-bath for 3 minutes and then placed at 37°C in a water-bath for 2 hours. Sucrose (50 mg) was added by the end of the heating period, followed by two drops of 0.05 % of bromophenol blue solution. A portion (50 µl) of sample solution was placed on the top of the gel. The

Table 5. Solution required for preparation gel electrophoresis SDS.

Solution	Composition
Acrylamide	22.2 g Acrylamide, 0.6 g Bis-acrylamide, deionized water to 100 ml.
Gel buffer	7.8 g $NaH_2Po_4.H_2O$, 36.8 g $Na_2HPo_4.7H_2O$, 2g SDS, deionized water to 1000 ml pH (0.2 M, 7.2).
Ammonium presulfate	15 mg/ ml deionized water.
Sample buffer	Sodium phosphate buffer pH (0.01 M, 7.2), 2 % of SDS/2-mercaptoethanol.
Resevoir buffer	1 part buffer (0.2 M, pH 7.2), 1 part water.

Table 6. Mixing ratios in gel solution SDS by volume (ml).

Solution	7.5 % Gel
Acrylamide	10.0
Water	3.4
Gel buffer	15
Ammonium presulfate	1.5
TEMED	0.045

current of the electrophoresis power supply (Bio-Rad) was 6 mA per and the electrophoresis was run for 6 h, using phosphate buffer (0.1 M, pH 7.2).

3.8.2.4. Staining and Destaining

The gels were removed from the tubes after the electrophoresis was completed and immersed in a fixative solution containing (40% methanol and 7% acetic acid) for 24 hours. The gels were immersed for 3h, in a staining solution Coomassie Brilliant Blue (0.025%) prepared in solution containing 40% methanol and 7% acetic acid. The gels were rinsed with deionized water and transferred to a destaining solution (10% acetic acid) and kept until the background became clear. The gels were stored in a solution of 7% acetic acid.

3.9. Liquid Chromatography

Liquid chromatography was performed in two different systems: (1) Conventional Chromatography and (2) Fast Protein Liquid Chromatography (FPLC). In conventional chromatography the fractions were collected on a fraction collector 2211 Superrac (LKB Co.) using microprepex persistaltic pump (model 2132) uvicords (model 2138) and a recorder from (Fisher, recordall seriers 5000). The proteins fractions were monitored at 280 nm.

An FPLC system (Pharmacia Co.) with a controller LCC-500 Plus that has a programmable memory to allow the control of chromatographic procedures, modify separation parameters, evaluate and print-out data from the chromatography. FPLC has several advantages: (a) high recovery of biological activity, (b) compatibility with aqueous buffer and salts solution, (c) high speed, high resolution and high capacity and (d) analytical and preparative possibilities. FPLC was composed of two P-500 pumps. Persitaltic pump P-1 was used to pump the sample through the valve MV-7

which is designed for application of automatic injections. Valve MV-8 is motorized for use as an automated selection valve for columns, solvents and samples that are controlled by LCC-500 Plus without removal of any columns from the system. Other components are a UV-M monitor with flow cell for protein detection at 280 nm, a FRAC-100 fraction collector and a REC-482 strip chart recorder. The samples and buffers were filtered through a 0.22 μm filter (Millipore) and degased using the same filter under vacuum prior to their use with FPLC system. The system was designed in a special way to protect the column and pumps are made from stainless steel to resist corrosion.

3.9.1. Ion-Exchange on DEAE-cellulose

The partially purified fraction (3) precipitate by ammonium sulfate 20 to 50% of saturation, was solubilized in sodium phosphate buffer (0.5 M pH 7.4; buffer A) and filtered through 0.45 μm type HA millipore. The sample was loaded on DEAE-cellulose (Sigma Chemical Co.) column (1.6 x 30 cm) pre-equilibrated with buffer (A). A linear gradient elution was used for the elution of active protein; a mixture of A and B (buffer A containing 0.5 M sodium chloride) was used for this purpose. The flow rate was adjusted to 30 ml per hour, and recorder rate was 0.25 cm per minute. The fractions of 2 ml/tube were collected and the absorbance of elute fractions was measured at 280 nm.

3.9.2. Ion-Exchange Chromatography on Mon Q

Mon Q HR 5/5 is a strong anion exchange column, prepacked with chemical and physical stability, which can be used in aqueous media in the pH range of 2-12. For this column, the protein loading capacity is normally 20-25 mg/column. Enzyme activity recoveries are normally greater than 80 % (Pharmacia Handbook, 1989). The

partially purified extract (fraction 3) was separated on Mon Q HR 5/5 column (0.5 x 5 cm) ion-exchange chromatography using FPLC system (Pharmacia Co.). The column was pre-equilibrated with sodium phosphate buffer A (5 mM, pH 7.4) and the sample was solubilized in the same buffer. A gradient of buffer A and buffer B (buffer A containing 1 M sodium chloride) was used. The system was programmed to elute 2 ml of buffer A, perform a gradient was run (0-100 %) of buffer B in a total volume of 20 ml, then buffer B was run 100 % in a volume of 2ml, followed by 3 ml of buffer A only. The elution was performed at a flow rate of 1 ml/min. Fractions of 1 ml were collected, dialysed, concentrated by ultrafiltration CX-30 cartidge (Millipore) and lyophilized.

3.9.3. Gel Filtration Chromatography

Superose-12 are prepacked low pressure gel filtration chromatography columns. The low pressure was recommended for high resolution. Gel properties include a cross-linked agraose based medium optimized for high performance gel filtration of biomlecules and being stable in the pH range of 1-14. The protein sample loaded should not contain more than 5-10 mg. Exclusion limit is globular proteins with molecular weight over 2 x 10^6 (Pharmacia Instruction, 1989). The isozyme fractions obtained by ion-exchange chromatography on Mono Q column were subjected to Superose-12 HR 10/30 gel filtration column using the FPLC. The column was equilibrated with sodium phosphate buffer (5 mM, pH 7.4) containing 0.1 M sodium chloride and the samples were solubilized in the same buffer. Elution was performed at a flow rate of 0.3 ml/min and 1 ml/tube were collected. The separated fractions were filtered through P-D10 column cartidge and concentrated by ultrafiltration immersible CX-30 cartidge . All other conditions were the same as those described for the initial ion-exchange chromatography.

The separated fractions from gel filtration were subjected to Superose 12 column for re-gel filtration. All other conditions were the same as those described for the gel filtration chromatography. Fractions of 1 ml/tube were collected, concentrated using ultrafiltration immersible cartidge CX-30 lyophilized and subjected for analysis.

3.10. Kinetics Studies of Lipoxygenase Isozymes

3.10.1. Substrates Preparation

Linoleic acid, monolinolein, dilinolein and trilinolein (Sigma Chemical Co.) were used as substrates for measurement of lipoxygenase isozymes activity. The substrates were prepared in sodium phosphate buffer (0.2 M, pH 7.0) according to the method described previously (Axelrod *et al.*, 1981). The final concentration of each substrate was 10×10^{-3} M. All the condition used were as described previously for the partially purified enzyme.

3.10.3. Maximum Absorbance

The maximum absorbance of linoleate hydroperoxides, resulting from isozymes action, was determined spectrophotometrically. The absorbance was scanned at the range of 230 to 240 nm using Beckman DU-65 spectrophotometer. All the conditions for the assay were as described previously for isozyme assay and substrate preparation.

3.11. Scanning of Electrophoresis

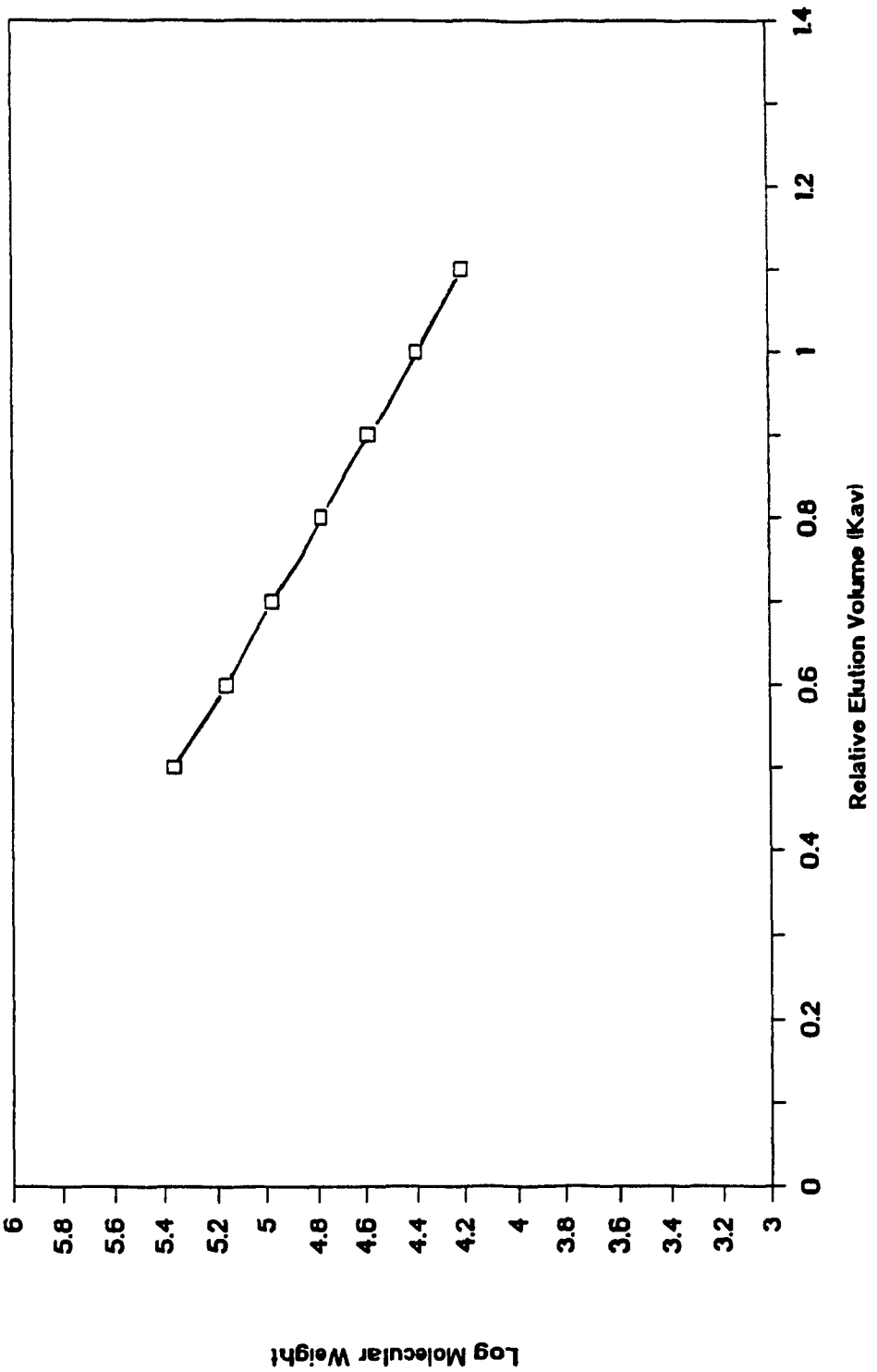
Electropherograms of lipoxygenase isozymes from SDS-PAGE disc gel electrophoresis were scanned using Beckman CDS-200 computing densitometer system. The system was composed for the fluorescence mode of a reflectance filter

(position 6). The instrument is equipped with two operator selectable slit sizes that differ both in width and length. The small slit which has a width of 0.3 mm was employed for the reflectance mode at 600 nm. The R_f of samples were compared to those of standards.

3.12. Molecular Weight Determination on Superose-12

The molecular weight of lipoxygenase isozymes were also determined on gel filtration superose 12 10/30 column, equilibrated with 0.1 M NaCl in 5 mM sodium phosphate buffer (pH 7.4). The molecular weight of protein standards, obtained from Sigma Chemical Co., were cytochrome *c* (Mr 12,400), carbonic Anhydrase (Mr 29,000), egg albumin (Mr 45,000), serum bovin albumin (Mr 66,000), soybean lipoxygenase (Mr 97,400,), yeast alcohol dehydrogenase (Mr 150,000), B-amylase (Mr 200,000) and blue dextrane (Mr 2,000,000). Standard solutions were injected in 500 ul from each standard, with flow rate 0.3 ml/min. The protein standard was prepared as 1 mg sample/2ml buffer and was measured at 280 nm as shown in Figure 4. The elution volume collected between the time of injection and the center of the elution peak. The elution volume for Blue dextrane is equal to the column void volume (V_o). Blue dextrane was prepared as fresh solution (1.0 mg/ml) in the eluent buffer. The rate of solubilization of the Blue dextrane was increased by heating the buffer to 50°C, before adding the blue dextrane.

Fig 4. Standard curve for molecular weight determination using FPLC.


Log Molecular Weight
6
5.8
5.6
5.4
5.2
5
4.8
4.6
4.4
4.2
4
3.8
3.6
3.4
3.2
3
0
0.2
0.4
0.6
0.8
1
1.2
1.4
Relative Elution Volume (Kav)

CHAPTER IV

RESULTS AND DISCUSSION

4. Purification of Canola Lipoxygenase

4.1. Partial Purification

Literature screening showed conflicting reports regarding to the presence of lipoxygenase in the rapeseed. These reports have suggested that lipoxygenase was probably present in rapeseed but could not detected (Franke and Freshe, 1954; Bronsiz *et al.*, and Appleqvist, 1972). The fragility of the enzyme and the complexity of its isolation and purification are some of the factors which could have contributed to that conflict. The procedure used for isolation and purification of lipoxygenase from Canola seed was similar to those used for lipoxygenase from other sources, e.g. peas (Eriksson and Svensson, 1970; Haydar and Hadziyev, 1973;, Haydar *et al.*, 1975; and Klein, 1976), immature English peas (Chen and Whitaker, 1986) and French bean (Kermasha and Metche, 1986). However, some modifications were made in order to develop a reliable method, since one of the objectives of this study was to purify an active lipoxygenase.

A typical diagram for the isolation and purification of lipoxygenase from Canola seed is shown in Figure 5. Purification scheme for the active lipoxygenase extract from Canola is given in Table 7. The results (Table 7) showed that 83% of Canola lipoxygenase was located in the fractions precipitated by ammonium sulfate at 0 to 20 % and 20 to 50 % of saturation. The low recovery of lipoxygenase activity (29%) in the 50 to 100% of saturation fraction may reflect the fact that the addition of ammonium sulfate at high concentration can result in a drop in pH (5.5-5.9) even in a well buffered system (Kermasha and Metche, 1986; Schwimmer, 1981). The results also show the presence of relatively higher enzymatic activity (53%) in the 20 to 50 % ammonium sulfate precipitation (fraction 3). The lipoxygenase activity in fraction 3 had increased 4-fold

Fig 5. Diagram of extraction of lipoxygenase from Canola seed.

