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by

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Two enzyme forms (PAO-I and PAO-II) of porcine liver aldehyde oxidase (aldehyde: oxygen oxidoreductase, EC 1.2.3.1) were purified to homogeneity using affinity chromatography. The heat treatment and ammonium sulfate fractionation steps resulted only in a partial purification. Both enzyme forms, PAO-I and PAO-II, have similar pIs of 5.8 and molecular weight of 262,000 and 255,000 daltons, respectively.

The two enzyme forms exhibited different substrate specificities. Compared to PAO-II, PAO-I showed higher affinity for the medium-chain aldehydes (pentanal and hexanal) which are mainly responsible for the greeny and beany off-flavor of soybean products. The PAO-I enzyme form, which is more specific in utilizing medium-chain aldehydes, could be used for reducing the off-flavors associated with soybean proteins.

PAO-I was selected to study pH and temperature stability. Purified PAO-I was stable between pH of 7.1 and 10.7 and at temperatures up to 45 °C. Energy of denaturation for PAO-I (158.1 kJ/mol · K^{-1}) was more than three-fold higher than the energy of activation (47 kJ/mol · K^{-1}). The enzyme was more stable at alkaline pHs than at neutrality and repeated freezing and thawing did not inactivate the enzyme.

The two pK values of 6.2 and 11.3 of PAO-I indicated the presence of histidine and guanidine residues in the ES complex. The estimated pK values of 7.5 and 9.9 for the n-pentanal binding to the free enzyme suggested the involvement of cysteine, lysine, tyrosine, and arginine as possible amino acid residues.

Gas chromatography analysis showed that more headspace volatiles were present in the water extract of soy proteins at pH 7.0 than at pH 9.0. Schiff-base formation between aldehydes and soy protein increased at alkaline pH and reduced the amount of headspace volatiles at pH 9.0. The incubation of a soy protein extract and PAO-I reduced the headspace pentanal and hexanal by as much as 90%. The sensory panelists perceived lower beany flavor ($p<0.01$) of the aldehyde oxidase treated soy protein extract.

To My Love, Monica

and My Heart, Kunal

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INTRODUCTION

The current annual world production of soy protein ingredients (flours, concentrates and isolates) for food is estimated at about 1 billion pounds with 75% produced in the United States alone. The total production of soybean protein ingredients is roughly equivalent to about 1.5 billion pounds of 44% protein meal. More than 95% of the soybean meal produced annually in the U.S. (approximately 45 billion pounds) is used to supply protein in animal feeds. Soybean meal constitutes the largest portion of the monetary value of soybeans. About 75-80% of the soybean protein ingredients production is sold as edible flour, the balance is sold as protein concentrates and isolates. About 50 million pounds of the ingredients are marketed in speciality feeds and pet foods (Greiner, 1990).

As much as $1.50/lb is added to the market value of soybean meal when it is processed and sold as protein ingredient. Food-grade soybean flour normally contains more than 50% protein and is prepared by dehulling and extracting the soybean oil. Soybean flour is processed into protein concentrates containing greater than 65% protein by acid washing, by extracting with 60-75% ethanol, or by heating and water washing to remove soluble sugars. About 50-60% of the alcohol extracted protein concentrate is sold as speciality feeds and pet foods, while the balance is texturized to be used as meat extenders. Soybean flour can be processed into protein isolates containing more than 90% protein by extracting it with alkali and then recovering the protein by isoelectric precipitation. Only about one-third of the starting defatted meal is recovered as a protein isolate. Food-grade flour sells for about $0.15-0.20/lb; texturized flour, $0.35/lb; concentrates, $0.50-0.60/lb; texturized

concentrates, 0.60/lb; isolates, $1.20-1.25/lb; and spun fibers, $1.50/lb. Industrial-grade soy protein sells for $0.85-0.90/lb (Greiner, 1990).

Despite considerable consolidation among the processors of soy protein in recent years, production of edible protein ingredients continues to grow only at about 15% per year. Most of the protein is used to replace more expensive animal protein in meats and to a lesser extent in dairy-type products. About 80-90% of the edible soybean protein products are consumed in meat products, either as extenders or functional ingredients. Soy protein has also been shown to reduce oil uptake in fried products. A consumer preference for low fat foods is expected to increase the market for low/reduced fat products, which could create an additional market for soy protein.

Prospects for continued growth of soy protein market are good, especially in export markets to Japan, where animal protein is expensive, and in domestic and export markets for foods targeted to meet growing consumer concerns over diet and health. A recent trend in the consumer preference for "cholesterol-free" food could help increase the usage of plant proteins over traditional animal proteins.

Soybeans are also used in oriental soyfoods such as tofu, soymilk, tempeh, miso, natto, and soy sauce, whose combined market has grown from retail sales of approximately $150 million in 1986 to $500 million in 1990. The projected retail sales of these soyfood products are estimated at $1 billion by 1997.

The soybean protein in human foods suffers from a poor image due to objectionable flavor, presence of anti-nutritional trypsin inhibitors, flatus-causing oligosaccharides, and compounds that elicit allergic reactions. The off-flavor associated with soy proteins is the

single most technical impediment in the increased usage of soy proteins in human foods. The Iowa Agriculture and Home Economic Station estimates that an additional value of 1 ¢/ lb to soy flour and 46 ¢/bu to soybean seed can be added by reducing off-flavor of soybean and/or its products. A market for an additional 31 million bushels of edible soybeans worth $14.3 million/year can be expanded as a result of flavor improvement of soybean products. The low cost (compared with sodium caseinate) and the high nutritional value of soy proteins have been motivating researchers to resolve the flavor problem.

Literature Review

Off-flavor Formation

The off-flavor in soy-proteins is caused by aldehydes, ketones, furans and alcohols. Sessa and Rakis (1977) reported that 3-*cis*-hexenal, 2-pentyl furan and ethyl vinyl ketone were the major contributors to the green-beany flavor of soybeans. In another study, medium-chain aldehydes (pentanal, hexanal and heptanal) were found to be the major class of compounds contributing to the "beany and grassy flavor" of soy proteins (Kinsella and Damodaran, 1980). The production of off-flavor compounds by lipoxygenase may be important for plant physiology. The presence of the off-flavor compounds, such as hexanal, has been attributed to the inhibition of microbial growth *in vitro,* particularly in fungal species (Hamilton-Kemp *et al.*, 1992).

Mattick and Hand (1969) identified ethyl vinyl ketone as the compound responsible for the beany odor and flavor in soy products. These authors reported that the formation of ethyl vinyl ketone was due to enzymatic action in unblanched soy product. Cowan *et al.*

(1973) concluded that the action of lipoxygenase on linoleic acid was the chief factor in the production of off-flavor compounds in partially processed soybeans. The authors suggested a need for complete processing of soybeans, i.e., total inactivation of lipoxygenase. Axelrod *et al.* (1981) also found that these off-flavor compounds were generated by peroxidation of linoleic and linolenic acid by lipoxygenase. The three soy lipoxygenase isozymes (L-1, L-2 and L-3) have different pH optima of pH 9.0, pH 6.6 and pH 6.8, respectively, in the peroxidation of linoleic acid. The formation of off-flavor compounds has also been attributed to the incomplete removal of lipids in soy flour causing autoxidative decomposition products (off-odor compounds) of soy lipids (Hsieh *et al.*, 1981).

Soy Proteins and Mechanism of Flavor Binding

Several studies were conducted to determine the type of binding and nature of the off-flavor compounds to the soy proteins. Therefore, it is important to briefly discuss the nature of soy proteins and the flavor-ligand binding to soy proteins.

Soy Proteins Soybean seeds have two main storage proteins, glycinin and β-conglycinin. The acid precipitable fraction of soybean seed proteins (pH 4.8) contains 34% glycinin and 27% β-conglycinin. The remaining proteins include off-flavor causing enzyme lipoxygenase (EC 1.13.11.12), which could be as high as 1% of the total protein.

Glycinin contains six subunits, each of which comprises an acidic and basic polypeptide. The C-terminal region of the acidic chain is strongly hydrophilic in contrast to the hydrophobic C-terminal region of the basic polypeptide (Plietz *et al.*, 1987). In the

native state, the acidic and basic polypeptide chains are linked by a single disulfide bond (Nielsen, 1985). Wolf (1993) reported that fully reduced glycinin contained 41 sulfhydryl groups/mol. Dickinson *et al.* (1990) showed that the acidic polypeptide of glycinin were more important than the basic domains in keeping the molecule in solution.

According to Than and Shibasaki (1976), β-conglycinin is composed of several isomeric forms (B_0-B_6), containing three subunits (α, α' and β) in different ratios. The molecular weights of the subunits are 57, 58, and 42 kDa for α, α' and β subunits, respectively. The N-terminal amino acids of α, α' and β subunits were identified as Val, Val and Leu (Hirano *et al.*, 1987). The β-conglycinin exists in various structures at different pH and ionic strength. At ionic strengths greater than 0.5 or at pHs lower than 4.8 the protein existed in globular conformation (trimer "monomer"). The β-conglycinin formed a 9S hexameric "dimer" (Nielsen, 1995) at low ionic strength ($I<0.2$) or between the pH 4.8 to 11.0. Deshpande and Damodaran (1990) reported that β-conglycinin had a higher surface hydrophobic topology than other legumin-like proteins, with a sedimentation coefficient of 7S.