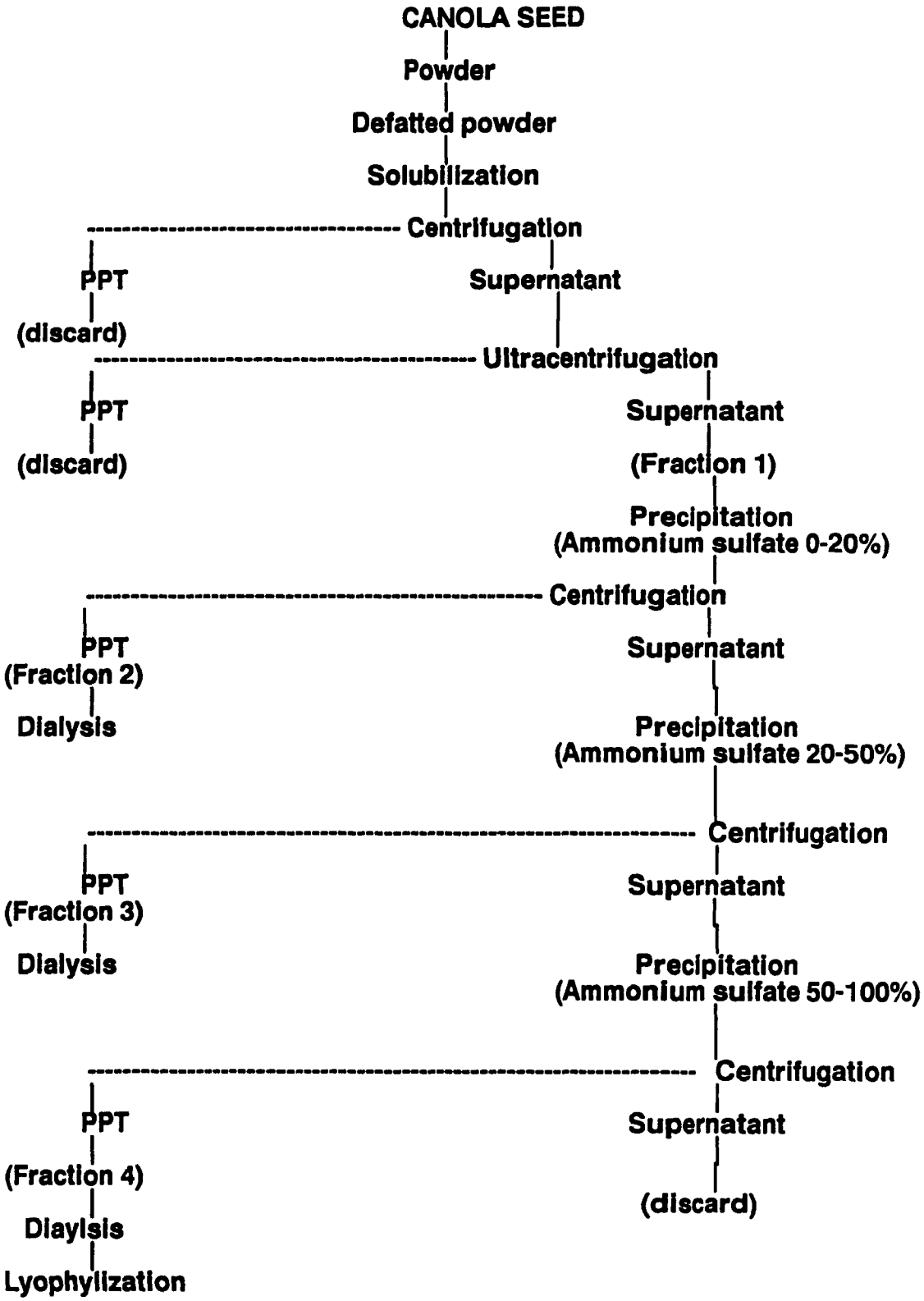

CANOLA SEED
Powder
Defatted powder
Solubilization
Centrifugation
PPT
(discard)
Supernatant
Ultracentrifugation
PPT
(discard)
Supernatant
(Fraction 1)
Precipitation
(Ammonium sulfate 0-20%)
Centrifugation
PPT
(Fraction 2)
Dialysis
Supernatant
Precipitation
(Ammonium sulfate 20-50%)
Centrifugation
PPT
(Fraction 3)
Dialysis
Supernatant
Precipitation
(Ammonium sulfate 50-100%)
Centrifugation
PPT
(Fraction 4)
Dialysis
Lyophylization
Supernatant
(discard)

Table 7. Purification scheme for lipoxygenase extract from Canola seed.

Fraction	Total protein[a] (mg)	Total activity[b] (units)	Specific activity[c]	Recovery (%)	Purification (fold)
Crude Extract (Fraction 1)	2019	7571	3.75	100	0
0-20% ammonium sulfate precipitation (Fraction 2)	1743	9150	5.25	120	1.4
20-50% ammonium sulfate precipitation (Fraction 3)	1350	20250	15	267	4.0
50-100% ammonium sulfate precipitation (Fraction 4)	986	8035	8.15	80	2.1

[a]Protein was determined according to the method of Hartree (1972), a modification of Lowry *et al.*, using bovine serum albumin as standard.

[b]Unit of activity is defined as A per min, where A is equal to 0.001 absorbance at 234 nm.

[c]Specific activity is defined as A/mg protein/min, where A is equal to 0.001 absorbance at 234 nm.

when compared to that in the crude extract (fraction 1). These results are in agreement with those reported for peas lipoxygenase (Haydar and Hadziyev, 1973) and for French bean enzyme (Kermasha and Metche, 1986). The high activity of lipoxygenase in fraction 3 is also very close to those reported for lipoxygenase from pea (Eriksson and Svensson, 1970), for immature English pea lipoxygenase (Chen and Whitaker, 1986), from dried split pea seeds and snap beans (Klein, 1976) and from dried winged beans (Van Den *et al.*, 1982a).

Higher plants generally contain various compounds, such as phenols, which act as enzyme inhibitors (Cheetham, 1985). Due to the relatively high content of phenolic compounds in Canola seed and the likelihood of interference by phenolic compounds, work was also directed at quantitating the removal of phenolic compounds found in the enzymatic extracts of the Canola seed. A significant decrease in the amount of phenolic compounds was found in the enzymatic extracts when compared to the full-fat Canola seed. The results (Table 8) show that the 20-50% ammonium sulfate precipitated protein fraction (fraction 3) contained only one third and the phenolic compounds present in the defatted Canola seed and half of those found the crude extract (fraction 1). The decrease in the amount of phenolic compounds in the partially purified enzymatic extract (fraction 3) could be an important factor in the removal of the inhibitory effect on lipoxygenase activity, since phenolic compounds can form several types of bonds with proteins (Loomis, 1974). The greatest difficulty encountered in the extraction of plant proteins is perhaps due to the action of plant phenolic compounds. Most plant tissue contains a wide variety of phenolic compounds (Anderson, 1968). However, repetitive centrifugation (3 times) and particularly ultracentrifugation at 200,000xg was used to remove potential inhibitors present in Canola extract. Many workers have suggested that lipoxygenase was probably present in rapeseed but could not detected because of an inhibitor (Appleqvist, 1972). Phenolic compounds, such as catechol, can interact with

Table 8. Total phenolic compound in the defatted Canola seed and lipoxygenase extracts.

Sample	Phenolic compounds (%)[a]	(%)[b]
Defatted Canola seed	7.6	100.0
Crude enzymatic extract (Fraction 1)	4.8	63.1
20-50% ammonium sulfate precipitation (Fraction 3)	2.5	32.8

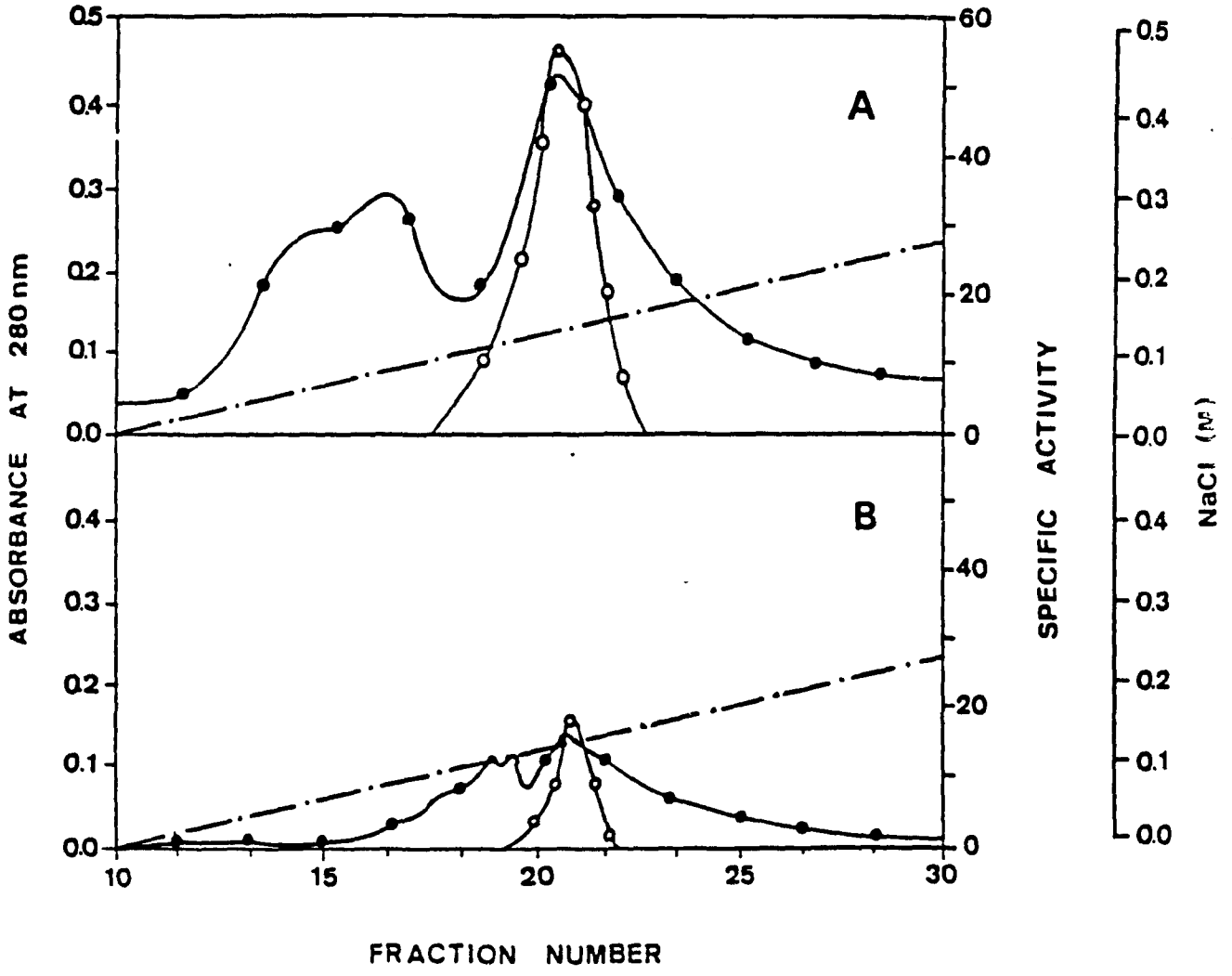
[a]Percent phenolic compounds (dry weight basis).

[b]Percent of phenolic compounds relative to initial phenolic content in Canola seed.

proteins and subsequently inhibit enzymatic activity (St-Angelo *et al.*, 1979), however the mechanism of inhibitor is not well understood. This may also implies, the elimination of other enzymes, such as peroxidase, that utilize the same substrate (unsaturated fatty acids) as lipoxygenase, or the elimination of other inhibitors from the enzyme extract suspension (Kermasha and Metche, 1986). Kermasha and Metche (1986) showed that the ultracentrifugation at 115,000xg for 3 h increased the activity of lipoxygenase of *Phaselous vulgaris* by 30 %

Preliminary purification of the partially purified extract (fraction 3) was performed on DEAE-cellulose ion-exchange chromatography. Comparative DEAE-cellulose ion-exchange chromatograms of commercial soybean lipoxygenase (Sigma Chemical Co.) and the partially purified enzyme (fraction 3) extract of Canola seed are shown in Figure 6. The results show similar ion-exchange chromatogram profiles of the two enzymes preparations. The two chromatograms demonstrate the presence of a major peak and two minor peaks. However, the two minor peaks in the enzyme prepared from Canola seed were better separated than those from commercial soybean. The lipoxygenase activity profile indicates the presence of a single enzymatically active fraction in both Canola extract and commercial soybean lipoxygenase. The results obtained from ion-exchange chromatography on DEAE-cellulose were similar to those obtained with immature English peas lipoxygenase (Chen and Whitaker, 1986), commercial soybean lipoxygenase (Stevens *et al.*, 1970) and pea lipoxygenase (Arens *et al.*, 1973; Eriksson and Svensson, 1970; Haydar and Hadziyev, 1975). It has been noted that hemoproteins such as peroxidase, catalase and cytochromes which are strong lipid peroxidizers could be separated from lipoxygenase by the DEAE-cellulose liquid chromatography (Eriksson and Svensson, 1970).

Fig 6. DEAE-cellulose gradient ion-exchange chromatograms of (A) commercial soybean lipoxygenase, and (B) partially purified lipoxygenase extract (fraction (3, obtained from Canola seed; (●-----●) protein profile,(o-----o) lipoxygenase activity profile.


A
B
ABSORBANCE AT 280 nm
SPECIFIC ACTIVITY
NaCl (M)
FRACTION NUMBER
0.5
0.4
0.3
0.2
0.1
0.0
60
40
20
0
10
15
20
25
30

4.2. Purification of Lipoxygenase Isozymes

The partially purified enzyme extract (fraction 3) was further purified by successive separations on ion-exchange chromatography (Mono Q HR 5/5 column) and gel filtration chromatography (Superose-12 HR 10/30 column), using Fast Protein Liquid Chromatography (FPLC) System. The scheme of purification procedure, used for the separation and purification of lipoxygenase isozymes, is given in Figure 7.

The results (Fig. 8) show that the FPLC gradient ion-exchange chromatography on Mono Q resulted in a major fraction, with two shoulders, (FII) and two smaller fractions (FI and FIII). Howevere, FIII shows the highest level of lipoxygenase activity. Each fraction (I, II and III) was collected separately and subjected to chromatography and re-chromatography on Superose-12 gel filtration column.

Figure 9A shows the profile of fraction I obtained by the Mon Q column ion-exchange chromatography. The application of fraction I to the Superose-12 gel filtration column resulted in one fraction with two shoulders showing a single peak of activity (Fig. 9B). The re-gel filtration of this fraction on Superose-12 column resulted into two well-separated fractions. The determination of lipoxygenase activity showed that only the second fraction was containing such activity (Fig. 9C).

A similar purification procedure was performed with fraction II. Figure 10A shows the separated fraction II was then applied to Superose-12 column and resulted in two peaks, major and minor (Fig. 10B). These two peaks were subjected to a second gel filtration chromatography and the results show the presence of two isozymes IIA (Fig. 10C) and IIB (Fig. 10D). Fraction III resulted by Mon Q column (Fig. 11A) was purified on Superose-12 column and this resulted in a well-separated fraction (Fig. 11B). Re-

Fig 7. Diagram of purification lipoxygenase isozymes.

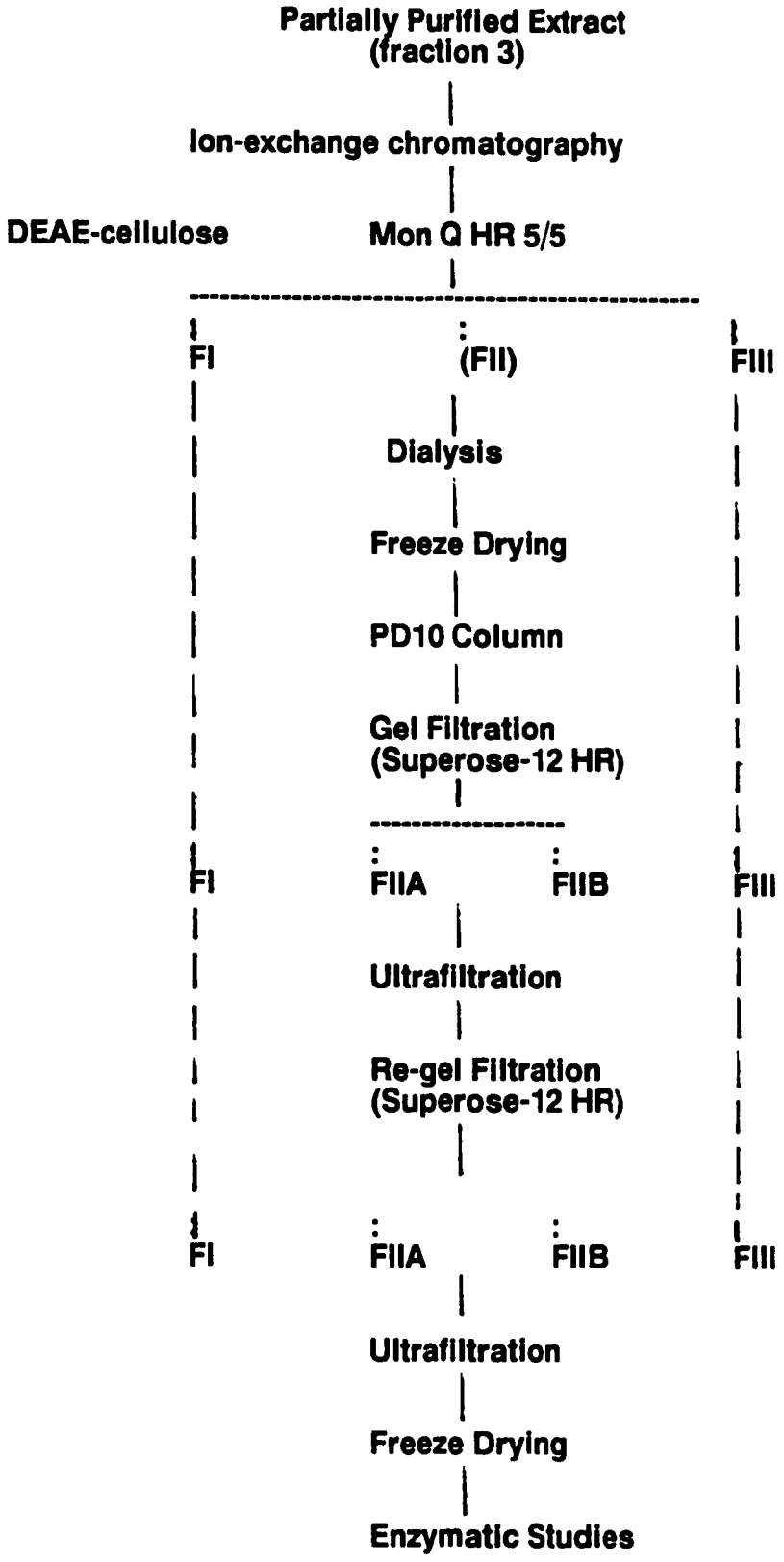

Partially Purified Extract
(fraction 3)
Ion-exchange chromatography
DEAE-cellulose
Mon Q HR 5/5
FI
(FII)
FIII
Dialysis
Freeze Drying
PD10 Column
Gel Filtration
(Superose-12 HR)
FI
FIIA
FIIB
FIII
Ultrafiltration
Re-gel Filtration
(Superose-12 HR)
FI
FIIA
FIIB
FIII
Ultrafiltration
Freeze Drying
Enzymatic Studies

Fig 8. The profile of partially purified (fraction 3) purification by ion-exchange chromatography column (Mon Q HR 5/5) gradient using the FPLC system; (o-----o) protein profile, (●-----●) lipoxygenase activity profile.

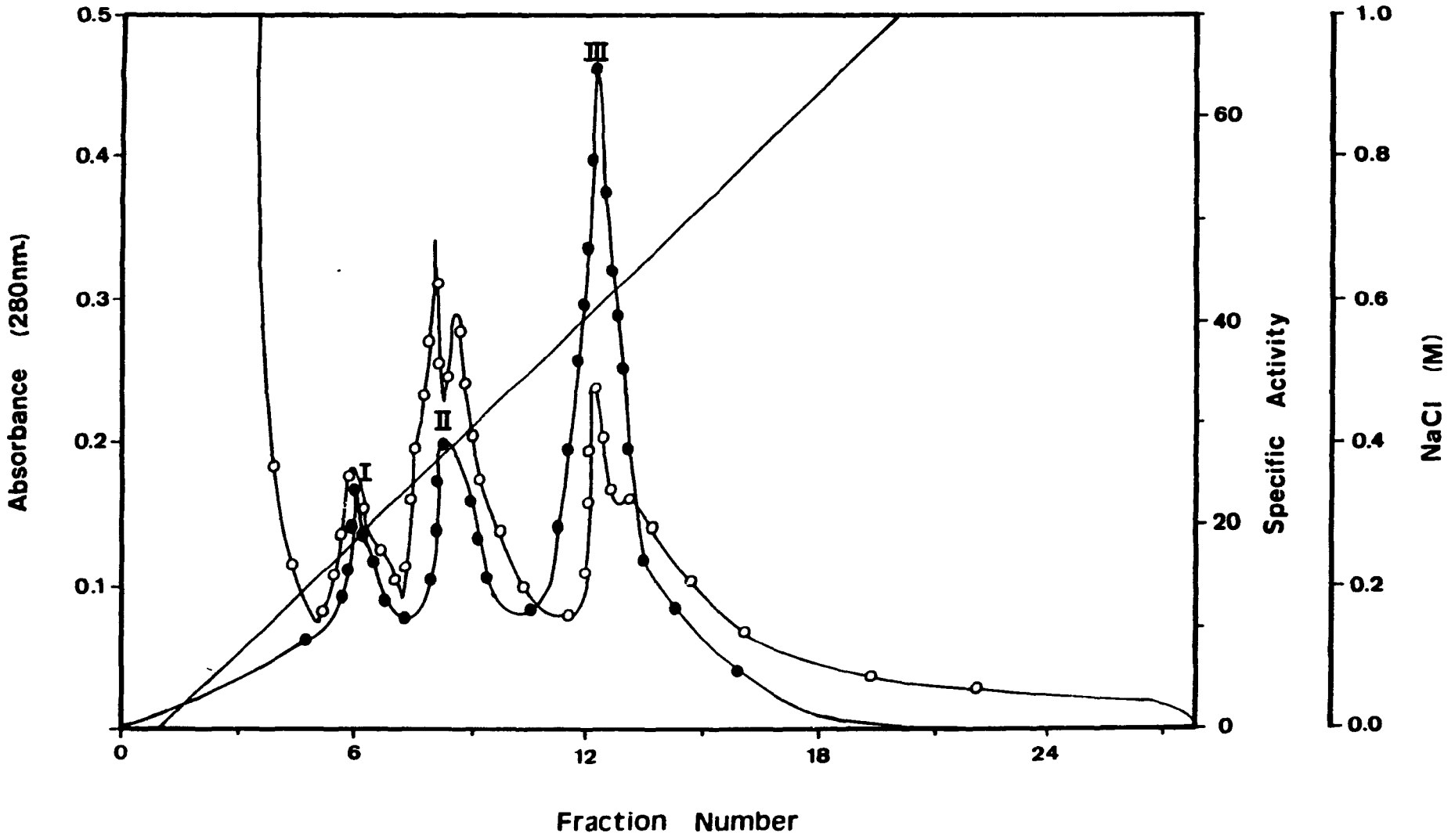

Absorbance (280nm)
0.5
0.4
0.3
0.2
0.1
I
II
III
0
6
12
18
24
Fraction Number
60
40
20
0
Specific Activity
1.0
0.8
0.6
0.4
0.2
0.0
NaCl (M)

Fig 9. The profile of isozyme I purification (A) by ion-exchange (Mon Q 5/5), (B) gel filtration (Superose-12 HR 10/30), and (C) re-gel filtration chromatography (Superose-12 HR 10/30) using the FPLC system; (o-----o) protein profile, (●-----●) lipoxygenase activity profile.

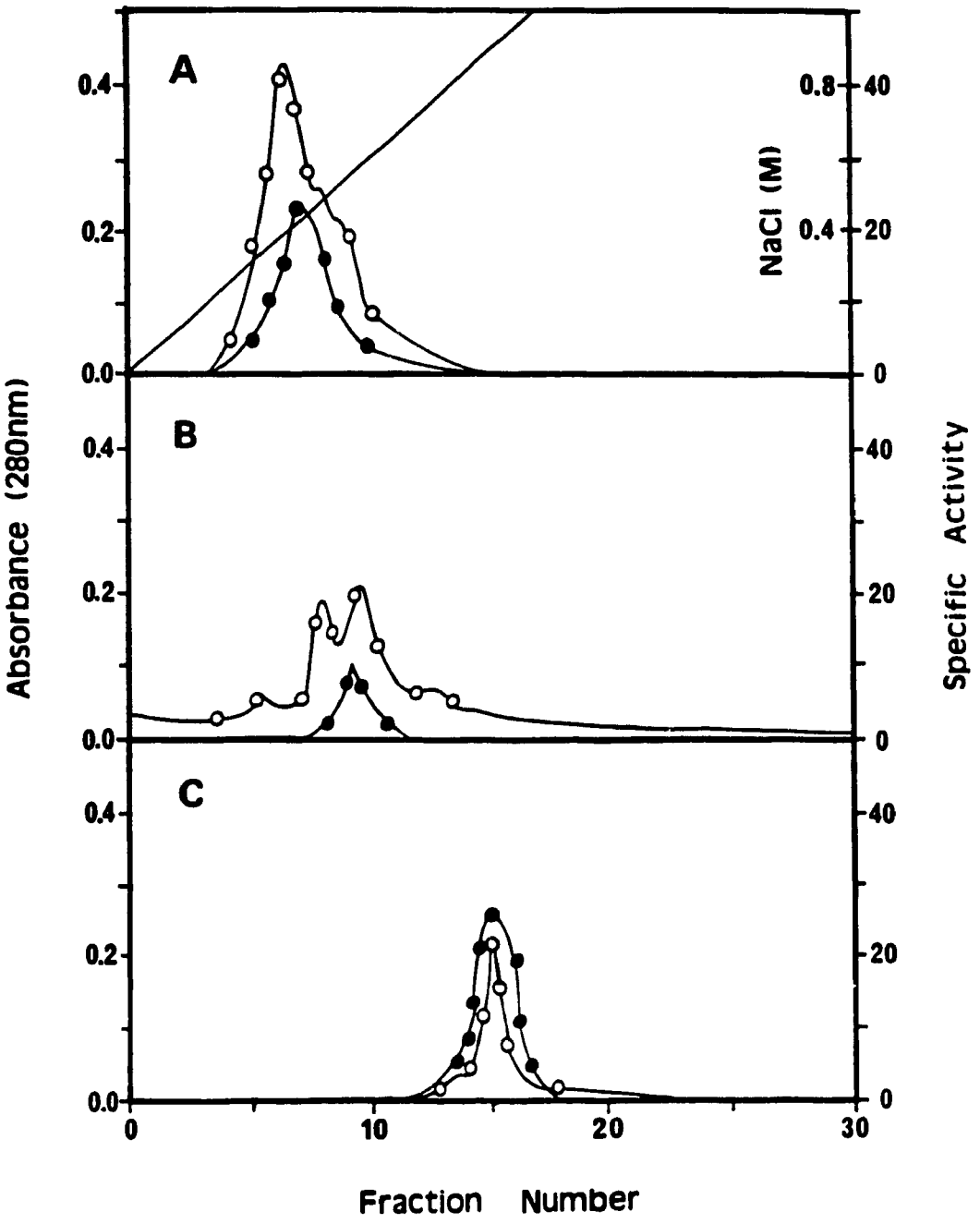
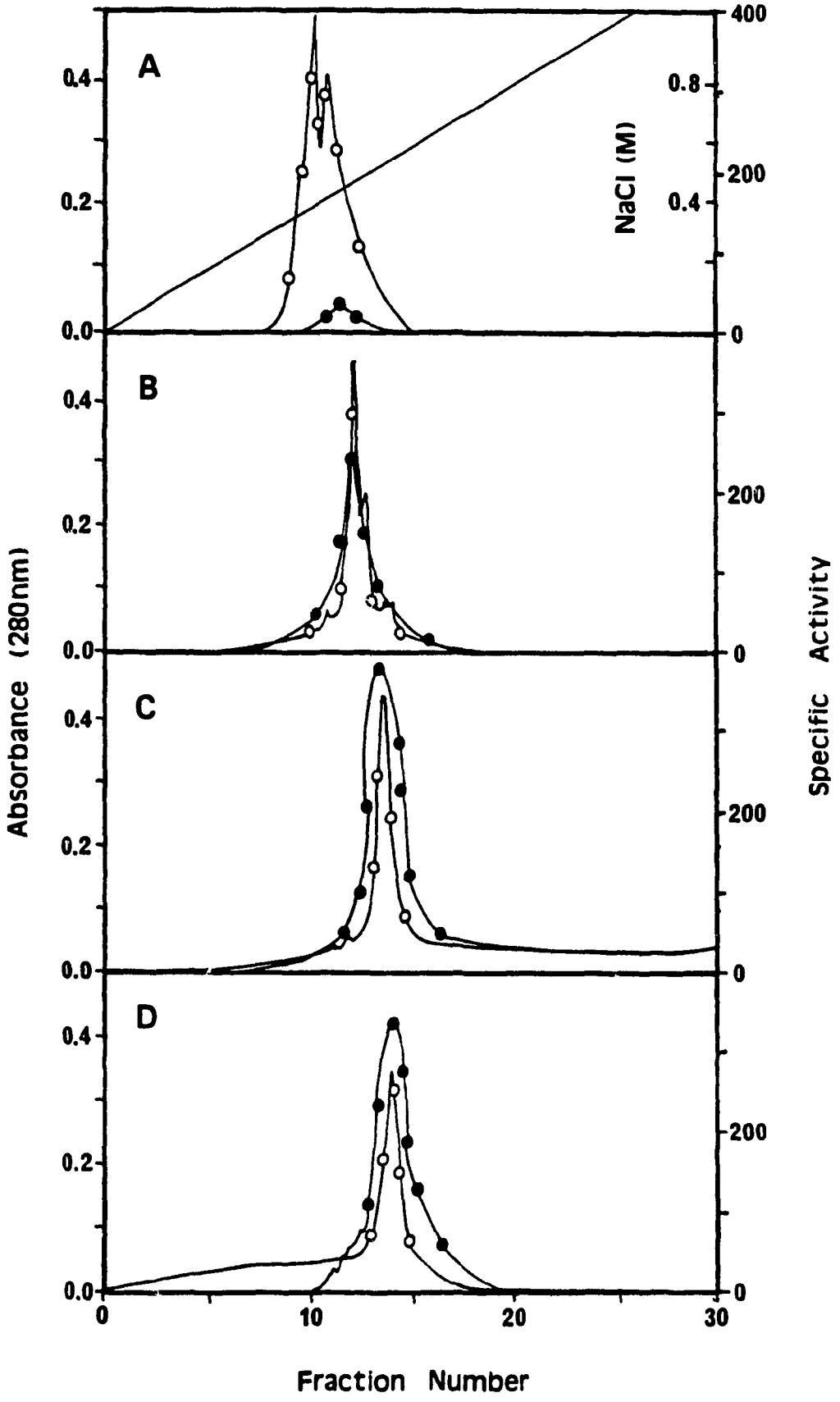

A
B
C
Absorbance (280nm)
NaCl (M)
Specific Activity
Fraction Number
0.0
0.2
0.4
0.8
0
20
40
0
10
20
30

Fig 10. The profile of isozyme II purification (A) by ion-exchange (Mon Q 5/5), (B) gel filtration (Superose-12 HR 10/30), (C) isozyme IIA re-gel filtration chromatography (Superose-12 HR 10/30), and (D) isozyme IIB re-gel filtration chromatography (Superose-12 HR 10/30 using the FPLC system; (o-----o) protein profile, (●-----●) lipoxygenase activity profile.


A
B
C
D
0.4
0.2
0.0
0.8
0.4
400
200
0
NaCl (M)
Absorbance (280nm)
Specific Activity
0
10
20
30
Fraction Number

-chromatography of fraction III on gel filtration resulted by a high purified isozyme (Fig. 11C).

The ion-exchange chromatography of isozymes I, II and III resulted, respectively, by 22, 28 and 67-fold purification. On the other hand, the gel filtration chromatography of fraction I, IIA, IIB and III resulted, respectively, by 25, 104, 89 and 240-fold purification. The results (Table 9) show the recovery of lipoxygenase isozymes obtained by ion-exchange was 19% for isozyme I, 23% for isozyme II and 57% for isozyme III, whereas that for isozymes resulted from the chromatography and re-chromatography on Superose-12 was 5% for isozyme I, 22% for isozyme IIA, 19% for isozyme IIB and 52% for isozyme III.

Regarding the number of isozymes separated by conventional chromatography, the literature showed that three isozymes were reported for peanut lipoxygenase (Sanders *et al.*, 1975) and for wheat germ lipoxygenase (Nicolas *et al.*, 1982). However, the number of isozymes varied according to the source of lipoxygenase and these differences could be attributed also to the chromatographic conditions and techniques (Haydar *et al.*, 1975; Klein, 1976; Yoon and Klein, 1979; Stevens *et al.*, 1970). Using DEAE-cellulose ion-exchange chromatography, the degree of purification for pea lipoxygenase was 73 and 33-fold (Arens *et al.*, 1973; Eriksson and Svensson, 1970), for pea seeds lipoxygenase was 23.4-fold (Haydar and Hadziyev, 1973) and for wheat lipoxygenase was 102-fold (Wallace and Wheeler, 1975). The Sephadex G-150 gel filtration was also used for the purification of the enzyme; the degree of purification was 86-fold for pea lipoxygenase (Eriksson and Svensson, 1970) and 42.3 and 57-fold, respectively, for rice lipoxygenase-2 and lipoxygenase-3 (Ida *et al.*, 1983).

Fig 11. The profile of isozyme III purification (A) by ion-exchange (Mon Q 5/5), (B) gel filtration (Superose-12 HR 10/30), and (C) re-gel filtration chromatography (Superose-12 HR 10/30) using the FPLC system; (o-----o) protein profile, (●-----●) lipoxygenase activity profile.