Another important soy protein is lipoxygenase (EC 1.13.11.12), which catalyzes the hydroperoxidation of polyunsaturated lipids containing *cis,cis*-1,4-pentadiene moieties, resulting in medium chain aldehydes and alcohols (Matoba *et al.*, 1985 a). The carbonyl production by L-1 (Grosch and Laskawy, 1975) is less than the lipoxygenase isozymes L-2 and L-3 at pH 6.5 to 7.0 (natural pH of soybean extract) (Matoba *et al.*, 1985a). Zhuang *et al.* (1991) found that the L-2 isozyme was not only most effective in the formation of C_6-aldehydes from polyunsaturated fatty acids but also was able to oxidize triglycerides.

Binding of Off-flavor to Soy Proteins The knowledge of the parameters involved in the off-flavor binding to soy proteins is important before one can solve the flavor problem by the means of enzymatic treatment. Solm *et al.* (1973) reported that soy proteins in a native state were compactly folded, forming interior hydrophobic and exterior hydrophobic regions. The authors found that the binding of flavor-ligand to soy protein was of a hydrophobic nature. The breakdown of tertiary structure resulted in exposing more hydrophobic regions that were buried inside. Gremli (1974) found that off-flavor compounds preferentially adsorbed to soy proteins in the following order: unsaturated aldehydes, saturated aldehydes, ketones, and alcohols. According to these authors, there was more reversible than irreversible binding of off-flavor causing aldehydes.

Beyler and Solm (1974) investigated the binding of several flavor compounds to soy proteins using an equilibrium dialysis method. The authors concluded that the nature of binding was both electrophobic and hydrophobic. The binding constants of 2-butanone, 1-butanol, phenol, and vanillin to soy proteins were increased when the pH was raised from 4.5 to 7.0 at a constant temperature of 20 °C. The overall binding constant K ranged from 0 to 10^4 M^{-1}, increasing in the order of phenylalanine, aniline, dimethylpyrazine, carboxylic acids, alcohols, ketone and aldehydes.

Damodaran and Kinsella (1980) studied the binding of 2-heptanone, 2-nonanone, and nonanal to bovine serum albumin in an aqueous model system. These investigators showed that there was no formation of Schiff bonds between nonanal and the ϵ-amino group of lysine and that the binding was hydrophobic in nature. Another study by this group investigated the thermodynamics of binding of 2-heptanone, 2-octanone, 2-nonanone and

nonanal to soy proteins (Damodaran and Kinsella, 1981a). The binding was reduced due to a decrease in hydrophobic interactions that resulted from the decrease in the carbon chain length of the flavor ligand. Temperature also affected the binding of flavor ligands to soy proteins. The binding affinity was reduced from 2000 M^{-1} at 5 °C to 930 M^{-1} at 25 and 45 °C. The reduced binding was speculated to be due to the structural rearrangements of the various subunits of soy proteins at a higher temperature. The nature of binding was concluded to be hydrophobic in nature by these investigators. Damodaran and Kinsella (1981b) also noted that 2-nonanone had a smaller affinity for glycinin than β-conglycinin due to higher number of disulfide bonds in glycinin. They speculated that the disulfide bond network in glycinin was rendering the hydrophobic group inaccessible to 2-nonanone. In addition, when 3-D structure was unfolded with urea, the binding affinity of 2-nonanone to soy proteins was decreased. The authors hypothesized the removal of the bound off-flavors from the soy proteins could be achieved by reversibly altering the quaternary structure.

The nature and thermodynamics of the off-flavor binding to soy protein in a dry system (without water) was investigated by Aspelund and Wilson (1983). They performed a gas chromatographic analysis of hydrocarbons, ketones, aldehydes, methyl esters, and alcohols at various temperatures to determine the strength of adsorption of flavors onto soy protein by heat of adsorption data, Gibbs free energy and entropy of adsorption parameters. The Gibbs free energy decreased with the increase of the incubation temperature, indicating that the adsorption was more favorable at lower temperatures. The binding of alcohols to the soy isolate was the strongest among the volatiles studied, due to the formation of two hydrogen bonds per molecule of alcohol and nonspecific van der Waals forces.

Hydrocarbons showed the weakest binding affinity because they formed only nonspecific hydrophobic bonds. In addition to van der Waal interactions, aldehydes, ketones and esters formed one hydrogen bond and the binding affinity of these compounds was lower than alcohols.

O'Keefe *et al.* (1991) investigated the thermodynamics of binding of volatile off-flavor ligands butanal, pentanal, hexanal, octanal, 2- and 3-hexanone, 2- and 5-nonanone, hexanol, and hexane to soybean glycinin and β-conglycinin in aqueous model systems. The binding affinity of all flavor ligands was greater for glycinin than β-conglycinin at 5, 20 and 30 °C. The binding affinity of the aldehydes to glycinin increased with increasing chain length but remained constant for β-conglycinin. The binding of hexane did not take place above 5 °C. The authors speculated that this was due to the structural changes at the lower temperature that allowed interaction with exposed non-polar groups. The number of binding sites on glycinin was greater at 5 °C than at higher temperature for ketones, hexanol and hexane. The number of binding sites for hexanol was lower than that of hexanal and 2-hexanone in both glycinin and β-conglycinin at 5, 20, and 30 °C temperatures. The binding constants for straight chain aldehyde ligands increased as the chain length increased, which was the opposite of Damodaran and Kinsella's (1981) study. The equilibrium binding constants for glycinin were smaller at 20 and 30 °C than at 5 °C. The authors reported an increase in surface hydrophobicity at 5 °C compared to 20 °C, which was assumed to be a consequence of protein unfolding, increase in strength or increase in number of binding sites at the lower temperature.

In a companion study by O'Keefe *et al.* (1991) the equilibrium binding of hexanal to soybean glycinin and β-conglycinin was evaluated at several buffer conditions. A positive cooperativity was also observed for the binding of hexanal to both proteins. The affinity of hexanal binding was slightly greater for β-conglycinin than for glycinin: 303 ± 30 and 270 ± 24 M^{-1}, respectively in 0.3 M Tris buffer, but a reverse trend was observed when NaCl and NaN_3 were included in the buffer.

Cooray (1992) studied the binding of ^{14}C-heptanal to glycinin and β-conglycinin. According to this study, hydrophobic interactions were the sole contributor to the tight binding of the ^{14}C-heptanal to the soy protein in an aqueous medium. Entrapment of aldehyde and Schiff base formation with the ϵ-amino group of lysine were not the major contributors to the strong interaction of ^{14}C-heptanal with soy proteins. The interaction between ^{14}C-heptanal and both soy proteins was not completely reversible due the presence of a significant level of tight binding.

Since the enzymatic treatment of soy protein with aldehyde oxidase to reduce off-flavors take place in an aqueous environment, hydrophobic interaction would be the major type of binding. It is expected that initially only the free aldehydes in the aqueous soy protein solution would be available for conversion to carboxylic acid by aldehyde oxidase.

Technology Review of Reduction and Removal of Off-Flavors from Soybean Proteins

Mustakas *et al.* (1969) and Smith and Circle (1978) carried out rapid inactivation of lipoxygenase by using wet or dry heat treatment. The heat treatment resulted in protein denaturation, i.e., a low nitrogen solubility index (NSI) value, and in a cooked and toasted

flavor that was not desirable. Protein denaturation is usually measured by using NSI or protein dispersibility index (PDI) values (Wolf and Cowan, 1977).

Man *et al.* (1989 and 1994) used acid treatment with HCl, H_3PO_4, and tartaric acid to inactivate lipoxygenase in soy flour. These investigators obtained a bland soy flour after the acid treatment, but the protein dispersibility index (PDI) decreased to 49% compared with the control. Furthermore, the neutralization of soy flour after acid treatment resulted in a accumulation of salt in the flour, which was unacceptable to the sensory panelists. Srinivas *et al.* (1992) used hexane containing 3 and 5% (v/v) acetic acid. The authors achieved a 55-63% reduction of the beany flavor and complete inactivation of lipoxygenase. However, NSI was reduced by 39% compared with the hexane-extracted meal (control) containing 5% acetic acid.

Eldridge *et al.* (1971) used hexane:methanol (75:25), hexane:ethanol (82:18) and hexane:isopropanol (80:20) solvent mixtures effectively in removing the off-flavors. But organic solvent mixtures significantly reduced the NSI values and hence denatured the soy protein.

Baker *et al.* (1979) used ethanol, methanol, and isopropyl alcohol at temperatures ranging from 30 to 75 °C to extract off-flavors from soy flour. The best flavor scores were obtained when ethanol azeotrope (92.7%) was used at 60 °C. The proteins were denatured when the extractions were made with 70% alcohol rather than with the azeotrope. Methanol caused the most denaturation and isopropyl alcohol the least. These treatments did not completely eliminate the off-flavor and denatured the protein. An additional problem with the solvent extraction approach is the requirement to eliminate all residual solvent from the

product, which would increase the processing cost and might cause further denaturation of soy proteins.