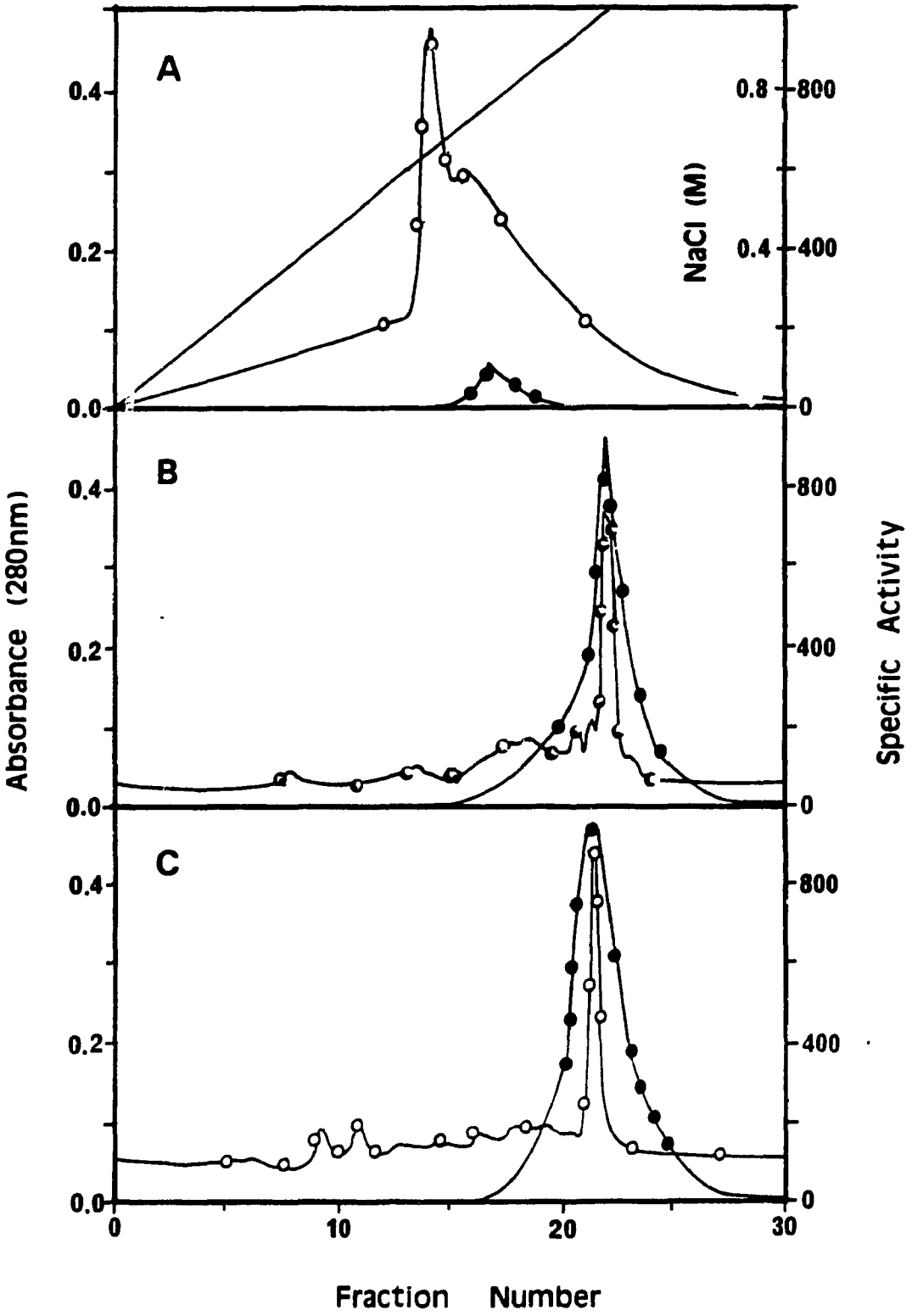

A
B
C
0.4
0.2
0.0
0.8
800
400
0
NaCl (M)
Absorbance (280nm)
Specific Activity
0
10
20
30
Fraction Number

Table 9. Purification scheme for lipoxygenase isozymes

Fraction	Total protein (mg)	Total activity	Specific activity[a]	Recovery (%)	Purification (fold)
Crude Extract[b]	2440	9150	3.75	100	0
Partially Purified[c]	1632	24480	15	267	4
FPLC Ion-Exchange Chromatography (Mon Q 5/5)					
Fraction I	23.97	2013	84	22.0	22
Fraction II	72.60	7623	105	83.3	28
Fraction III	22.83	5708	250	62.4	67
FPLC Gel Filtration Chromatograhy (Superose 12 10/30)					
Fraction I	0.474	44	93	0.5	25
Fraction IIA	2.562	999	390	10.9	104
Fraction IIB	3.420	1139	333	12.4	89
Fraction III	0.246	221	900	2.4	240

[a]Is defined as A/mg protein, where A is equal to 0.001 absorbance at 234 nm or 238 nm.

[b]Fraction 1 after ultrcentrifugtion

[c]Fraction precipitated with ammonium sulfate at 20-50% of saturation (fraction 3).

The overall of these results (Table 9) shows that the successive separations of enzyme extracts on ion-exchange and gel filtration chromatographies, using FPLC system, resulted in a high degree of purification of lipoxygenase isozymes.

4.3. Properties of Lipoxygenase Enzyme.

4.3.1. Lineweaver-Burk Plots

Lineweaver-Burk plots (Lineweaver-Burk, 1934) were used to demonstrate the effect of substrate concentration on initial velocities of hydroperoxidation of linoleic acid catalyzed by the enzyme. The K_m value is known to designate the enzyme affinity towards its substrate. Linoleic acid was used for kinetic determination for the activity of the partially purified enzyme (fraction 3) and lipoxygenase isozymes. For the determination of apparent K_m values for the activity of the lipoxygenase partially purified extract, linoleic acid concentrations were varied from 0.05 to 1 mM. The maximum activity was observed with 0.50 mM of substrate. The apparent K_m value was determined from the best straight line. The K_m value for the partially purified enzyme (fraction 3) was 2.0×10^{-4} M (Fig. 12). This value was close to that reported for lupin seed lipoxygenase (2.4×10^{-4} M; Olias and Valle, 1988) but lower than those reported for pea lipoxygenase (2.3×10^{-3} M; Haydar and Hadziev, 1973), for broad bean lipoxygenase (2.8×10^{-3} M; Al-Obaidy and Siddiqi, 1981), for tomato lipoxygenase (1.42×10^{-3} M, Zamora *et al.*, 1987) and for avocado lipoxygenase (7.2×10^{-2} M; Marcus *et al.*, 1988). The obtained value was higher than those reported for wheat lipoxygenase (5×10^{-6} M; Irvine and Anderson, 1953) and soybean lipoxygenase (1.35×10^{-4} M; Holman, 1947).

Lineweaver-Burk plots were also calculated for lipoxygenase isozymes. These values are either similar or close to K_m values reported for lipoxygenase isozymes from other sources. The K_m value for isozyme I was 5.5×10^{-4} M (Fig. 13) which is similar to that reported for soybean lipoxygenase-2 (Andrawis *et al.*, 1982) and close to that

Fig 12. **Lineweaver-Burk plot for partially purified lipoxygenase activity from Canola seed extract (fraction 3) using linoleic acid as substrate.**

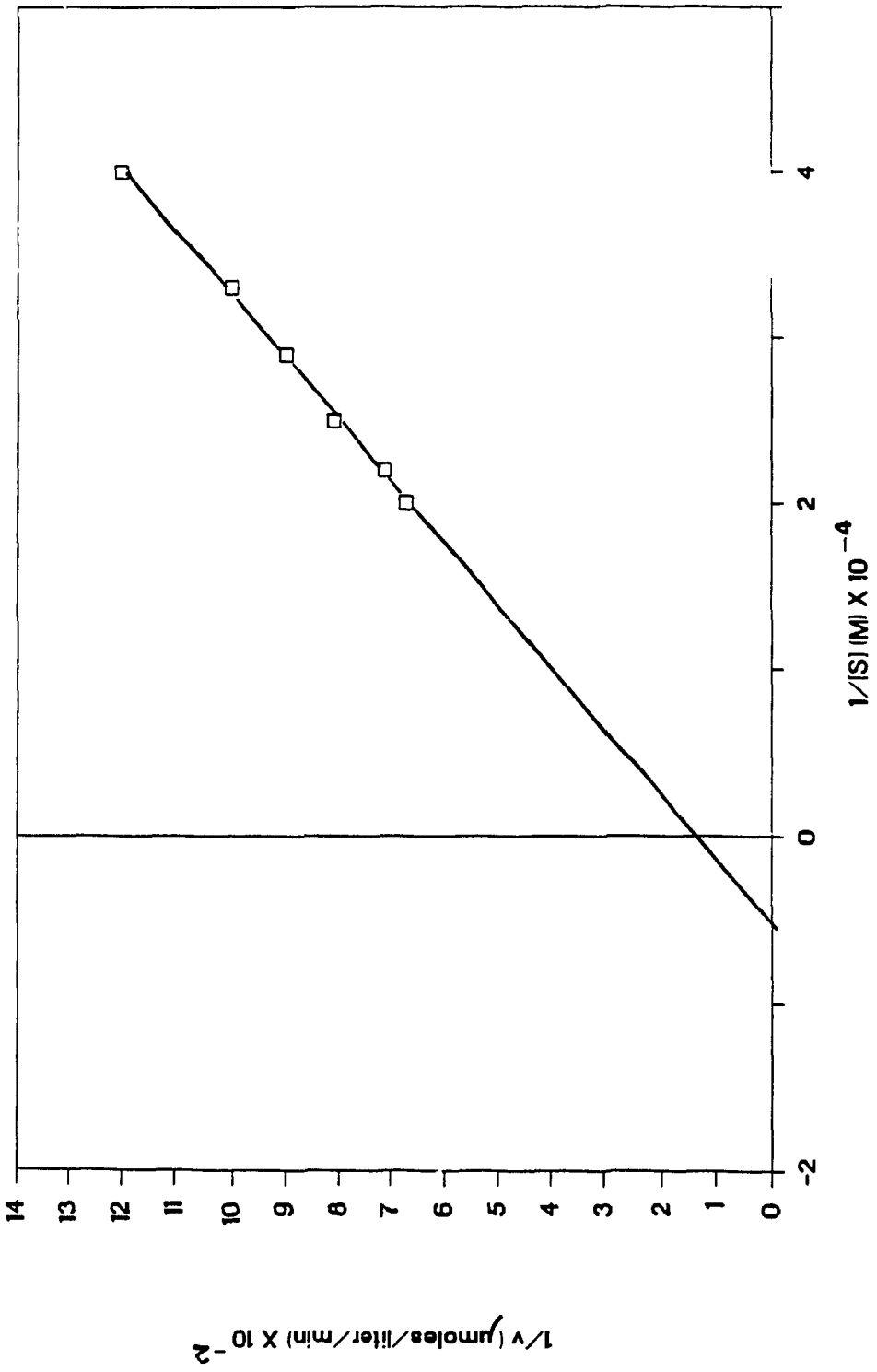
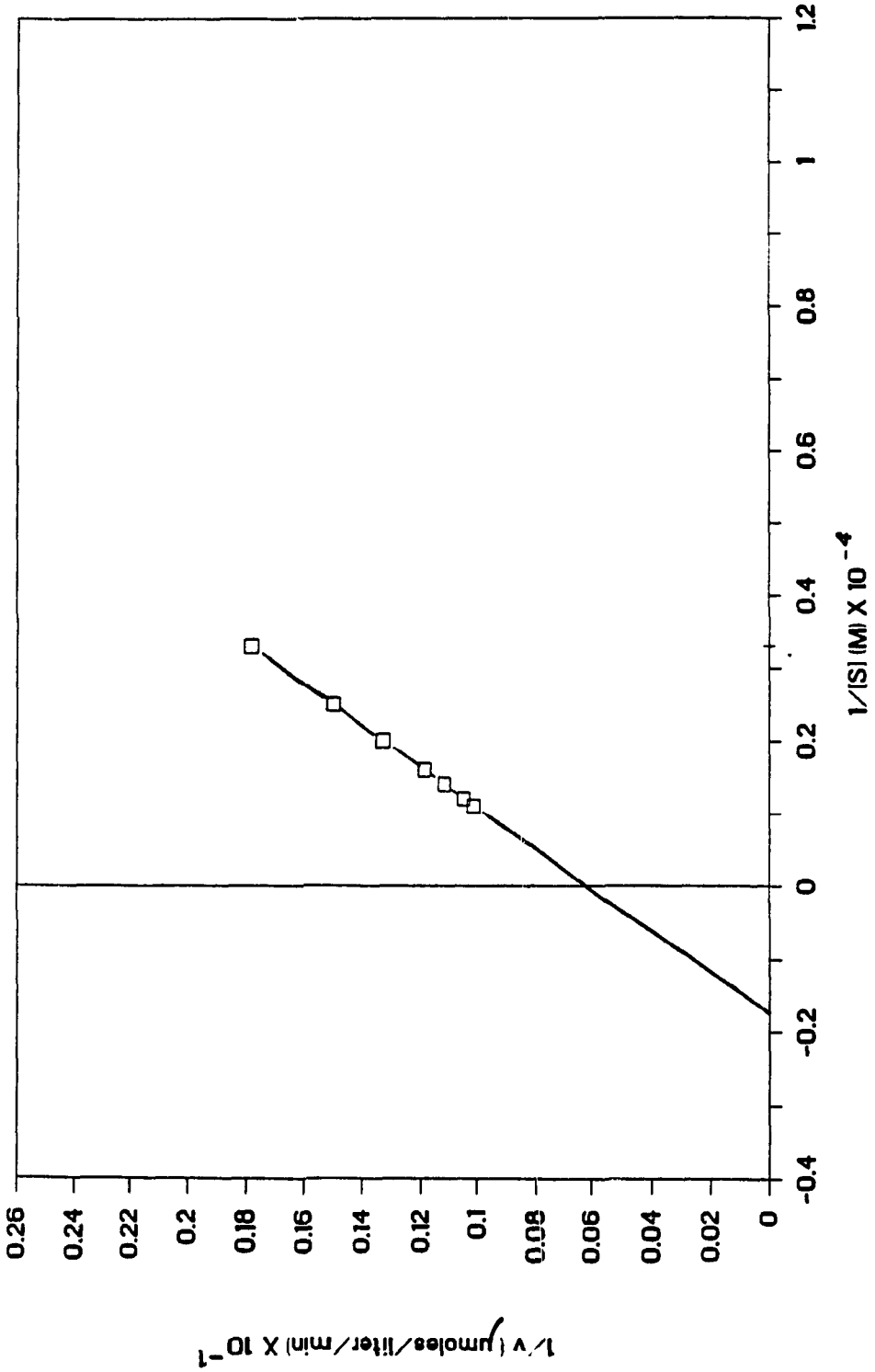

1/v (μmoles/liter/min) X 10⁻²
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
-2
0
2
4
1/[S] (M) X 10⁻⁴

Fig 13. **Lineweaver-Burk plot of lipoxygenase isozyme I.**


1/v (μmoles/liter/min) X 10⁻¹
0.26
0.24
0.22
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1
1.2
1/[S] (M) X 10⁻⁴

reported for small faba beans lipoxygenase (5.7×10^{-4} M; Eskin and Henderson, 1974). The K_m value of isozyme IIA is 3.5×10^{-4} M (Fig. 14) which is identical to that reported for rice bran lipoxygenase (Shastry and Raghavendra, 1975) and close to those results reported for soybean enzyme (3.8×10^{-4}; Tapple *et al.*, 1952 and Allen, 1968). Isozyme IIB is showed a K_m value of 4.0×10^{-4} M (Fig 15) this value is close to that reported for FI winged bean lipoxygenase (4.4×10^{-4} M ;Van Den *et al.*, 1982b). For isozyme III, the K_m value is 3.8×10^{-4} M (Fig. 16); this value is close to that reported for winged bean lipoxygenase FII (3.7×10^{-4} M; Van Den *et al.*, 1982b). The discrepancy is K_m values probably due to difference in enzyme source and assay methods (Klein, 1976). The K_m value for lipoxygenase depends on pH and critical micell concentration (Allen 1968) and probably has little physiological significance.

4.3.2. Effect of pH on Enzyme Activity

The effect of pH on lipoxygenase activity was investigated. The partially purified Canola seed lipoxygenase showed a sharp pH optimum activity at 7.5 (Fig. 17). This result is identical to that reported for snap bean (Klein, 1976) and close to the optimum pH of French bean (7.3; Kermasha and Metche, 1986), pea seeds (7.2; Haydar and Hadziyev, 1973) and avocado (7.1; Marus *et al.*, 1988). However, the pH value is higher than those reported for tomato fruit lipoxygenase (6.3-6.5; Bonnet and Crouzet, 1977), for germinating sunflower seed lipoxygenase (6.2; Leoni *et al.*, 1985), for wheat lipoxygenase (6.5; Irvine and Anderson, 1953), for eggplant lipoxygenase (6.5 Grossman *et al.*, 1972), for lupin lipoxygenase (6.1; Olias and Valle, 1988) and for apple lipoxygenase (6.0; Kim and Grosch, 1979). Galliard and Phillips (1971) noted that the partially purified lipoxygenase from potato tubers has an optimum pH at 5.5 to 6.0 and the enzyme was inactive at pH 9.0.

Fig 14. Lineweaver-Burk plot of lipoxygenase isozyme IIA.

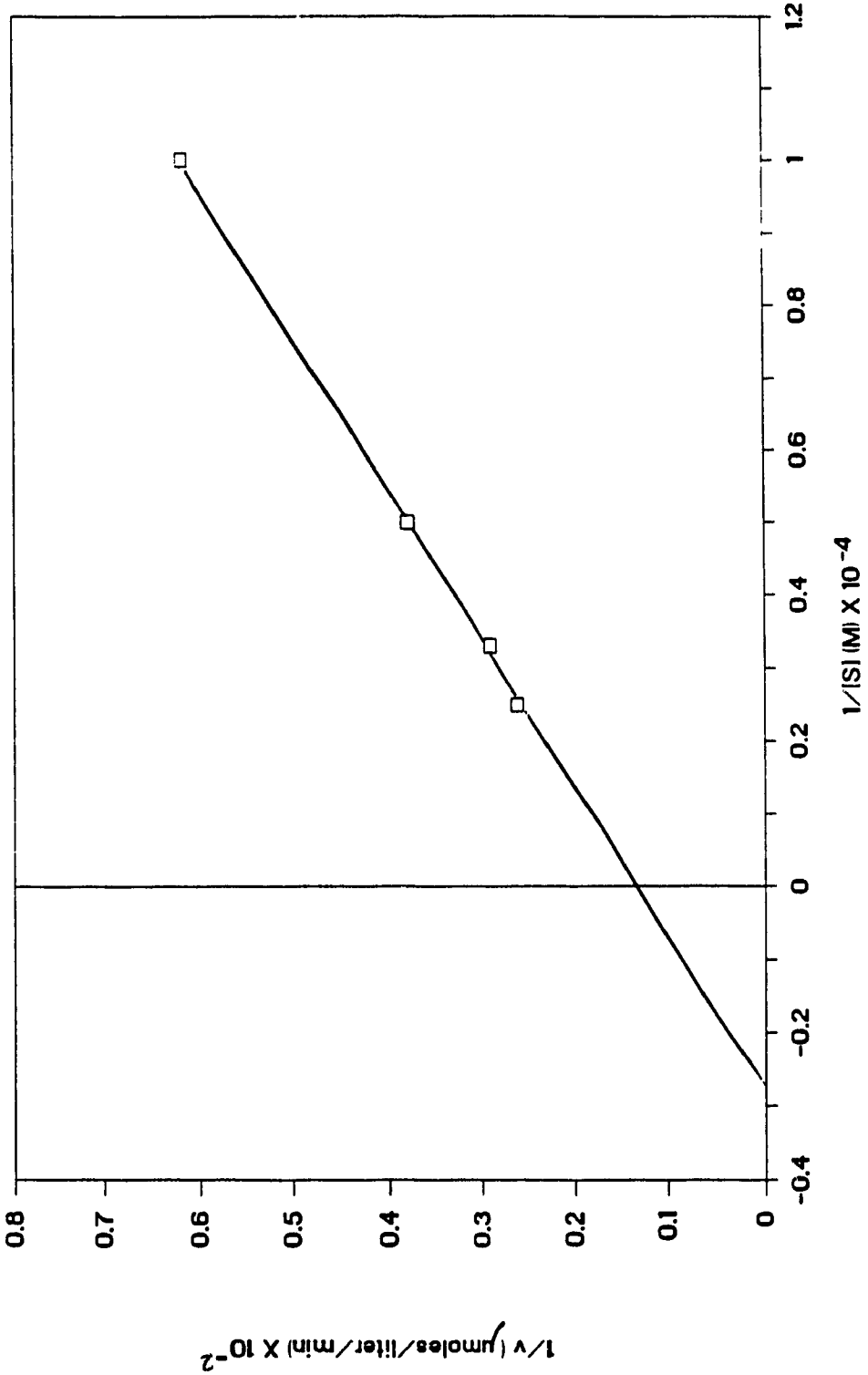

1/v (μmoles/liter/min) X 10^-2
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1
1.2
1/[S] (M) X 10^-4

Fig 15. **Lineweaver-Burk plot of lipoxygenase isozyme IIB.**

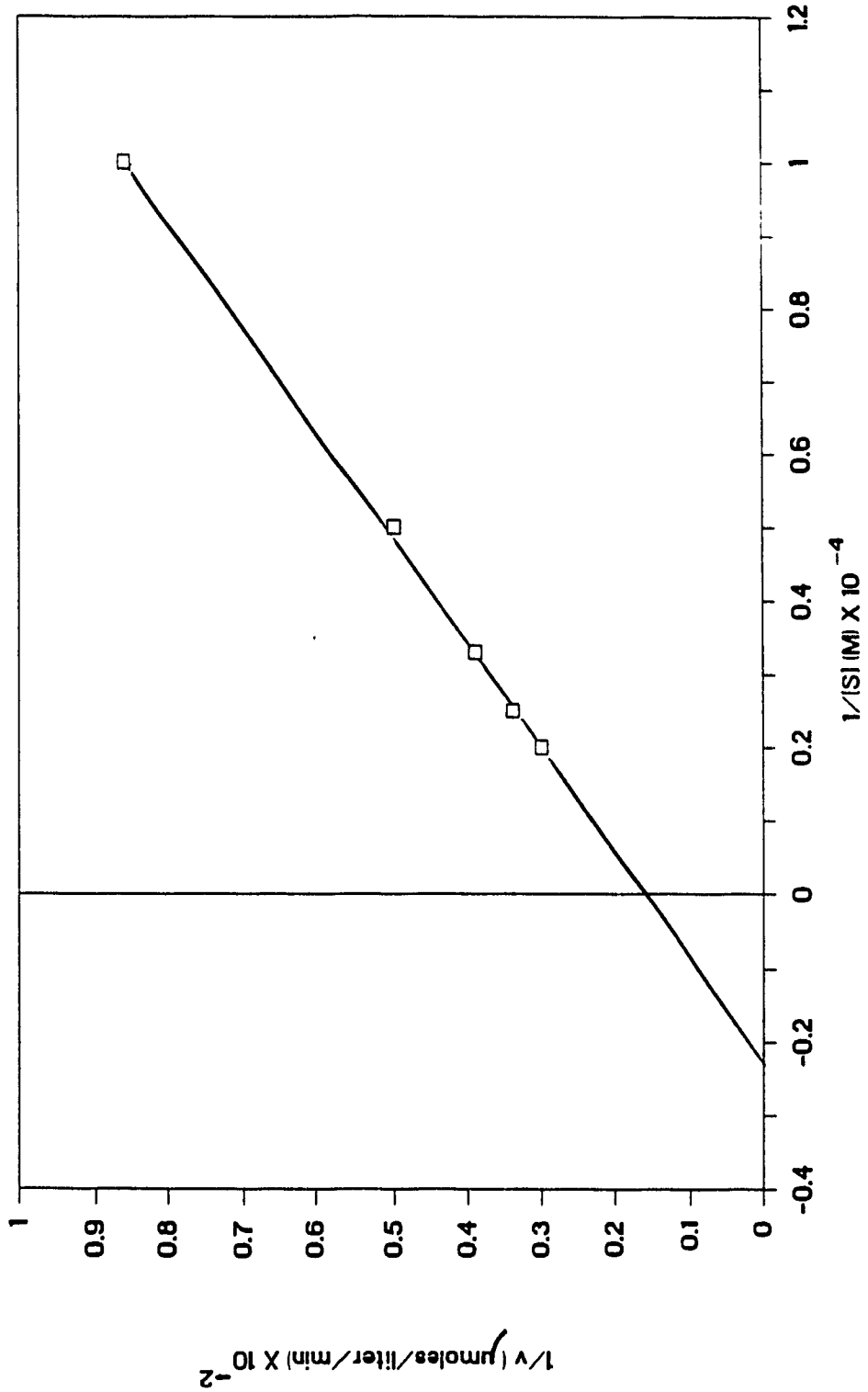

1/v (μmoles/liter/min) X 10⁻²
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1
1.2
1/[S] (M) X 10⁻⁴

Fig 16. Lineweaver-Burk plot of lipoxygenase isozyme III.

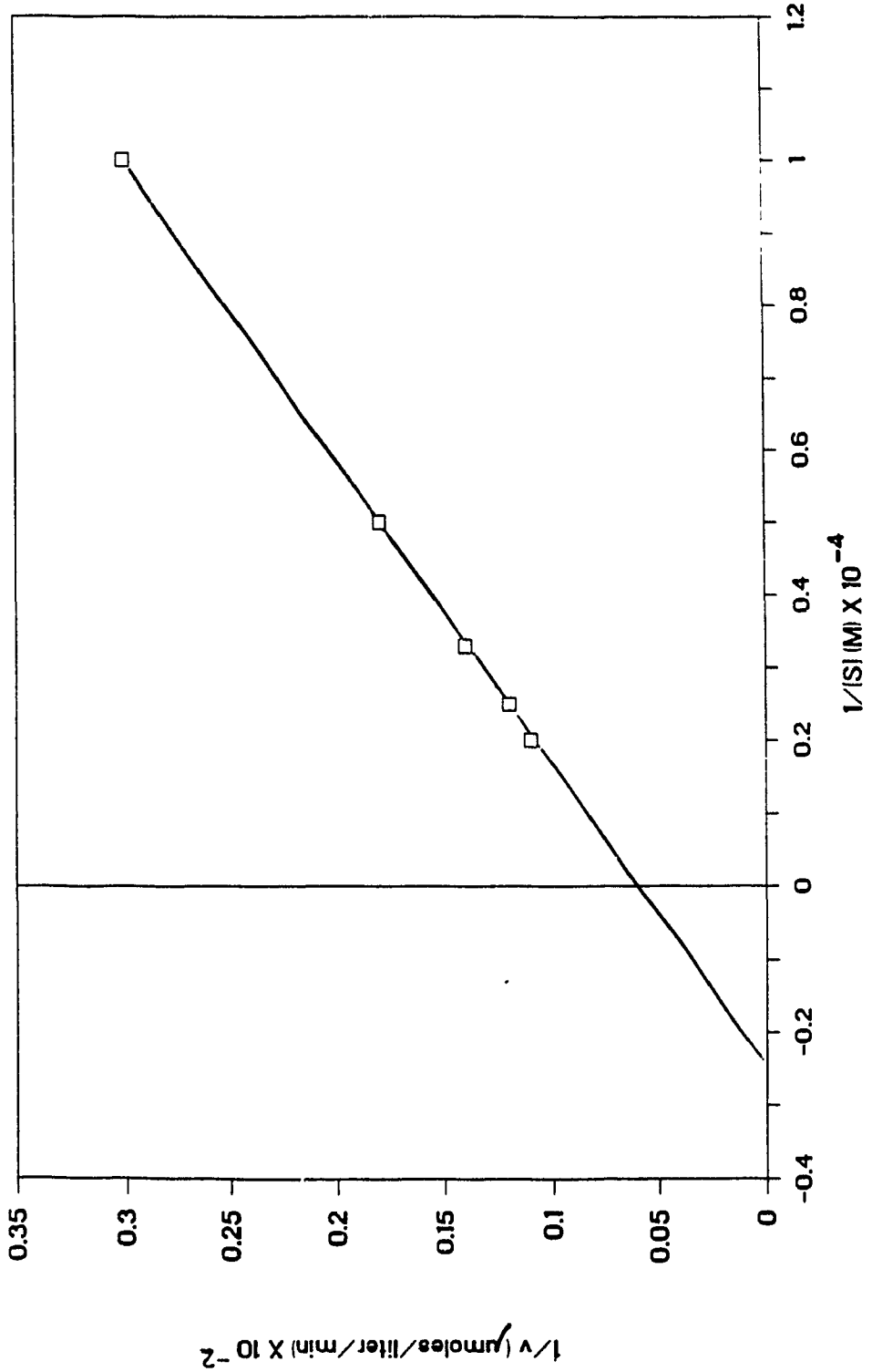
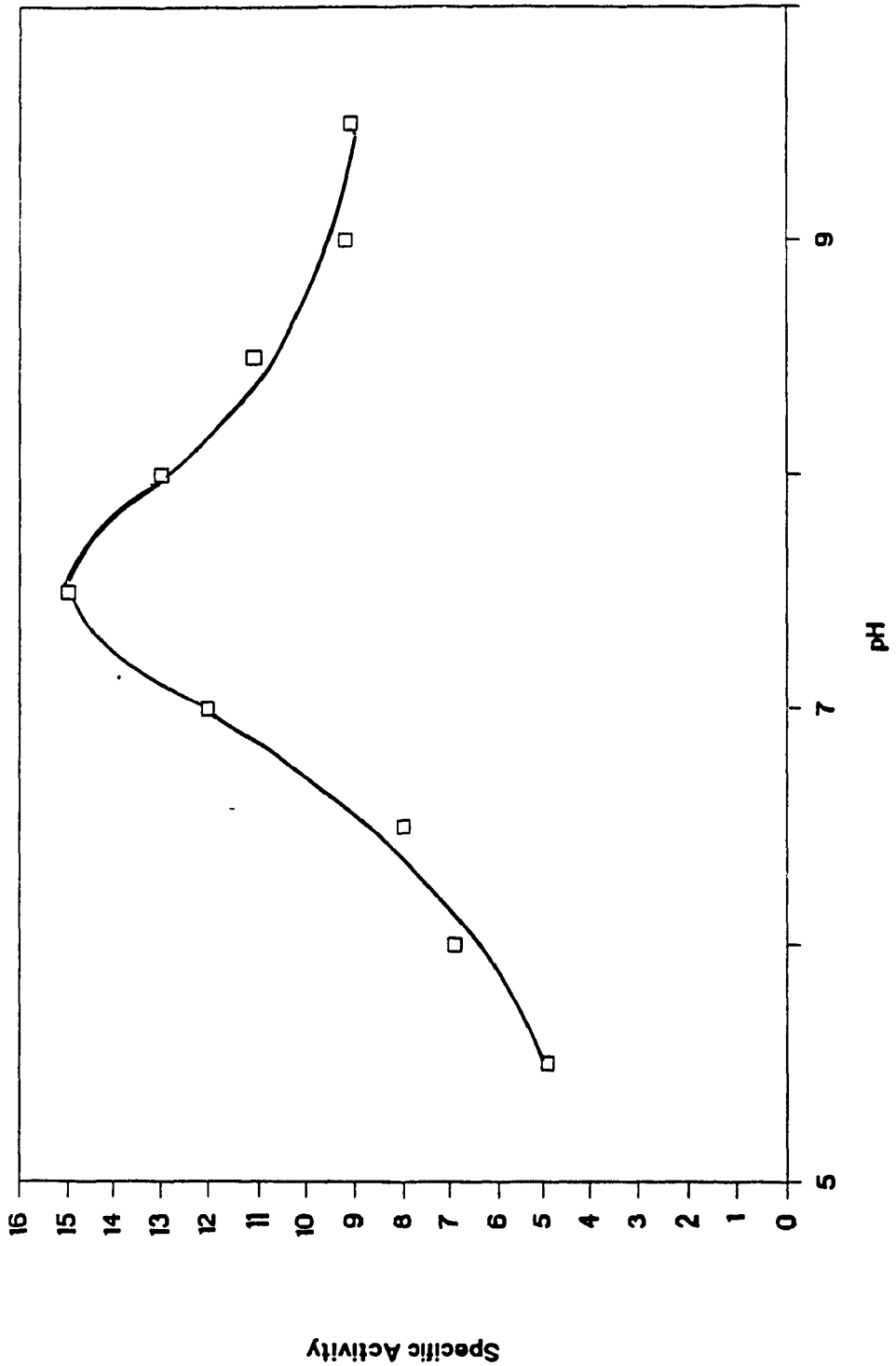

1/v (μmoles/liter/min) X 10 ⁻²
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1
1.2
1/[S] (M) X 10⁻⁴

Fig 17. **The effect of pH on lipoxygenase activity of Canola seed extract.**


Specific Activity
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
5
7
9
pH

The optimum pH of Canola lipoxygenase isozymes was also studied and showed certain similarity to those reported from other sources. The results (Fig. 18) showed that the maximum activity for isozyme I was observed at pH 6.5; this result was similar to those obtained for soybean lipoxygenase-2 (Andrawis *et al.*, 1982) and small faba beans) and (Eskin and Henderson, 1974), for wheat germ lipoxygenase-2 and -3 (Nicolas *et al.*, 1982) and wheat germ lipoxygenase-2 (Wallace and Wheeler, 1979). The results were also close to that reported for pea lipoxygenase-2 (6.8; Reynolds and Klein, 1982). On the other hand, the optimum pH for isozyme IIA, IIB and III was 6.0. These findings were identical to those reported for winged bean lipoxygenase FI (Van Den *et al.*, 1982) and wheat germ lipoxygenase-1 (Wallace and Wheeler, 1979) and close to those reported for soybean lipoxygenase-2 (6.2; Deil and Stan, 1978) peanut lipoxygenase-2 and -3 (6.2; Sanders *et al.*, 1975) and for pea lipoxygenase-2 (6.3; Arens *et al.*, 1973).