An approach to mask the off-flavor of textured soy proteins with chicken soup broth was investigated by Malcolmson *et al.* (1987). The masking was successful at a soy protein level less than 16%. However, at 16% soy protein level, there was no effect of increased chicken broth concentration on the off-flavor masking. Therefore, the masking of soy protein off-flavor did not prove to be successful. In another study, Wang *et al.* (1974) attempted to eliminate off-flavor by fermenting soy milk using *Lactobacillus acidophilus* and *L. bulgaricus*. The beany flavor was masked slightly, but a complete flavor masking was not achieved.

Eldridge *et al.* (1986) used supercritical carbon dioxide (SC-CO_2) technology to improve the flavor profile of soybean flakes without impairing protein solubilities (NSI and PDI). These authors used different temperatures (80-100°C), pressures (72.1-84.4 MPa), and moisture contents (5-13.5%) for SC-CO_2 extraction of full-fat soybean flakes. A constant moisture of the sample during the extraction was maintained by adding a water-saturated glass-wool plug at the inlet of the extractor. They observed that the presence of moisture in the flakes caused denaturation of lipoxygenase that led to improved flavor. However, at higher moisture content (11.4%) and temperatures above 80°C, protein solubility index (NSI) was reduced. According to these authors, high protein solubility and good flavor scores can be obtained at pressures greater than 83 MPa, temperatures about 80°C, and moisture levels between 10.5 and 11.5%. The usual grassy/beany and bitter flavors of hexane-defatted soybean flours were minimally detectable when extracting under these conditions. The

lower lipoxygenase activity was also responsible for reducing the development of undesirable flavors at 37 °C during the two-month storage time.

Use of SC-CO_2 has also been attempted by this investigator (1991 and 1995) and Ooi (1994) at Iowa State University. The SC-CO_2 at 27.6 MPa and 40 °C was effective in removing the off-flavor from soy flour and soy protein isolate. The sensory panelists perceived a significant reduction in the beany flavor of SC-CO_2-treated soy protein. Panelists also significantly preferred the SC-CO_2-treated soy protein compared with the untreated control. The effects of liquid-CO_2 (L-CO_2) and SC-CO_2/ethanol mixture on the off-flavor were also studied. We found that L-CO_2 was the least effective, followed by SC-CO_2/ethanol and SC-CO_2. Although the headspace gas chromatography of SC-CO_2/ethanol mixtures showed more reduction of off-flavor-causing volatiles than SC-CO_2 treated soy protein isolate, the sensory panelists did not prefer the off-flavor caused by the residual ethanol bound to proteins. The desorption of off-flavor volatiles by SC-CO_2 suggests that a substantial amount of these volatiles are weakly bound. These results agree with the study of Aspelund and Wilson (1983) work that aldehydes did not bind as strongly to soy isolate in the dry state as only van der Waals' forces and one hydrogen bond were involved in binding. The hydrophobic nature of SC-CO_2 also suggests that SC-CO_2 removed the volatiles, which were bound by hydrophobic interaction those reported by Cooray (1992). The SC-CO_2 treatment was effective in removing soy off-flavors without impairing protein functionality. The high capital cost involved in developing a SC-CO_2 extraction process for off-flavor removal from soy proteins cannot be justified.

Molecular biology was used as a tool to modify soybean seed to obtain soybeans with improved flavor (Kitamura, 1984). The author isolated mutant varieties of soybean seeds that were lacking either one or two lipoxygenase isozymes. But these mutants did not result in a successful crop variety because of lower yield. In addition, the beany and grassy off-flavor were again generated by auto-oxidation when the seeds were crushed. Another method using a molecular biology approach involved the removal of the genes encoding lipoxygenases (L_1, L_2, and L_3) (Davies *et al.*, 1987). Removal of L_2 isozyme from genetically engineered cultivars resulted in a significantly less beany and rancid flavor. However, the L_2-null soybean preparation had a more dairy and cerealy flavor; the genetic modification method was not very efficient in producing a bland product. In addition, auto-oxidation of the soybean oil still remained a problem in the lipoxygenase-null variety of soybean seed. Moriera *et al.* (1993) bred different varieties of soybean seeds to produce lipoxygenase (LOX) null varieties. These investigators reported no effect of LOX 1 on hexanal amount in soy protein. LOX 2 was found to be the most important isozyme in the formation of hexanal followed by LOX3.

An enzymatic treatment using aldehyde dehydrogenase (ALDH) enzyme (Chiba *et al.* 1979a) was successful in removing the green and beany flavor from the aqueous suspension of soy-protein isolate. Aldehyde dehydrogenase effectively removed bound as well as free aldehydes from soy extract. However, this approach would be impractical and uneconomical because NAD^+ is required as a cofactor for the enzyme reaction, which makes the process expensive.

Nomura *et al.* (1988) attempted to suppress off-flavors in defatted soymilk using the immobilized acetic acid bacterium. *Acetobacter aceti* IFO 3284, entrapped in *k*-carrageenan gel had an intracellular aliphatic aldehyde oxidase enzyme that oxidized hexanal at 50 °C and pH 5.0. The immobilized cells were used to suppress off-flavors in ten batches of defatted soymilk without any loss in enzyme activity. In addition, the aldehyde oxidase activity of the immobilized cells was preserved for 65 days at 5 °C. Although these results indicate the feasibility of off-flavor reduction using immobilized aldehyde oxidase, the pH optima of 5.0 is questionable as soy proteins start precipitating at that pH. This study did not elaborate on the amount of off-flavor reduction and the process time, which are very critical factors in evaluating the commercial feasibility of such a process.

The oxidation of aldehydes in soybean extract by bovine liver aldehyde oxidase (aldehyde:oxygen oxidoreductase, EC 1.2.3.1) was investigated by Takahashi *et al.* (1979). Their work showed that this enzyme used dissolved oxygen as an electron acceptor and catalyzed the irreversible conversion of aldehydes to acids. These authors partially purified the bovine liver aldehyde oxidase using ammonium sulfate fractionation and hydrophobic interaction chromatography (Octyl-Sepharose resin). Only fourfold purification after Octyl-Sepharose chromatography was achieved in this study. A low initial activity of aldehyde oxidase present in crude homogenate was attributed to the presence of catalase activity which interfered with the coupled assay involving the oxidation of *o*-dianisidine. The partially purified aldehyde oxidase lost 31% activity after five days storage at 4 °C at pH 9. However, up to 83% activity was retained for similar time-temperature storage conditions when 0.1 mM dithio threotol (DDT) was included in the storage buffer. This preparation of bovine

liver aldehyde oxidase showed a broad substrate specificity. The authors reported K_m of 0.006 and 20 mM for n-hexanal and acetaldehyde, respectively, indicating a higher affinity of this enzyme for medium chain aldehydes compared with shorter chain ones. Takahashi *et al.* emphasized the importance of catalase presence in the reaction mixture to prevent oxygen limitation. The result of the sensory analysis indicated that beany odor was reduced substantially. The rate of reaction of aldehyde oxidase on the aldehydes bound to soy protein was low. Apparently aldehyde dehydrogenase (Chiba *et al.*, 1979) is a better enzyme that can act upon bound aldehydes, but the reaction rate for the conversion of bound aldehydes was only 0.4 times of the free aldehydes rate in the water extract of soybean. The cause of this difference between AOX and ALDH is unknown.

In another study by this group (Sasaki *et al.*, 1982) bovine liver mitochondrial aldehyde dehydrogenase was used to reduce aldehydes in defatted soybean extract. The aldehyde level increased during incubation in a flour-water suspension with enzyme at pH 6.5, while it decreased markedly at alkaline pHs. This aldehyde eliminating activity was observed in concentrated suspension (1:5 flour to water ratio) but was not in a suspension prepared as 1:20 flour to water ratio. ^{14}C-Labelled acetaldehyde added to a 1:5 flour to water extract was converted to acetic acid. The acetaldehyde level decreased by 70% after 5 hours of incubation at 25 °C and pH 9.3. The presence of NAD^+ as a cofactor was required in this study to for enzyme activity making the process expensive.

Aldehyde Oxidase

Properties of Aldehyde Oxidase A number of studies have been carried out to purify and characterize aldehyde oxidase (EC 1.2.3.1) from various sources, such as rabbit liver, pig liver, mouse liver, turkey liver, and drosophila. Mahler *et al.* (1954) studied aldehyde oxidase properties in detail and reported the pH optimum of pig liver aldehyde oxidase was between pH 6.8 and 7.9. The same authors also reported that AOX exists as a monomer and in several polymeric forms with identical physical, chemical, and catalytic properties and with the same charge to mass ratio but different molecular weights. Although Mo^{3+} is an essential element involved in the electron transport sequence of AOX, the activity of AOX did not increase by the addition of molybdenum as an aqueous solution of molybdic trioxide when oxygen or dyes were used as electron acceptor. However, the presence of Mo^{3+} either bound to the enzyme or added externally was necessary for the interaction with cytochrome c as electron acceptor. A non-heme iron was also present in pig liver aldehyde oxidase. The iron-sulfur complex has been shown to be a part of catalysis, but the removal of iron did not result in the complete inactivation of AOX (Mahler, 1954).

Palmer (1962) and Rajagopalan and Handler (1968) reported that aliphatic aldehyde oxidase obtained from pig and rabbit livers oxidizes several aliphatic aldehydes, aromatic heterocycle compounds such as *N'*-methynicotinamide, and many purines. Bray (1975) and Rajagopalan (1980) screened aliphatic aldehyde oxidase enzyme from various mammalian sources as flavoproteins of dimeric structure with molecular subunits of 135-150 kDa. The term "aliphatic" means that the aldehyde oxidase catalyzed the oxidation of aliphatic aldehydes. However, the aldehyde oxidase from mammalian sources also catalyzed aromatic

aldehydes as well.

Branzoli and Massye (1974) demonstrated that the active form of an aldehyde oxidase from rabbit liver had a persulfide group present in the active site. Coughlan (1977) determined that active center of the aldehyde oxidase from rabbit liver had a molybdenum iron/sulphur complex. An active center cysteine residue was a source of cynolyzable sulphur. Coughlan confirmed the importance of sulphur by conducting the cynolysis of the active site sulphur that resulted in the inactivation of aldehyde oxidase. Inactivation of the same AOX also occurred upon treating the enzyme with menandione, methanol and arsenic.

Bray *et al.* (1982) concluded from the electron-paramagnetic-resonance spectroscopy of the molybdenum center of the aldehyde and xanthine oxidases that both had essentially the same environment among different species. The difference in substrate specificity of the two oxidases was primarily due to the structural differences in the regions of the active centers concerned solely with substrate binding, rather than the difference in the catalytically important molybdenum sites. Rajagopalan (1987) noted that molybdenum was an essential element for the activity of aldehyde and xanthine oxidase, especially for the electron transport reaction.

Various compounds are inhibitory to aldehyde oxidase activity. Triton X-100, amyltal, estradiol, menadione, antimycin A and cyanide are just a few from the long list of inhibitors of AOX (Rajagopalan and Handler, 1964). Rajagopalan and Handler noted markedly different effects of several inhibitors on the reduction of various electron acceptors. This strongly suggests the existence of multiple points of the electron donation from the enzymes.

One should be careful when considering the substrate specificities of various sources and isozymes of aldehyde oxidase. Different mammalian aldehyde oxidase show different substrate specificity. For example, Palmer (1962) has shown the pig liver aldehyde oxidase is specific for medium chain-length aliphatic aldehydes (C2-C6). However, the rabbit liver aldehyde oxidase (Rajagopalan *et al.*, 1962) was more specific for *N'*-methylnicotinamide and many other purines. Crawford *et al.* (1982) and Deobald and Crawford (1989) reported an aromatic aldehyde oxidase from *Streptomyces viridosporous*. The aromatic aldehyde oxidase as distinguished by Crawford oxidized many aromatic aldehydes, such as benzaldehyde, salicyldehyde, terephthaldehyde, vanillin, and verataraldehyde to their corresponding aromatic acids. However, the aliphatic aldehydes were not oxidized. The molecular weight of aromatic aldehyde oxidase from *Streptomyces viridosporous* was estimated to be 80,000 Da, different than the aliphatic aldehyde oxidase (250-270 kDa). Therefore, the difference in aromatic aldehyde oxidase from *S. viridosporous* and mammalian aldehyde oxidases is that aromatic AOX oxidizes only aromatic aldehydes while the mammalian AOX oxidizes both aliphatic and aromatic aldehydes.

The substrate specificities and molecular weights clearly indicate that there is a significant difference in the aliphatic and aromatic aldehyde oxidase. In this study, we will be focusing on the aliphatic aldehyde oxidase as our substrates of interest are the medium chain aliphatic aldehydes.

Purification of Aldehyde Oxidase Several approaches were used in the purification and characterization of aldehyde oxidases from the various mammalian sources.

Rajagopalan *et al.* (1962) purified a rabbit liver aldehyde oxidase. A heat treatment followed by ammonium sulfate (50% saturation) precipitation was used to remove tissues and to precipitate unwanted proteins. Acetone fractionation (43-50%), calcium phosphate gel chromatography and alumina gel chromatography resulted in 93-, 290-, and 400-fold purification over the heat-treated extract. However, the authors reported contamination of aldehyde oxidase with other proteins at every step of purification. Therefore, the preparation was not very homogeneous. The purified preparation of the aldehyde oxidase showed broad substrate specificity ranging from acetaldehyde to salicylaldehyde as well *as N'*-methylnicotinamide to quinine.

Palmer (1962) studied the purification and properties of pig liver aldehyde oxidase. The AOX was purified by using reverse ammonium sulfate fractionation, heat treatment, and ion-exchange chromatography on DEAE-cellulose and hydroxylapatite chromatography. The authors acknowledged the presence of many protein impurities in the purified AOX. The enzyme was stable in the pH range from 9.0 to 9.5, but the activity was lost at neutral pH. Ammonium ion increased the storage stability of AOX. The maximum activity of AOX was reported at pHs between 8 and 9 when dicholorophenol indophenol (DIP) and ferricynides were used as electron acceptors. The V_m value was the highest for butrylaldehyde and the K_m was the lowest for heptanal.

One of the most cited purification works is that of Felsted *et al.* (1973). Hog liver aldehyde oxidase was purified using heat treatment, ammonium sulfate precipitation, acetone fractionation, alumina C_γ, DEAE-cellulose, and preparative acrylamide electrophoresis. A 115 fold purification and 6.8% activity yield was achieved based on the clarified crude liver

homogenate. The authors reported a 99% purity of the preparation. These investigators estimated the molecular weight as 270,000 Da for the hog liver AOX. The hog liver aldehyde oxidase polymerized upon storage to a molecular weight of 540,000 Da and higher. Therefore, thiol reagents at a concentration of 2 mM were essential to prevent the aggregation of AOX. The use of higher than 5 mM concentration of thiol reagent led to the inhibition of AOX activity. The pH optimum was 10.5 for the hog liver aldehyde oxidase as determined by using *N'*-methylnicotinamide.

Crawford *et al.* (1982) and Deobold and Crawford (1989) produced another class of aldehyde oxidase which was classified as aromatic aldehyde oxidase. Aromatic aldehyde oxidase was produced by *Streptomyces viridosporous* and partially purified by ammonium sulfate precipitation and DEAE-cellulose ion-exchange chromatography. This intracellular aromatic AOX oxidized aromatic and α,β-unsaturated aromatic aldehydes to corresponding acids. An extracellular aromatic aldehyde oxidase was produced on agar containing insoluble dehydrodivanillin. The extracellular form of this enzyme oxidized the aldehydes groups in lignin. The enzyme was partially purified (five-fold) using gel filtration and DEAE-Sepharose ion-exchange resin.

To our best knowledge Stell *et al.* (1989) and Warne and Stell (1990) obtained the highest purity of an aldehyde oxidase by using affinity chromatography. Rabbit liver was homogenized, heat-treated and then precipitated with ammonium sulfate to get the crude preparation of the enzyme. The crude preparation of AOX was further purified by using affinity chromatography on a Benzamidine-Sepharose 6B column at pH 9. The adsorbed AOX was eluted by using benzamidine-containing buffer. This single affinity step resulted

in a 38-fold increase in the purity over the crude preparation with a 84% recovery of enzyme activity. Further purification with a mono-Q ion-exchange resin and liquid chromatography with fast protein liquid chromatography (FPLC) resulted in highly purified aldehyde oxidase preparation.

Characterization Studies Mahler *et al.* (1954) and Mahler (1955) reported the pH optimum for a pig liver aldehyde oxidase (PAO) to be between pH 7.0 and 8.0. The reaction rates at pHs 5.0, 6.0, 9.0, and 10.0 were 10, 50, 50, and 40% of the maximal rate, respectively. The enzyme specificity was the highest for acetaldehyde and it decreased with increasing aldehyde carbon chain length. PAO was completely inhibited by *p*-chloromecurylbenzoate, a sulfhydryl inhibitor, which indicated the importance of sulfhydryl group in the catalysis of aldehydes by PAO.

Palmer (1962) reported some kinetic studies of PAO. PAO had the highest specificity for C_4 aliphatic aldehyde. Although the Michaelis constant was lower for pentanal (1.25 mM) and heptanal (1.3 mM) than butanal (25 mM), their V_m values of pentanal and heptanal (4.1 and 0.54 μmol aldehyde oxidized/min/mg protein, respectively) were smaller than that of butanal (20.6 μmol aldehyde oxidized/min/mg protein). The pH optima for PAO was 9.0. This pH and V_m data indicates that the two PAOs examined by Mahler (1955) and Palmer (1962) are different in their characteristics. According to Palmer (1962), the kinetic analysis of PAO inhibition by 1,10-phenathroline demonstrated that the Mo^{3+} did not function as a binding site when cytochrome *c* was used as a substrate instead of aldehydes. His results suggested that both the Mo^{3+} site and the cytochrome *c* binding site

must be very close to one another on the surface of PAO. The binding of the inhibitor at a distant site might have induced conformational changes in the protein that resulted in decrease in affinity of enzyme for its substrate. Palmer's work also suggested a partial competitive inhibition of activity of aldehyde oxidase by aldehydes. When aldehydes were used as substrate, the excess of aldehydes caused PAO substrate inhibition. Palmer ascribed this inhibition to the presence of a sulfhydryl-aldehyde complex which blocked the access of the enzyme to the substrate. Stell *et al.* (1989) showed that the inhibition of rabbit liver aldehyde oxidase with *p*-dimethylaminocinnamaldehyde was purely competitive in nature at pH 9, whereas at pH 7 it was a mixed type-competitive and noncompetitive inhibition.