4.3.3. Fatty Acids Analysis Canola Lipid

The fatty acids analysis of endogenous Canola seeds lipid extract, performed by gas chromatography, is summarized in Table 10. The results show the presence of high percentage of oleic acid (51.3%) and linoleic acid (35.2%). The overall analysis is close to that reported in the literature (Eskin, 1987).

4.3.4. Substrate Selectivity

The substrate specificity of Canola lipoxygenase extract (fraction 3) was assayed for free linoleic acid, linolenic acid, monolinolein, dilinolein, trilinolein and the endogenous Canola lipid extract used as substrates. The results (Table 11) indicate that the partially purified Canola extract (fraction 3) has higher specificity for linoleic acid compared to that for linolenic acid. These results agree with those reported for winged bean

Fig 18. Optimum pH for lipoxygenase isozyme I (□ ----- □), isozyme IIA (O ----- O), isozyme IIB (● ----- ●) and isozyme III (Δ ----- Δ).

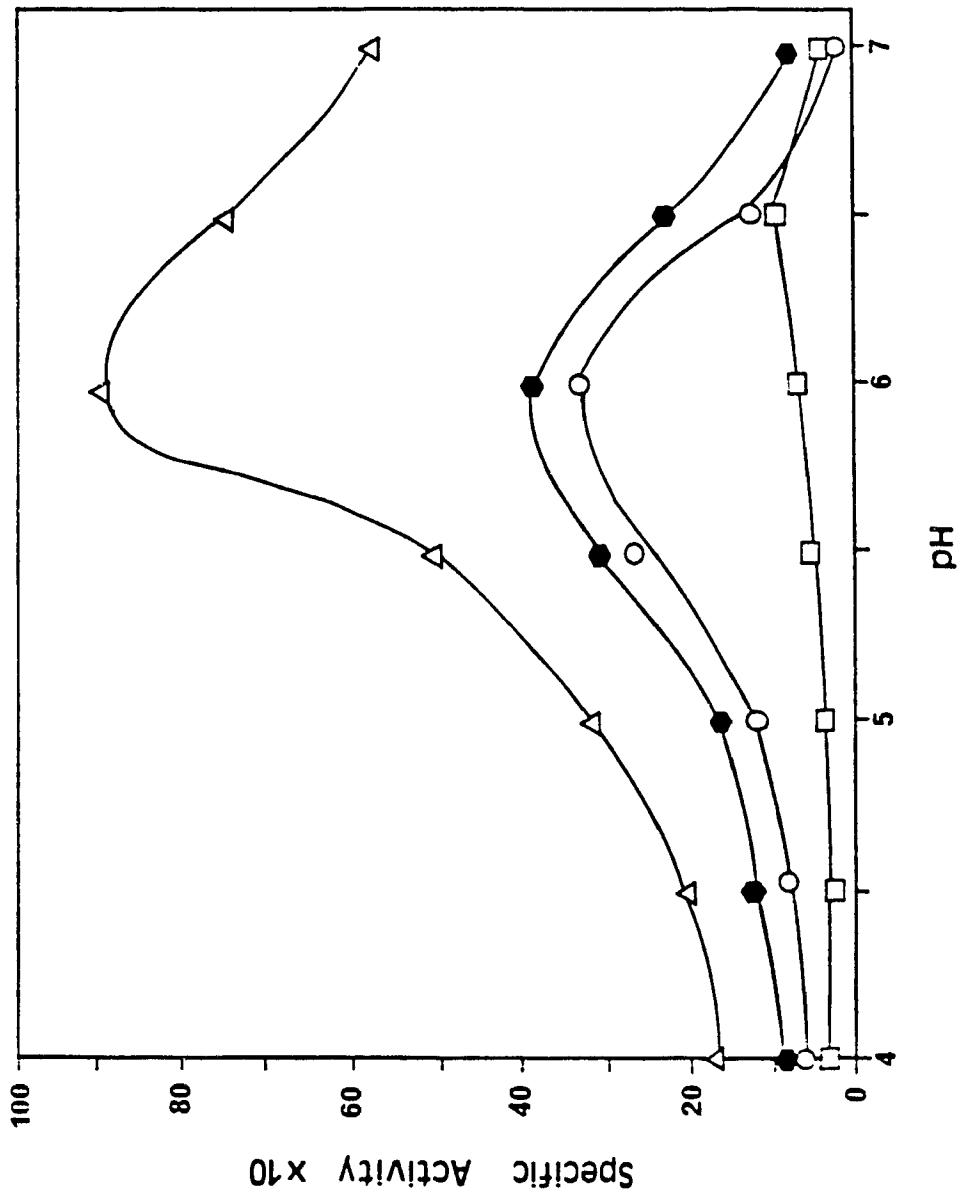

Specific Activity x10
100
80
60
40
20
0
4
5
6
7
pH

Table 10. Fatty acid composition of Canola lipid extract.

	Relative (%)[a]	
Fatty acid	**Canola[b]**	**Reference[c]**
16:0	6.8	4.0
18:1	51.3	55.0
18:2	35.2	26.0
18:3	6.7	10.0

[a]Percent of each fatty acid relative to the total fatty acids content in the lipid fraction.

[b]Fatty acid analysis of Canola (Brassica napus var. Westar) used in this study.

[c]Fatty acid analysis of Canola (Brassica napus var. Regent), previously reported by Eskin (1987).

Table 11. Substrate selectivity of the partially purified lipoxyenase of Canola seed extract (fraction 3).

Source of Lipoxygenase	Specific activity[a]					
	Linoleate	Linolenate	Monilnolein	Dilinolein	Trilinolein	Canola oil
Canola[b]	19.1	5.7	8.5	7.25	1	16
Soybean[c]	58.5	51.4	28.3	25	4.5	8

[a]Specific activity is defined as A/mg protein/min, where A is equal to 0.001 absorbance at 234 nm.

[b]Partially purified Canola lipoxygenase obtained by precipitation with ammonium sulfate at 20-50 of saturation (fraction 3).

[c]Commercial soybean lipoxygenase (Sigma Chemical Co.).

lipoxygenase (Van Den *et al.*, 1982). On the other hand, commercial soybean lipoxygenase demonstrates enzymatic specificity with both linoleic and linolenic acids (Table 11); these findings are similar to those reported for lipoxygenase from potato tuber (Pinsky *et al.*, 1973) and germinated sunflower seed (Leoni *et al.*, 1985). The results (Table 11) also show that fraction 3 has greater specificity for linoleic acid as substrate when compared with linoleic esters. The specificity of lipoxygenase toward the esters linoleic acid was investigated. The observed order of activity was as follows: linoleic> monolinolein> dilinolein> trilinolein. These results are similar to those reported for the partially purified lipoxygenase from French bean lipoxygenase (Kermasha and Metche, 1986). Eggplant lipoxygenase showed the order of activity with substrates as: linolenic> linoleic> dilinolein> trilinolein (Grossman *et al.*, 1972). However, these findings are different from those reported for asparagus (Ganthavorn and Powers, 1989) which showed that the asparagus lipoxygenase was active with monolinolein as well as linoleic acid, but was less active with dilinolein and trilinolein. Apple lipoxygenase showed higher activity with linolenic acid when compared to linoleic acid (Kim and Grosch 1979). Eskin and Henderson (1974) reported the partially purified lipoxygenase from small faba beans demonstrated higher activity with linoleic acid when compared with linolenic acid, however, the enzyme shows similar trends in activity towards monolinolein, dilinolein and trilinolein.

On the other hand, Canola lipoxygenase showed higher specificity for the endogenous lipid extract compared to that for linoleic acid and its esters (monolinolein, dilinolein and trilinolein). The greater activity of lipoxygenase observed with the endogenous lipid extract could be explained by the concept of preferred conformational enzyme-endogenous substrate reaction which may be related to the charge and polarity of the substrate (Bild *et al.*, 1977). However, this higher activity was not due to the

presence of free fatty acids whose content in Canola lipid is relatively low (0.5-0.8%; Eskin, 1987).

The substrate specificity of lipoxygenase isozymes is shown in Table 12. Isozyme I displayed preferential activity for monolinoleate followed by a tendency for monolinoleate and trilinoleate. However, there was no enzymatic activity observed for both isozymes I and IIA towards free linoleic acid. Isozyme IIB showed common activity towards free linoleate as well as monolinoleate, dilinoleate and trilinoleate. On the other hand, isozyme III showed significant preferential activity towards free linoleate. Reynolds and Klein (1982) indicated that Type-1 pea lipoxygenase was active on methyl linoleate and trilinolein but to a less extent than its activites on linoleic acids. Pea lipoxygenase-1 was active with trilinolein but was less active on linoleic acid (Reynolds and Klein, 1982). Pea lipoxygenase-1 effectively catalayzed the oxidation of linoleate, linolenate and trilinolein substrates but exhibited much lower activity than type-2 pea lipoxygenase (Yoon and Klein, 1979). The activities of winged bean isozymes FI and FII using linolenic acid as substrate were, respectively 23% and 17% of those resulted when linoleic acid was used as substrate (Van Den *et al.*, 1982b); however, monolinolein, trilinolein, linoleic methyl ester and linoleic ethyl ester were not oxidized by the enzyme preparations. For the two fractions of the purified soybean lipoxygenase, one showed preferential activity for the free fatty acid while the other was for the methyl ester (Veruhe and Franke, 1972). It has been suggested that there are apparently two different types of lipoxygenase, namely the free fatty acids lipoxygenase and the triglyceride lipoxygenase (Koch *et al.*, 1958).

4.3.5. Effect of Cyanide

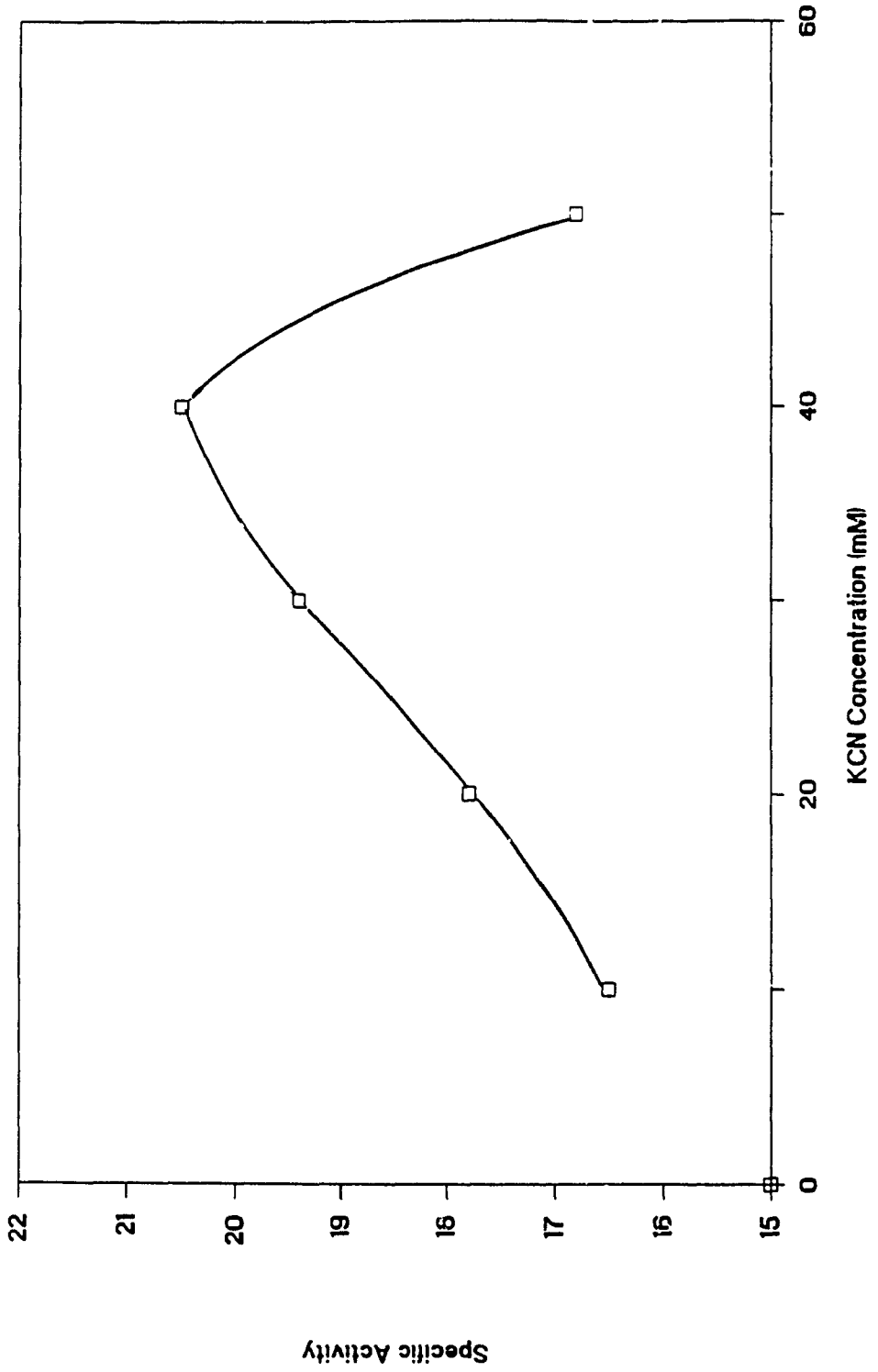
The results (Fig. 19) indicate that the addition of cyanide to the partially purified enzyme extract (fraction 3) resulted in an increase in the apparent enzyme activity,

Table 12. Substrate selectivity of lipoxygenase isozymes of Canola seed.

	Specific activity[a]			
Isozyme	**Linoleate**	**Monlinolein**	**Dilinolein**	**Trilinolein**
I	0.0	0.68	0.59	0.00
IIA	0.0	0.02	0.12	0.03
IIB	0.06	0.10	1.17	0.08
III	0.98	0.5	0.13	0.03

[a]Is defined as A/mg protein, where A is equal to 0.001 absorbance at 234 nm or 238 nm.