Rajagopalan and Handler (1964) studied the substrate binding site of aldehyde oxidase from rabbit liver. The authors showed that K_m for acetaldehyde and nonquartenary aromatic substrates increased with pH up to 10.0. But the K_m for *N'*-methylnicotinamide was 8×10^{-5} M at pH 10.0 compared with 3×10^{-4} M at pH 7.8. The uncharged substrates, such as purines, salicylaldehyde, acetaldehyde, and quinone, exhibited a marked inhibition of activity when present at a concentration 50 to 100 times the K_m value. The products formed by the oxidation were not inhibitory in any instance. These authors postulated the following mechanism of oxidation: The reaction occurred with a removal of hydride ion from the carbon to be "oxidized" with a replacement by the hydroxyl ion from the medium. The aldehyde oxidase provided a molybdenum atom serving as a Lewis acid. Molybdenum acted as initial acceptor of electrons from substrate to make this process work. The authors reported the presence of sulfhydryl, lysine, and arginine groups in the active site of the rabbit liver aldehyde oxidase.

Sensory Evaluation of Aldehyde Treated Soy Extract Takahashi *et al.* (1979) reported a reduction of beany-odor from water extract of soybean by bovine liver aldehyde oxidase. The odor scores were low when menandione (inhibitor to PAO activity) was added. The odor score of aldehyde dehydrogenase treated soy extract samples in the presence of NAD^+ were higher than the aldehyde oxidase treated sample. These authors neither reported the level of significance of sensory analysis nor the amount of enzyme required.

Statement of the Problem

The present work focuses on the removal of the off-flavor from soy proteins by using aliphatic aldehyde oxidase (aldehyde:oxygen reductase, EC 1.2.3.1) purified from pig liver. The bovine hepatic enzyme was able to remove the off-flavors from soy proteins (Takahashi *et al.*, 1979), but the study was neither complete in terms of enzyme purity nor in the characterization studies. The authors used a very crude form of aldehyde oxidase. The fourfold purification is not enough to study the stability of this enzyme. Other proteins present in the enzyme preparation might stabilize the enzyme, resulting in incorrect stability data for this enzyme. The study also lacked the qualitative analysis (SDS-PAGE) of enzyme purity, which is also important in gathering molecular weight information for the aldehyde oxidase. The enzyme cannot be well characterized unless a homogenous enzyme preparation is obtained. Enzyme characterization is necessary in order to determine the optimal pH for stability, maximal rate, and temperature stability data. In addition the energy of activation and denaturation data are also necessary to evaluate the commercially feasibility of AOX. All these studies were lacking in the work of Takahashi *et al.*. There is also a need to

determine the V_m and K_m at several pHs in order to obtain suitable process conditions and to interpret the active site amino acid residues. A storage stability study of this enzyme in the highly purified form is also important. The storage stability data is important commercially in evaluating the process feasibility. The sensory analysis data with statistical information is also necessary. Takahashi *et al.* did not perform statistical analysis in their sensory analysis data.

The porcine hepatic aldehyde oxidase has not been investigated for its efficacy for off-flavor removal. This enzyme has not been well purified and characterized in the literature. Therefore, this work focussed on the characterization of this enzyme to determine pH, temperature optima, substrate specificity, and energy of activation and denaturation. An attempt was also made to determine the possible amino acid residues involved in catalysis. The application of purified porcine hepatic aldehyde oxidase was also attempted in the reduction of off-flavors from soy proteins in a model system.

MATERIALS AND METHODS

Purification of Porcine Liver Aldehyde Oxidase

Porcine liver (Landrace x Yorkshire x Hampshire x Duroc hog of 180 days old) was obtained from the Iowa State University Meat Laboratory. A 150-200 g piece of pig liver was sliced into small pieces and homogenized using a tissue homogenizer (Tekmar, Cincinnati, OH) in the extraction buffer (4 X) containing 1.15 % KCl and 0.1 mM EDTA (Fisher Scientific, Fair Lawn, NJ) at pH 7.8 (Takahashi *et al.*, 1979). The pH of the extraction buffer during homogenization was continuously adjusted with 1 M NaOH. The homogenized sample was centrifuged at 30,000 x g for 30 minutes. The supernatant (crude extract) was used for aldehyde oxidase purification and the pellet was discarded. The unused part of the liver was stored frozen at -70 °C until further use. In the subsequent crude enzyme preparations, frozen liver was used as the source for aldehyde oxidase. The initial aldehyde oxidase activity recovered from fresh as well as frozen porcine livers were compared. There was no significant difference in the activity yield of aldehyde oxidase from fresh and frozen liver.

The crude extract was divided into several 250-mL Erlenmeyer flasks containing 100-mL extract which were placed in an 80 °C-bath (Fisher Scientific, Pittsburgh, PA) with continuously stirring. As soon as the temperature reached 54 °C, the flasks were removed from the 80 °C-bath and the samples were held for 4 min in a 54 °C-water-bath. The heat-treated extract was immediately placed in an ice-water bath to bring the temperature to 5 °C, centrifuged at 30,000 x g for 30 min and the pellet was discarded. The supernatant was

collected and an aliquot was saved for measurement of aldehyde oxidase activity and protein content determination.

The heat-treated extract was subjected to ammonium sulfate (Fisher Scientific, Fair Lawn, NJ) fractionation. Both solid ammonium sulfate and saturated ammonium sulfate solution were used and no difference in AOX activity was observed. A step-wise ammonium sulfate fractionation was performed and a 35 to 55% cut resulted in the optimal yield and purification of the enzyme. At both stages (35 and 55%) of fractionation, the ammonium sulfate pellet was obtained by centrifuging the solution at 30,000 x g for 45 min. An aliquot of supernatant was saved to measure protein and AOX activity. The ammonium sulfate pellet was frozen and stored at -18 °C. A part of the frozen ammonium sulfate pellet was solubilized in a glycine-NaOH buffer containing 100 mM glycine, 100 mM NaCl, 0.1 mM EDTA (I=0.2) (Fisher Scientific) and 2 mM L-cysteine (Sigma Chemical Company, St. Louis, MO) at pH 9.0 and dialyzed using dialysis tubing of 15,000 Da molecular weight cut-off (Spectrum, Houston, TX) against the same buffer using three volume (2 L each) changes. This ammonium sulfate fraction was used for ion exchange, gel filtration, preparative isoelectric focusing, and affinity chromatography.

The DEAE-Sephadex was purchased from Pharmacia LKB Biotechnology, Inc., (Piscataway, NJ), DEAE-Toyopearl 650-R from Supelco (Bellefonte, PA) and polyethyleneimine (PEI)-Acti disk from FMC Corporation (Pine Brook, NJ). The Sephacryl-300 HR gel filtration media was purchased from Pharmacia (Piscataway, NJ). The protease inhibitors, phenyl methyl sulfonyl fluoride, cystatin, and pepstatin A were purchased from Sigma Chemical Co. (St. Louis, MO).

Benzamidine-Sepharose 6B (Pharmacia) was used as affinity packing. The resin (50 mL) was packed in 15 cm x 2.5 cm chromatography column (Bio-Rad Labs, Hercules, CA). The dialyzed ammonium sulfate preparation was adjusted to a protein content of 10 mg/mL and a total of 60 mg protein was loaded on the column at a rate of 5 mL/hr by using a Rabbit peristaltic pump (Rainin Instrument Co., Inc., Woburn, MA). The column was then washed with 10 column volumes of glycine-NaOH buffer at a flow rate of 30 mL/hr. The aldehyde oxidase peaks (PAO-I and PAO-II) were eluted by using a 0-25 mM benzamidine gradient followed by a 100 mM benzamidine wash. After every five runs, the column was regenerated by washing the resin with 100 mM benzamidine containing 2 M NaCl. The flow rate during elution was set at 20 mL/hr. The eluted fractions (3.3 mL) were collected in 13 x 1 cm glass tubes by using a fraction collector (Isco, Inc., Lincoln, NE). The two PAO activity peaks were pooled and concentrated in a Centriprep concentrator (Amicon Inc., Baverly, MA) to a final volume of 2-mL (50 μg protein/mL). Concentration was necessary because the protein concentration in the eluted peak was very low (2-5 μg/mL). The protein peaks could not be detected by a UV_{280} detector, as benzamidine has a strong absorbance at 280 nm. The concentrated pools were dialyzed against glycine-NaOH buffer. Two aliquots of both the PAO-I and the PAO-II pools were stored at 4 °C and rest of the concentrated pool was stored at -18 °C.

The affinity chromatography was then scaled up on a 50 x 2.5 cm affinity column (Pharmacia). All the eluting conditions were the same as for the smaller column. The eluent was collected in 5-mL portions. The total time for chromatography was 72 h. For every 200 mg total protein of ammonium sulfate fraction loaded on this large affinity column about 250

and 350 μg total PAO-I and PAO-II enzyme were purified, respectively. There was no difference in PAO-I and PAO-II elution profile from the scaled-up affinity column compared with the smaller affinity column except the pool sizes for PAO-I and PAO-II were double in volume. The PAO-I and PAO-II fractions were concentrated and stored at -18 °C. The concentrated enzymes were stored in 2-mL batches to prevent possible loss in activity of PAO-I in the repeated freeze-thaw cycles if PAO-I was stored in a large batch. The thawed preparations were stored at 4 °C.