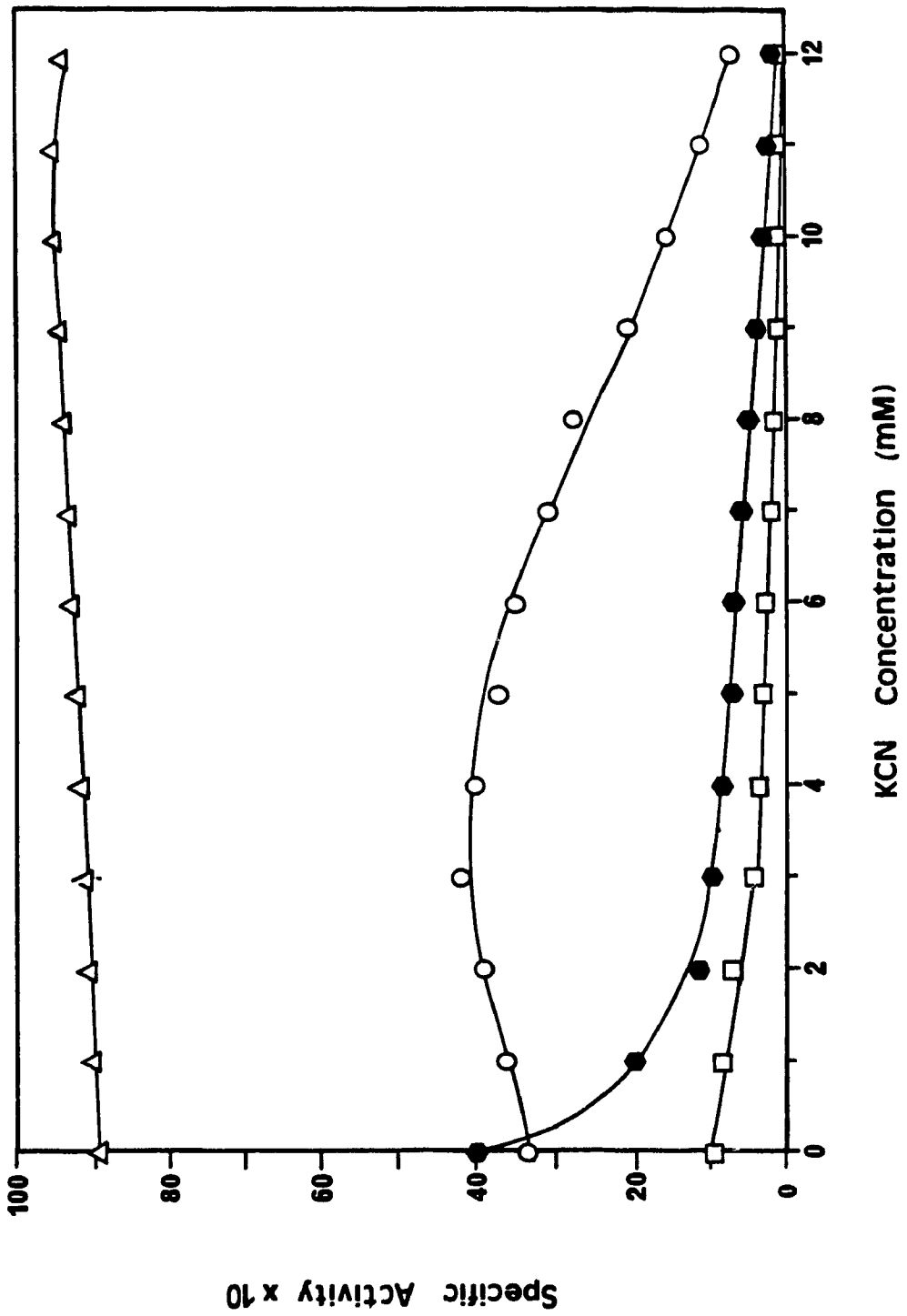
Fig 19. **The effect of cyanide concentration on the partially purified lipoxygenase activity from Canola seed extract (fraction 3).**


Specific Activity
22
21
20
19
18
17
16
15
0
20
40
60
KCN Concentration (mM)

however, concentration above 40 mM let to a decrease in lipoxygenase activity. These results are similar to those reported for the partially purified French bean lipoxygenase (Kermasha and Metche, 1986). Cyanide ion was reported to inhibit the partially purified lipoxygenase from peanut at concentrations greater than 1 mM (Siddiqi and Tapple, 1957) while De Lumen *et al.* (1978) reported that 50 % inhibition of green bean lipoxygenase was obtained at 20 mM cyanide concentration. Ganthavorn and Powers (1989) reported that 90 % of the asparagus lipoxygenase activity was inhibited by 5×10^{-4} M KCN whereas the 50 % ammonium sulfate pellet was less markedly inhibited by cyanide. The apparent increase in lipoxygenase activity in the presence of low concentrations of cyanide could be explained by the fact that the partially purified extract may contain other competing enzymes such as, catalase and peroxidases, which are also known to be inhibited by cyanide (Siddiqi and tapple, 1956). The addition of cyanide could initially inactivate catalase and peroxidase, preventing their action on linoleic acid substrate.

The effect of cyanide on enzyme activity showed (Fig. 20) that the activity of isozymes I and IIA were inhibited completely by the addition of 10 mM and 4 mM. The profile inactivation of Canola lipoxygenase isozymes I and IIA were similar to those reported for winged bean lipoxygenase FI and FII (Van Den *et al.*, 1982b). However, the addition of 3 mM and 10 mM KCN, respectively, to isozymes IIB and III increased the activity by approximately 20% and then decreased at higher concentration of cyanide (Fig. 20). On the other hand, peanut lipoxygenases L-2 and L-3 were inhibited completely by the addition of less than 1 mM cyanide but concentration below 14 mM had little effect on L-1 alkaline isozyme (Sanders *et al.*, 1975). However, contradictory results have been reported on the effect of cyanide ion on various lipoxygenase. Grossman *et al.* (1972) showed that was no inhibitory effect by cyanide on the eggplant

Fig 20. The effect of cyanide concentration on lipoxygenase isozyme I (□ ----- □), isozyme IIA (● ----- ●), isozyme IIB (○ ----- ○) and isozyme III (△ -----△).


Specific Activity x 10
0
20
40
60
80
100
0
2
4
6
8
10
12
KCN Concentration (mM)

partially purified lipoxyegenase. The conflicting reports suggested that it is difficult to demonstrate the real effect of cyanide on lipoxygenase activity.

4.3.6. Maximum Absorbance of End-Products

The linoleic hydroperoxides resulted from the enzyamtic activity were scanned at a range of wavelengths (230-240 nm). The results (Fig. 21) indicated that linoleic hydroperoxide resulted for enzymatic reaction of isozyme I, IIB and III absorbed at 234 nm, whereas those resulted by isozyme IIA absorbed at 238 nm. The absorbance at 234 nm was accepted as the most common wavelength used for the determination of lipoxygenase when linoleic acid is used as substrate (Klein, 1976; Chen and Whitaker, 1986; Axelrod *et al.*, 1981; Reynolds and Klein, 1982ab; Shastry and Raghavendra, 1975; Grosch and Laskawy, 1975). However, other wavelengths were also used. Al-Obaidy and Siddiqi (1981) as well as O'Reilly *et al.* (1969) measured the activity at 232.5 nm whereas Axelrod *et al.*, (1981) reported that the activity of lipoxygenase-2 was measured at 238 nm using arachidonic acid as substrate.

4.4. Electrophoresis

Electopherograms of disc gel electophoresis of the crude extract (fraction 1) and the partially purified lipoxygenase (fraction 3) from Canola seed as well as commercial soybean lipoxygenase (Sigma Chemical Co.) are shown in Figure 22. The results showed that the crude extract (fraction 1) was separated into two major and four minor bands (Fig. 22A) while the partially purified enzyme extract (fraction 3) shows the presence of one major and four minor bands (Fig. 22B). Comparative electrophoresis of commercial soybean lipoxygenase shows (Fig. 22C) the presence of four major and one minor bands.

Fig 21. **Maximum absorbance of hydroperoxides of linoleic acid resulted from the lipoxygenase reaction of isozyme I (□ ----- □), isozyme IIA (⬢ ----- ⬢), isozyme IIB (○ ----- ○) and isozyme III (△ ----- △).**

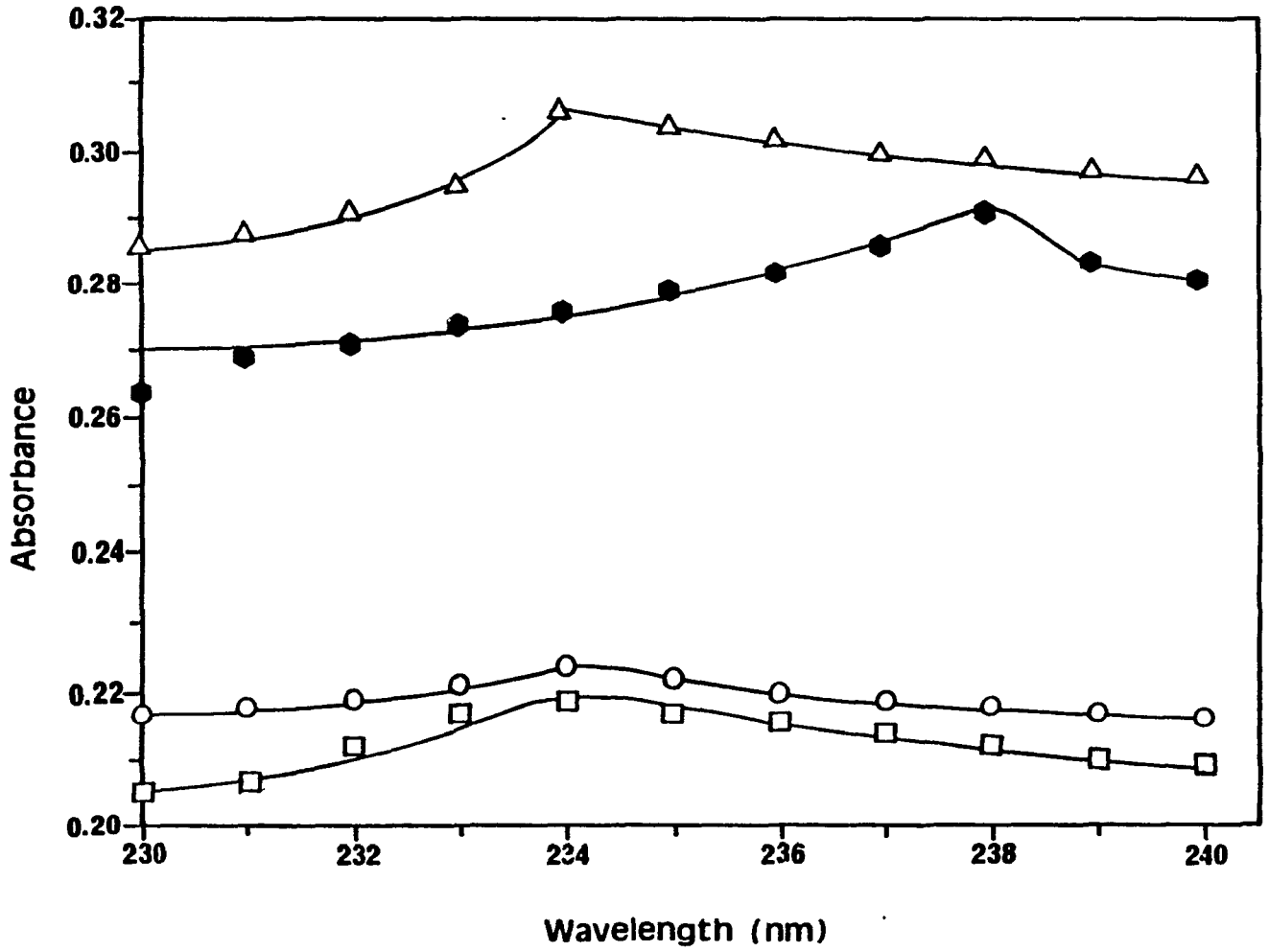
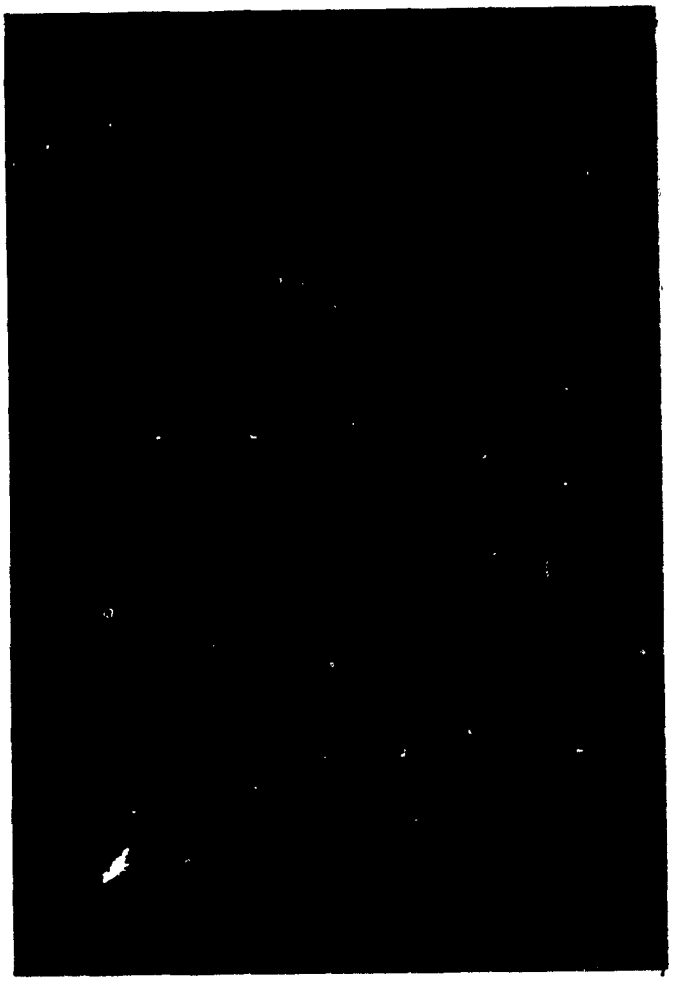

0.32
0.30
0.28
0.26
0.24
0.22
0.20
Absorbance
230
232
234
236
238
240
Wavelength (nm)

Fig 22. **Polyacrylamide (PAGE) disc electropherograms: (A) crude lipoxygenase of Canola seed (fraction 1), (B) partially purified lipoxygenase of Canola seed fraction 3) and (C) commercial soybean lipoxygenase.**



A B C

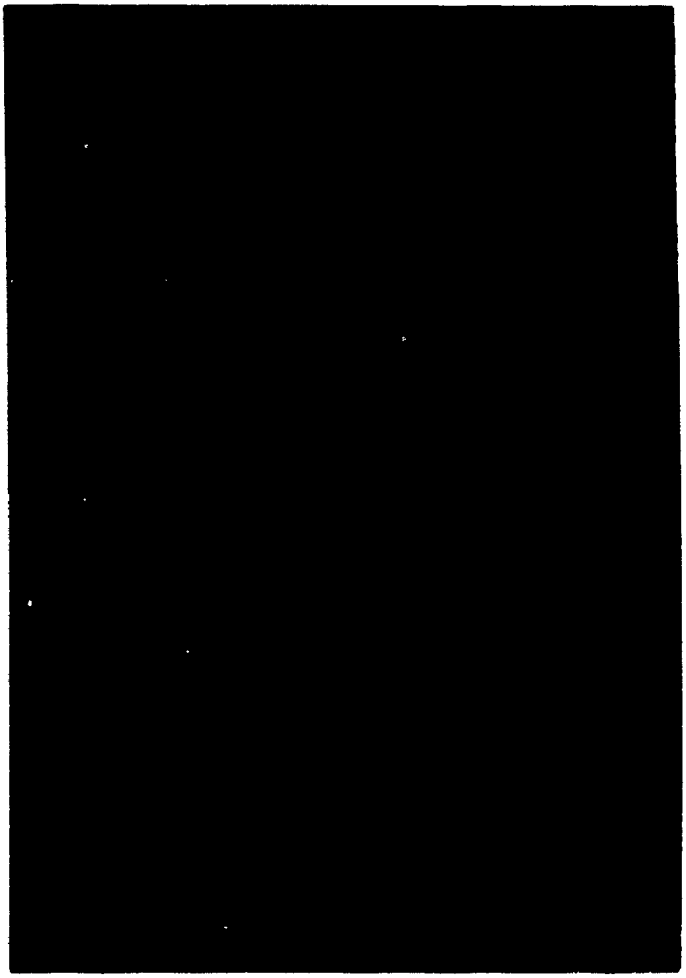
A chromogenic reaction on polyacrylamide-disc gel electrophoresis (Fig. 23) demonstrated that the enzyme activity in the crude extract (Fig. 23A) and the partially lipoxygenase purified (Fig. 23B) is associated with the major band in each gel. This chromogenic reaction confirmed the presence of lipoxygenase activity in Canola seed extract as compared to that of commercial soybean lipoxygenase (Fig. 23C).

SDS-PAGE (Fig. 24) electrophoresis of the crude extract (fraction 1), the partially purified enzyme extract (fraction 3) and the commercial soybean lipoxygenase were performed on gel electrophoresis. Crude extract lipoxygenase was separated into one major and nine minor fractions (Fig. 24A) and the partially purified extract into one major and three minor fractions (Fig. 24B). By comparison, the commercial soybean lipoxygenase was separated into four major fractions and at least four minor fractions (Fig. 24C). Haydar and Hadziyev (1973) showed the presence of three to four lipoxygenase bands in the pea seeds extract. Klein (1976) reported that lipoxygenase from dried split pea seed showed one major and two trace bands.

The homogeneity of lipoxygenase isozymes was examined by polyacrylamide gel electrophorseis and SDS-PAGE (Fig. 25). SDS-PAGE showed the presence of one single band for lipoxygenase isozyme I, IIA, IIB and III. The presence of a single protein band was reported by many workers for rice bran and embryo lipoxygenase-3 (Ohta *et al.*, 1986), for immature English pea lipoxygenase-1 (Chen and Whitaker, 1986), for pea lipoxygenase-1 (Reynolds and Klein, 1982) and for soybean lipoxygenase-2 (Christopher *et al.*, 1970 and Andrawis *et al.*, 1982).