Protein Assay

The protein content was determined by Bio-Rad protein micro assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as the protein standard (Pierce, Rockford, IL). The Coomasie blue dye binds to the basic and aromatic amino acids in the protein. Absorbance was read at 595 nm using a Beckman DU-50 spectrophotometer (Beckman Instruments Inc., Fullerton, CA).

Activity Assay

The activity assay was developed by modifying the assay of aldehyde oxidase activity used by Takahashi *et al.* (1979). The activity of AOX was determined by measuring a change in color due to oxidation of o-dianisidine dihydrochloride by the H_2O_2 produced in the reaction upon oxidation of 33 mM acetaldehyde or 3.3 mM pentanal substrates. A change in absorbance at 445 nm at 30 °C was continuously recorded. The assay mixture had a final composition in a final volume of 1-mL and pH 7.5 as follows:

100 mM Tris-HCl (Fisher Scientific)

0.3 mM o-dianisidine dihydrochloride (Sigma Chemical Co.)

7.8 μg peroxidase (Horseradish), (Sigma Chemical Co.)

33 mM acetaldehyde (Eastman Kodak Co, Rochester, NY) or 3.3 mM n-pentanal (Aldrich Chemical Co., Inc., Milwaukee, WI).

The reaction is shown as follows:

$$\text{-CHO} + H_2O + O_2 \xrightarrow{\text{PAO}} \text{-COOH} + H_2O_2$$

aldehyde → carboxylic acid

$$H_2O_2 + \text{o-dianisidine} \xrightarrow[445\text{ nm}]{\text{Peroxidase}} H_2O + \text{oxidized o-dianisidine (orange color)}$$

Activity measurement in the energy of activation study and in the effect of pH on PAO-I activity were conducted as a single-point assay. The reaction was carried out in a 3-mL buffer of choice containing PAO-I and substrate at a given temperature. A 200-μL aliquot of reaction mixture was withdrawn at various time intervals and boiled quickly to stop reaction. The color was developed by adding the boiled reaction mixture in a 400-μL assay buffer containing *o*-dianisidine and peroxidase at pH 7.5. The change in absorbance due to orange-brown color formation was measured at 445 nm.

A unit of aldehyde oxidase activity is defined as nmoles of H_2O_2 produced per min per mL of enzyme. A standard curve was prepared with known concentrations of H_2O_2.

Native Gel and Activity Staining Procedure

A non-denaturing polyacrylamide-gel-electrophoresis PAGE (native) (Maizel, 1971) was performed on 7.5 and 10% acrylamide gels at 150 volt using Mini-Protean II electrophoresis system (Bio-Rad). Crude, heat-treated, ammonium sulfate precipitated and affinity purified aldehyde oxidase preparations were loaded at a level of 25 to 280 μg protein. Bovine serum albumin, β-amylase, β-galactosidase (Sigma Chemical Co.) and a high-molecular weight prestained standard (Bio-Rad) were also loaded. The gel was run for 95 min (40 min more after the dye front leaved the gel) to achieve better separation. At the end of the electrophoresis, the gel was cut in two halves. One half was stained with Coomassie blue dye (Sigma Chemical Co.) to identify the protein bands. The second half of the gel was used for an aldehyde oxidase activity stain. The staining solution consisted of 100 mM Tris, 0.8 mg horseradish peroxidase, 0.6 mM *o*-dianisidine dihydrochloride and 66 mM acetaldehyde or 6.6 mM n-pentanal in a final volume of 30 mL. The orange-brown stain of aldehyde oxidase activity appeared after 30 to 60 min. The gels were photographed and dried.

Denaturing-PAGE

A 7.5% gel was used in SDS-PAGE (Laemmli, 1970) by following the Bio-Rad procedure for mini-Protein electrophoresis system (Bio-Rad). High-molecular weight protein standards of 45 to 200 kDa (Bio-Rad) and 45 to 205 kDa (Pierce) were used at 1 μg per lane. Similarly, each lane was loaded with aldehyde oxidase samples to a total protein concentration of 1 μg. The electrophoresis was performed at a constant voltage of 175 V for

1 h or until the dye travelled out of the gel. The gels were stained by using the silver stain procedure of Wray *et al.* (1981).

Isoelectric Focusing (IEF)

A mini IEF cell (Model 111) from Bio-Rad was used to determine the isoelectric points (Righetti, 1983) of PAO-I and PAO-II. A 5% polyacrylamide gel was cast using the Bio-Rad protocol. Biolytes (ampholytes) of pH 3.8-9.2 and pH 5.0-8.0 were purchased from Bio-Rad. Bovine milk β-lactoglobulin (pI 5.1), bovine erythrocyte carbonic anhydrase (pI 5.9), human erythrocyte carbonic anhydrase (pI 6.6), and horse heart myoglobin (pI 6.8, 7.2) were purchased from Sigma Chemical Company and used as IEF markers. The samples were dialyzed against glycine-NaOH buffer without salt. The samples (1-2.5 μL) were loaded and run at voltage increments of 100, 200 and 450 V for 15, 15 and 60 min, respectively. After the electrophoresis run, the IE-focussed proteins were fixed with a solution containing 12.5% trichloroacetic acid (Fisher Scientific), 4% sulfosalicylic acid (Sigma Chemical Co.) and 30% methanol (Fisher Scientific) for 1 h. The gel was then rinsed with nanopure water five times with gentle shaking to remove any residual trichloroacetic acid and sulfosalicylic acid. The gels were stained using silver staining. Any residual trichloroacetic and/or sulfosalicylic acid resulted in darkening of gel during staining. This problem was minimized by through washing of the gel. The distance travelled by the IEF markers were plotted against pI. The pI of PAO-I and PAO-II were determined using the standard curve with pI of standards.

PAO-I Characterization

pH Stability

The PAO-I was incubated for up to 60 min at 30 °C in the buffers (5-mL) ranging from pH 4.4 to 12.4. The pH of the buffers was measured after adding enzyme The buffers used were: 50 mM (Ionic strength, I=0.45) citric acid-citrate (pH 4.4, 4.9, and 5.3), 100 mM (I=0.4) phosphate (pH 5.9, 6.6, 7.1, and 7.6), 50 mM (I=0.15) sodium borate-HCl (8.1 and 8.6), 50 mM (I=0.15) sodium borate-NaOH (pH 9.3, 10.0, and 10.7), and 50 mM (I=0.15) disodium hydrogen phosphate-NaOH buffer (pH 11.5, 12.0, and 12.4). Five time points at 0, 10, 20, 30, and 40 min or 0, 15, 30, 45 and 60 min were used to incubate the enzyme at various pHs. Incubation buffers were chosen such that the temperature did not have appreciable effect on pH. An aliquot of 100-µL enzyme after incubating at a certain pH and time was brought back to the assay pH by adding 1-mL assay buffer, and the remaining activity determined. The enzyme assay pH of 7.5 was confirmed for each pH evaluated. The denaturation constant (*k) was* determined using eq. 1 (Dixon and Webb, 1979):

$$k = \frac{2.3}{t} \log \frac{A_o}{A} = 2.3 \; x \; slope \qquad (1)$$

where, A_o is the original activity and A is the activity after incubation.

Temperature Stability

The PAO-II was incubated between 20 to 65 °C at increments of 5 °C for various time intervals in 5-mL of 50 mM (I=0.15) sodium borate-NaOH buffer at pH 10.0. After

incubating at specific temperature, the PAO-I was brought back to assay temperature by warming or cooling the incubated sample to 30 °C. The denaturation constants (k) were determined using eq. (1). The energy of activation and denaturation was determined by using eq. 2 (Stauffer, 1989):

$$k = A \cdot e^{-\frac{E_a}{RT}} \quad (2)$$

where k is the rate constant, A, Arrhenius constant, R, universal gas constant, T, temperature in K, and E_a, energy of activation in kJ/mol · K^{-1}.

Effect of Oxygen Concentration on the Enzyme Assay and Comparison of Coupled Assay with Oxygen Uptake

A biological oxygen monitor, model 5300 (YSI Inc., Yellow Springs, OH) with Clark electrodes was used to determine the dissolved oxygen concentration (Chen and Whitaker, 1986). One aliquot of buffer was saturated with oxygen by bubbling air and the second aliquot was oxygen-free, which was achieved by bubbling the buffer by nitrogen for 30 min. These two buffers were mixed in different ratios to get 35, 22, 16, 9.4 and 1% oxygen which corresponds to 4.52, 2.84, 2.13, 1.21 and 0.13 mM oxygen, respectively at 30 °C.

The activity of PAO-I by the coupled assay involves the production of H_2O_2 followed by oxidation of o-dianisidine by peroxidase. This was compared by measuring the rate in oxygen consumption in converting aldehydes to carboxylic acid by using the oxygraph. The decrease in oxygen concentration was recorded on a chart recorder and the initial slope was

used to determine activity. The oxygen uptake in μmol was equal to (% O_2 uptake X 0.1)/2.15.

Effect of pH on the Rate of Reaction

The effect of pH on reaction rate was determined by carrying out the reaction at various substrate concentrations (Whitaker, 1972). The substrate concentrations were adjusted such that there was at least 100-fold difference in the lowest and the highest substrate concentrations. To determine pK of the prototropic groups present in the rate-limiting step (dissociation of ES complex) and on the free enzyme binding of substrate was determined by measuring initial rates at least eight substrate concentrations at pH 5.8, 7.05, 8.0, 9.0, 10.0, 11.0, and 12.0. The buffers used and their concentrations and ionic strengths were similar to that in pH stability study. Dixon-Webb plots (1979) were used to determine the pK*a* of amino acid residues in the ES complex by plotting log ($V^{H+}{}_m$) vs. pH and to determine the pK*a* of active site amino acid residues in the free enzyme, log ($V^{H+}{}_m/K^{H+}{}_m$) vs. pH was plotted.

The V_m and K_m were determined using a Wilkinson plot (Wilkinson, 1961) using Wilkinson program written in BASIC language. The first and second pK*a* for the ES complex were determined by using a Bell program rewritten in BASIC from Cleland's FORTRAN program (Stauffer, 1989). The two pK*as* for the binding of free substrate to the enzyme were determined by using the modified Bell program in BASIC based on the Alberty and Massey modification (1954).

Freeze-Thaw Stability

In a commercial process, the enzyme may be obtained by thawing a frozen enzyme preparation. This may result in multiple freeze-thaw cycles. Therefore, the affinity purified PAO-I was subjected to eleven freeze-thaw cycles to determine the enzyme stability during storage. A 1-mL aliquot of PAO-I was transferred to microfuge vials, frozen at -18 °C, and then thawed at room temperature and refrozen. This cycle was repeated eleven times. The residual activity of aldehyde oxidase was measured in duplicate.

Freeze Drying (Lyophilization)

Many of the enzyme preparations are stored in the lyophilized form for a longer storage life (Robyt and White, 1987). Easy use of lyophilized enzyme is preferred in industry. To determine the effect of freeze-drying on PAO-I, a Labconco (Sheboygan, WI) bench-top freeze dryer was used to freeze-dry 250 µg of PAO-I. The glycine-NaOH buffer containing PAO-I was placed in a 50-mL GC vial which was immersed in a bath containing acetone at -18 °C. Vacuum was applied after the completion of freezing to complete lyophilization. The lyophilized sample was then resuspended in a standard buffer (glycine-NaOH) to determine the protein and the activity remaining after lyophilization.

Off-flavor Removal from Soy Extract

Selection of Bean Variety

Two food-grade soybean varieties, organically grown Vinton 81 (crop year, 1994) and XLRB (mixed beans obtained from Nichii, Inc., Jefferson, IA), and one field bean (Land

O'Lakes, L-2949) (lipoxygenase positive) variety were chosen because of their widespread food use and/or objectionable beany flavor. Vinton 81 (food-grade) is used extensively in tofu preparation, while XLRB (food-grade) has been tested by the ISU sensory panelists as having a strong beany flavor. Field beans such as Land O'Lakes L-2949 are known to have a strong beany flavor. A high-speed flour mill (Magic Mill III Plus, Salt Lake City, UT) was used to grind the beans and produce soy flour. Dry-ice chips were added to prevent overheating and scorching of the flour. To determine which variety of beans produces the most intense beany flavor, an informal sensory panel of eleven panelists was formed. Three samples were randomly coded and the panelists were asked to rank the samples according to the beany flavor intensity. The sample coded for XLRB bean variety had the strongest beany-flavor, as perceived by the sensory panelists and was chosen for this study.

Cold Defatting of Flour

Cold defatting of XLRB soy flour (500 g) was performed at 25 °C to minimize protein functionality loss during defatting (O'Keefe, 1991). Equal quantities of flour and Skelly B (hexane) were placed in an Erlenmeyer flask. The mixture was stirred for 1 h and then the solvent was removed using a Buchner funnel with suction. This procedure was repeated five times or until the solvent was no longer yellow. The flour was then dried in the hood to remove residual hexane. The flour was then stored at 4 °C.

Preparation of Soy Extract

Soy extract was prepared by dissolving 10 g of defatted XLRB flour in 100-mL nanopure water and the pH of the extract was measured. The mixture was swirled at 200 rpm for 2 h to extract the maximal amount of the protein. The soy flour slurry was then centrifuged at 30,000 x g for 30 minutes. The supernatant was collected and used as water extract of soy proteins (1:10). The protein content of water extract of soy flour was determined by the Bio-Rad protein assay. The pH of the extract was adjusted to 7 and 9, respectively, using 1 M NaOH. To measure the amount of carboxylic acids and the residual aldehydes due to the action of PAO-I on the aldehydes in the headspace, the pH of the extract was brought to 2.0 using 5 M HCl.

For the headspace gas chromatography analysis, the PAO-I-treated soy protein extract was prepared by incubating 5-mL of soy extract with the enzyme. The amount of added enzyme was varied to obtain different ratios of PAO-I activity to soy protein. Initially, 1-mL aliquots were withdrawn at 0, 30, 60, 120, and 240 min intervals for headspace analysis. In the subsequent treatments, time intervals of 0, 15, 30, 45, and 60 min were found more suitable. The reaction was stopped by adding 5 M HCl.

Determination of PAO-I Activity in the Presence of Soy Protein

The effect of soy protein on enzyme activity was determined by incubating 0.3 % (w/w) suspension of PAO-I in the water extract of defatted soy flour at 4 °C for up to 12 h. An aliquot of the mixture containing PAO-I and soy protein was withdrawn at various time intervals and the enzyme activity was determined.

Gas Chromatography

Gas Chromatography Conditions

One mL external standard and soy protein extract were placed in 50-mL gas chromatography (GC) vials. The GC bottle was sealed with a Teflon septum and aluminum seal (Supelco, Bellefonte, PA) and then equilibrated for 2 h at 35 °C before head-space analysis in a Fisher Isotemp 500 series oven (Fisher Scientific, Pittsburgh, PA). The equilibrium headspace analysis of the control and PAO-I-treated water extract of soy flour was performed by using a Varian 3400 (Sunnyvale, CA) gas chromatograph equipped with a flame ionization detector (FID). Two different columns were used in this work: a 30-m long DBWAX fused-silica capillary column with 0.25-mm i.d. and 0.25-μ film (J&W Scientific Inc., Rancho Cordova, CA) and a 30-m long NUKOL fused silica capillary column with 0.32 mm i.d. and 0.25 μm film (Supelco). The column injector temperature and FID temperature were set at 230 °C for DBWAX and 210 °C for the NUKOL column. The DB-WAX column temperature was maintained at 40 °C for 10 min, then the temperature was changed to 200 °C at 10 °C/min and held at 200 °C for 10 min. An incremental temperature program was used for the NUKOL column. The initial temperature of the column was set at 40 °C, and then the temperature was raised to 100 °C at 15 °C/min. The next temperature increase was carried out in 10 min to achieve 150 °C followed by two more temperature increments of 2.5 °C/min and 12.5 °C/min, which resulted in 175 °C and 200 °C, respectively. The column was held at 200 °C for 2 min. The total running time for both the columns was 36 min. Throughout the GC analysis the flow rate of the carrier gas (H_2) was 4 mL/min. Nitrogen, used as a make-up gas, was delivered at a rate of 30 mL/min. The air and hydrogen flow

rates in the FID were set at 300 and 30 ml/min, respectively. The attenuation was set between 4-8, and the detector sensitivity was 10^{-12} amp/sec. The peak rejection value was set at 2000 integration units (IU), and the chart speed was set at 1.0 cm/min for all analyses. A time-temperature protocol used in this study is shown in Figure 1.

Sample Preparation

Two 50-mL GC-bottles containing the soy-extract and soy-extract/PAO-I mixture were analyzed. Total soy protein in the extract and PAO-I mixtures (200:1 and 1000:1 ratio of total soy protein to PAO-I) were incubated at 35 °C for various time intervals ranging from 15 to 240 min. A total of five injections in triplicate at various time intervals were made for each reaction. At the end of each incubation period, an aliquot of reaction mixture was withdrawn and the reaction was stopped by adding 5 M HCl. The treated extract and control samples were brought to pH 2.0. A 5.0-mL gas-tight Hamilton syringe (AllTech Associates Inc., Deerfield, IL) was used to inject a 25 µL to 2.5-mL headspace aliquot at a rate of 2 mL/min. After 2 min of cryofocusing (by placing the a loop of capillary column from injector side in a styrofoam cup containing liquid N_2), the temperature program was initiated, and the oven was turned on. The samples were incubated in the GC vials and the headspace GC analysis was carried out in triplicate. The decrease in the amount of aldehydes (C_2-C_7) and the concomitant increase in their corresponding fatty acids was followed by GC.