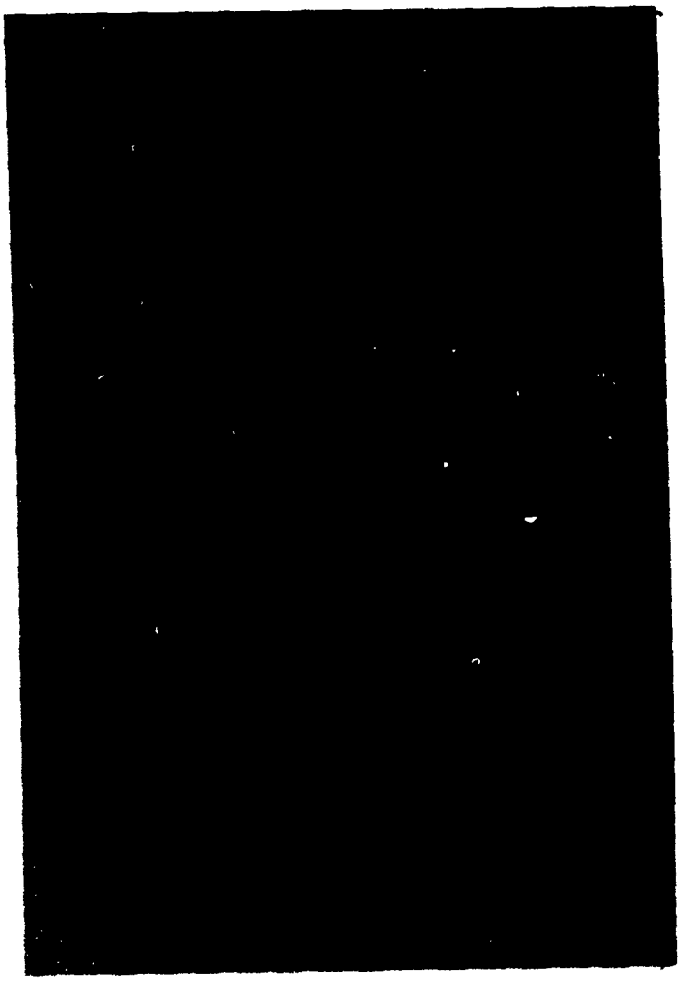
Lipoxygenase isozyme electorpherograms by SDS-PAGE disc gel electrophoresis were scanned at 600 nm using CDS-200 Computing Densitometer System. The results showed that a single peak was obtained for each isozyme (Fig. 26). Deil and Stan (1978) reported that the scanning at 600 nm of pclyacrylamide gel electorpherograms of

Fig 23. **Chromogenic electropherograms: (A) crude lipoxygenase of Canola seed (fraction 1), (B) partially purified lipoxygenase of Canola seed (fraction 3) and (C) commercial soybean lipoxygenase, incubated with linoleic acid used as substrate.**



A B C

Fig 24. **SDS-PAGE disc electropherograms: (A) crude lipoxygenase of Canola seed (fraction 1), (B) partially purified lipoxygenase of Canola seed fraction 3) and (C) commercial soybean lipoxygenase.**



A B C

Fig 25. SDS-PAGE disc electropherograms: (A) isozyme I, (B) isozyme IIA, (C) isozyme IIB and (D) isozyme III.

A B C D

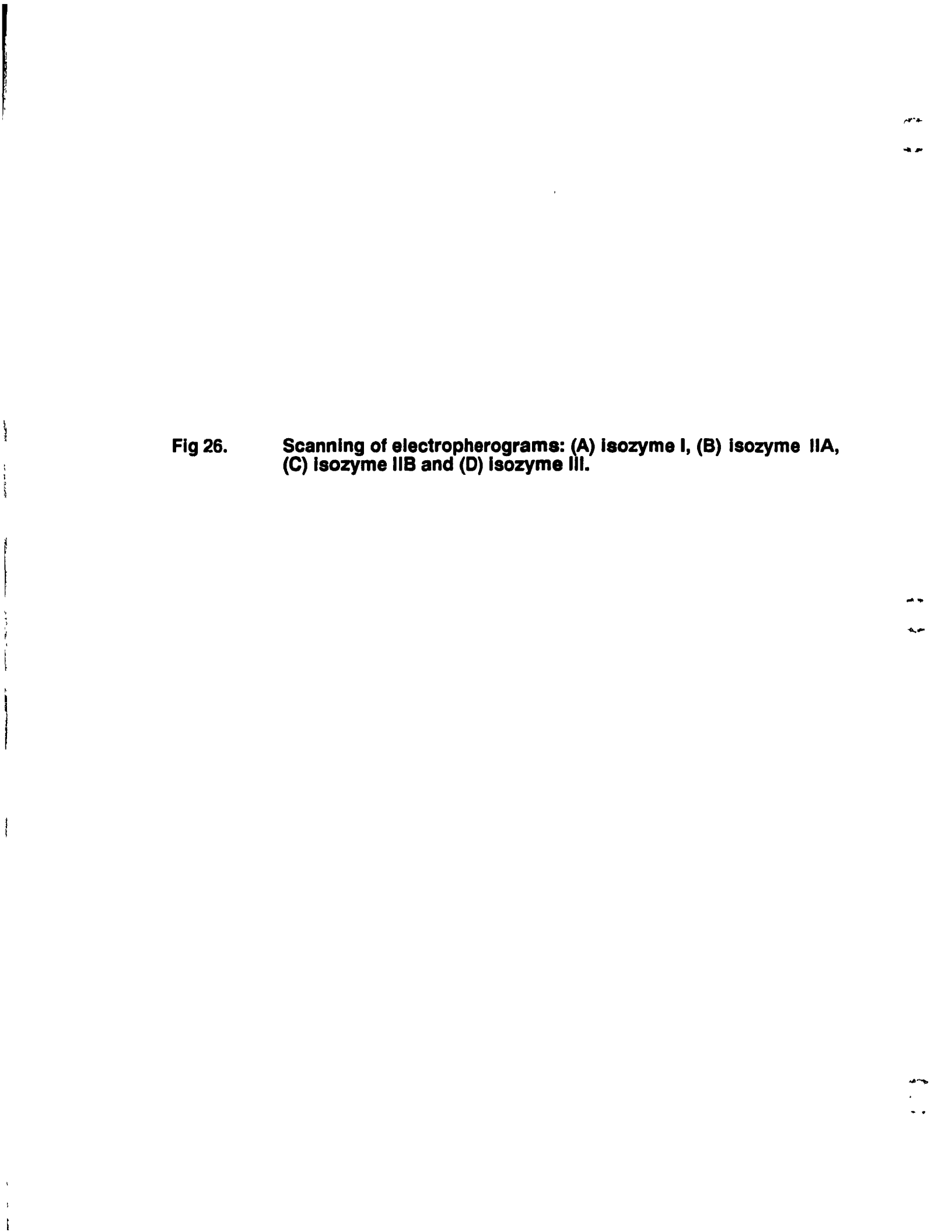
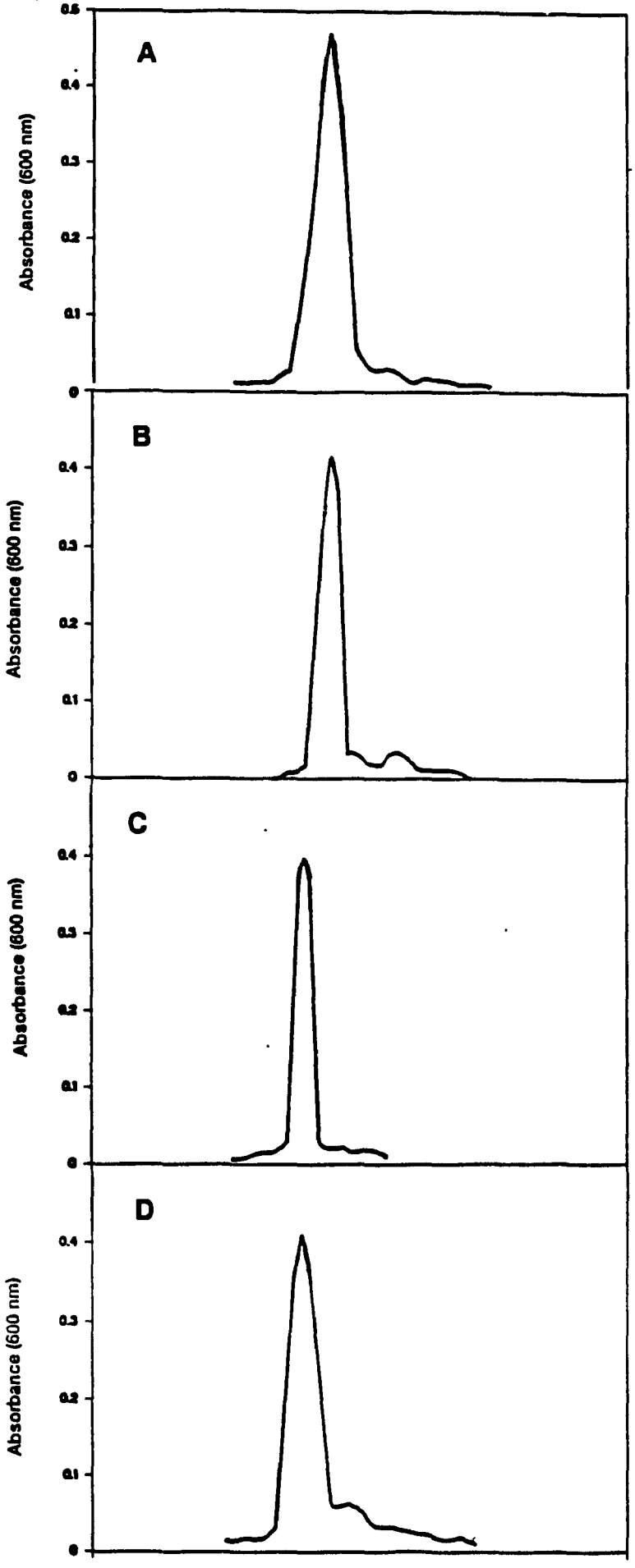


Fig 26. **Scanning of electropherograms: (A) isozyme I, (B) isozyme IIA, (C) isozyme IIB and (D) isozyme III.**


A
B
C
D
Absorbance (600 nm)
Absorbance (600 nm)
Absorbance (600 nm)
Absorbance (600 nm)

soybean lipoxygenase isozymes L-1 and L-2 resulted by a single peak for each isozyme. The presence of a single polypeptide chain was reported by many workers for rice bran and embryo lipoxygenase-3 (Ohta *et al.*, 1986), for immature English peas lipoxygenase-1 (Chen and Whitaker, 1986), for peas lipoxygenase-1 (Reynolds and Klein, 1982), and for soybean lipoxygenase-2 (Christopher *et al.*, 1970; Andrawis *et al.*, 1982). Nicolas *et al.* (1982) showed that wheat germ lipoxygenase isozymes, L-2 and L-3, were electrophometrically pure whereas L-1 was still contaminated by inactive proteins.

4.5. Molecular Weight Determination

The molecular weights of lipoxygenase isozymes were estimated, using Superose-12 HR 10/30 gel filtration column. The average molecular weight of isozyme I was 72,000; this results was close to those reported for peanut lipoxygenase L-1, L-2 and L-3 (73,000; Sanders *et al.*, 1975), for avocado lipoxygenase (74,000; Marcus *et al.*, 1988). Isozyme IIA was 106,000; this number was very close to those number reported for soybean lipoxygenase L-1 and L-2 (105,000; Diel and Stan, 1978), lower than that reported for wheat lipoxygenase isozymes L-3 (110,000; Nicolas *et al.*, 1982) and for green algae lipoxygenase (124,000; Beneytont *et al.*, 1989) but higher than that of number reported for immature English pea lipoxygenase (100,000; Chen and Whitaker, 1986). Isozyme IIB was 78,000; this number was similar to that reported for peas lipoxygenase-2 (Arens *et al.*, 1973) and close to that reported for winged bean lipoxygenase isozymes FI and FII (80,000; Van Den *et al.*, 1982). Isozyme III exhibited an apparent molecular weight of 62,000; this results was close to those obtained for pea lipoxygenase L-1 and L-2 (64,000 and 65,000; Yoon and Klein, 1979). However, there was some variation in the molecular weight of lipoxygenase in the literature as soybean enzyme is generally accepted to have a molecular weight of 100,000.

CONCLUSION

The isolation, purification and characterization of lipoxygenase enzyme from Canola seed (*Brassica napus* cv, Westar) were carried out. The partially purified enzyme showed an optimum activity at pH 7.5 and it's K_m value was 2×10^{-4} M. The activity of the enzyme extract was considerably greater with linoleic acid than with its esters; the observed order of activity was as follows: linoleic acid > monolinolein > dilinolein > trilinolein. The partially purified extract showed higher specificity for linoleic acid than for linolenic acid, however, the enzyme showed higher specificity for the endogeneous lipid extract than for linoleic acid. The addition of cyanide to the partially purified extract resulted in an increase in the apparent enzyme activity, however, concentrations above 40 mM led to a decrease in this activity. A chromogenic reaction on polyacrylamide-disc gel electrophoresis demonstrated that lipoxygenase enzyme activity is associated with the major band.

Conventional ion-exchange chromatography on DEAE-cellulose for the partially purified enzyme demonstrated the presence of a major and two minor protein fractions which were also found in a commercial soybean lipoxygenase form. However, the lipoxygenase activity profile indicates the presence of a single enzymatically active fraction in both Canola seed extract and commercial soybean lipoxygenase.

The purification of the partially purified by a successive chromatography on ion-exchange column (Mon Q HR) and gel filtration column (Superose-12 HR), using FPLC system, resulted into four well separated isozymes (I, IIA, IIB and III). SDS-PAGE gel electrophoresis and scanning for electrophoresis demonstrated the presence of a single protein band for each of the four isozymes. The molecular weights of isozymes I, IIA, IIB and III were, respectively, 72,000, 106,000, 78,000 and 62,000.

The optimum pH for lipoxygenase activity was 6.5 for isozyme I and 6.0 for isozyme IIA, IIB and III. The apparent K_m values for isozymes I, IIA, IIB and III were,

respectively, 5.5 x 10^{-4} M, 3.5 x 10^{-4} M, IIB 4 x 10^{-4} M and 3.8 x 10^{-4} M. The substrate specificity showed that isozyme I displayed a preferential activity for monolinoleate and dilinoleate, whereas isozyme IIA demonstrated showed a preferential activity for dilinoleate followed by a tendency for mono- and trilinoleate. However, there was no enzymatic activity observed by both isozymes I and IIA toward free linoleic acid. Isozyme IIB showed common activity towards free linoleate as well as mono-, di-and trilnoleate. Isozyme III showed siginficant a preferential activity towards free linoleate. The effect of cyanide on isozymes activity showed that the activity of isozyme I and IIA was inhibited completely with addition of 10 mM and 4 mM KCN, respectively. However, the addition of 3 mM and 10 mM KCN, respectively, to isozyme IIB and III increased the activity by approximately 20 % before being decreased at higher concentration of cyanide.

The lipoxygenase of Canola has not been studied either in terms of isolation or in terms of its physiochemical characteristics. The data gathered in this thesis demonstrated the presence of lipoxygenase activity in Canola seed. Overall, the results showed certain similarity to lipoxygenase from other beans, although a direct comparison was difficult.

This work has provided a better understanding for the role of lipoxygenase in the formation of hydroperoxides of polyunsaturated fatty acids in Canola and in turn may provide a means of controlling the oxidation process. This could result in efforts to improve the organoleptic quality of Canola oil.

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Publications

1. Khalyfa, A., Kermasha, S. and Alli, I. (1989). Purification and characterization of lipoxygenase from Canola seed (*Brassica napus* cv, Westar). *Can Inst. Food Sci. Technol.* 32nd Conference, June 4-7, Québec.

2. Kermasha, S., Khalyfa, A., Alli, I., and Lee, B.H. (1990). Purification and characterization of lipoxygenase isozymes from Canola seed (*Brassica napus* cv, Westar). Abstract acceptted to the Institute Food Technol. Conference, June 17-21, Anahiem, California.

3. Khalyfa, A., Kermasha, S. and Alli, I. (1990). Partial purification and characterization of lipoxygenase from Canola Seed (*Brassica napus* cv, Westar). Acceptted to the *J. Agric. Food Chem.*

4. Khalyfa, A., Kermasha, S., Alli, I., and Lee, B.H. (1990). Purification and characterization of lipoxygenase isozymes from Canola Seed (*Brassica napus* cv, Westar). In Preparation.