The volatile compounds were identified by using acetaldehyde, propanal, n-butanal, n-pentanal, n-hexanal, heptanal, acetic, propionic, butyric, pentanoic (valeric), hexanoic

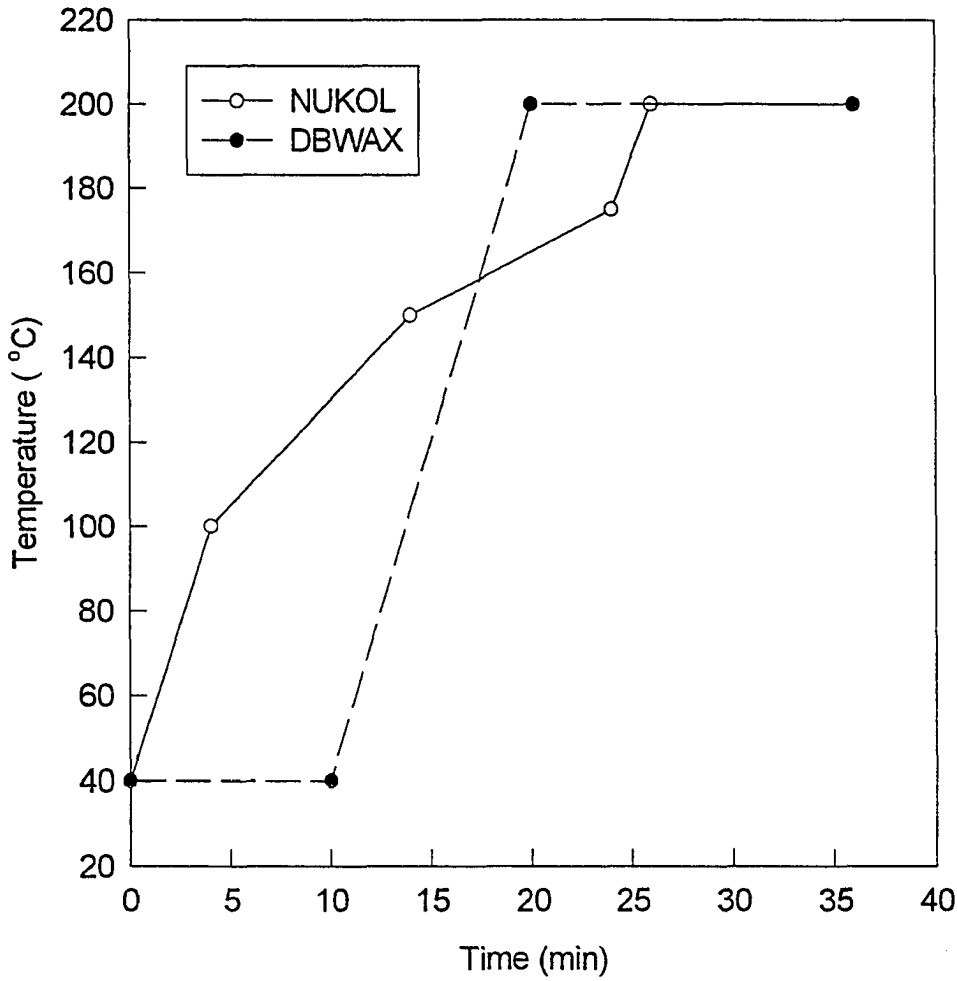

Figure 1. Time-temperature profile used in gas chromatography.

(caproic) and heptanoic acid as external standards. Various volumes (0.1-5 μL) of external standards were injected to generate peak area. This was used to correlate with the vapor pressure (to obtain moles of volatiles) data to find out the amount of volatiles in the control and enzyme-treated samples.

Determination of Vapor Pressure and Calculation of Mole Fraction of Aldehydes and Carboxylic Acids

The vapor pressures of aldehydes and carboxylic acids were determined by Clausius-Clapeyron equation and Nernst-Rankine equations (Montgomery, 1952) at 35 °C.

The Clausius-Clapeyron equation is given by:

$$\ln P = -\frac{\Delta H_{vap}}{RT} + A \qquad (3)$$

where P is the vapor pressure (Pa); ΔH_{vap} the heat of vaporization (J/mol); R the gas constant, 8.314 J/ mol · K; and T the absolute temperature (K).

The Nernst-Rankine equation is shown below:

$$P = \exp\left[A + \frac{B}{T} + C \ln T + D\, T^{E}\right] \qquad (4)$$

where P is the vapor pressure (Pa) and T is the temperature (K). The values of coefficients (A, B, C, D, and E were obtained from Daubert and Danner (1993).

After determining the vapor pressure, the number of moles left in the control and PAO-I treated soy-extract were calculated by using the ideal gas equation.

$$PV = nRT \tag{5}$$

where P is the vapor pressure(Pa); V the volume (mL) of GC vial; and n the number of moles.

The integration units of aldehydes and acids in the sample headspace were correlated with the nmoles by the standard curve. A 1-mL sample of pure aldehydes and carboxylic acid were placed in 50-mL GC vial and they were equilibrated for 1 h at 35 °C. The standard curve was prepared by injecting 0.5-5.0 μL of the headspace volatiles from the GC vials.

Sensory Analysis

A paired comparison test (O'Mahony, 1986) was conducted to determine the difference in the beany flavor in the control and PAO-I treated water extract of defatted soybean flour. Fifteen sensory panelists were chosen based on their ability to identify the beany-flavor. The panelists were asked to sniff the randomly coded control and PAO-I treated water extract of soybean. Standard tables (Meilgaard *et al.*, 1991) were used to determine the level of significance of the responses given by the sensory panelists.

Statistical Analysis

A student t-test was used to determine the level of significance throughout this study (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Purification

Purification of Aldehyde Oxidase

Fresh or frozen pig-liver was homogenized using a tissue homogenizer. The pH of the extract dropped as acids were released from the ruptured cells (Whitaker, 1972). The initial activity of aldehyde oxidase (AOX) of crude extract was determined by recording the decrease in absorbance at 600 nm due to the oxidation of 50 μM of reduced dichlorophenol indophenol (DCIP) dye by hydrogen peroxide produced at pH 9.0. The hydrogen peroxide was produced by the oxidation of acetaldehyde or pentanal by PAO. This assay was discontinued as change in absorption could not be directly correlated with suitable standards. The dye was not stable when hydrogen peroxide was used as standard. Therefore, the activity assay was changed to a coupled-enzyme assay involving PAO and horse-radish peroxidase. Oxidation of *o*-dianisidine dye by H_2O_2 resulted in orange-brown colored polymer. The H_2O_2 was produced by oxidation of aldehydes (acetaldehyde and n-pentanal) by porcine aldehyde oxidase at 445 nm wavelength. Takahashi *et al.* (1979) used wavelength of 436 nm to measure the orange-brown color. We found that the maximal absorption of the orange-brown color was at 445 nm wavelength for Beckman DU-50 spectrophotometer.

The tissue homogenate of porcine liver contains many endogenous substrates that may interfere in the activity assay. To correct the oxidation of *o*-dianisidine dihydrochloride by endogenous substrates, the blank contained the same aliquot of crude homogenate as in

sample cuvets. The crude homogenate was boiled to denature the AOX, and the assay was carried out. As shown in Table 1, the presence of catalase interfered in determining the initial activity of the crude homogenate. There was more than threefold increase in total activity of the heat-treated extract compared with the crude homogenate. Because catalase competes with peroxidase for H_2O_2, a true estimate of the aldehyde oxidase activity could not be made in the crude homogenate, heat-treated extract, and ammonium sulfate fraction. The presence of catalase was detected by adding hydrogen peroxide which was followed by rapid formation of bubbles from O_2 release in the crude homogenate, heat-treated extract and ammonium sulfate fraction. The preparation of bovine liver aldehyde oxidase of Takahashi *et al.* (1979) contained catalase and it was a problem in measuring AOX activity. Worthington (1988) reported that catalase was sensitive to freezing storage. The attempt to inactivate catalase in the crude and heat-treated homogenate by subjecting the extracts to freeze-thaw cycles did not succeed. Even after five freeze-thaw cycles the catalase was not completely inactivated. However, affinity chromatography step (discussed later) separated catalase from aldehyde oxidase.

After centrifugation, the crude extract was subjected to heat treatment. The supernatant after centrifugation was clear and substantial amount (15 mg/mL) of protein was precipitated. The purpose of heat treatment was to eliminate heat-sensitive proteases and other heat-labile enzymes. Fresh and frozen (-70 °C) porcine liver samples demonstrated no statistically significant difference ($p>0.05$) in the initial AOX activities of 1.3 units and 1.2 units, respectively. Catalase was detected in both preparations.

Table 1. Purification of aldehyde oxidase

Fraction	Volume (mL)	Total activity* (units)	Total protein (mg)	Specific activity (units/mg)	Fold Purification	% Yield
Crude extract	49.1	63.4	1225.8	0.05	1	100
Heat treatment	42.4	218.1	501.1	0.43	8	344
Ammonium sulfate fractionation (35-55% cut)	2.0	790.0	200.0	3.95	76 (1†)	1246 (100†)
Affinity chromatography (0-25 mM) gradient						
PAO-I affinity chromatography (8 mM benzamidine)	10.0	118.5	0.252	470.2	9042 (119†)	187 (15†)
PAO-II affinity chromatography (25 mM benzamidine)	10.0	158.0	0.360	438.9	8440 (111†)	249 (20†)

* Activity determined using acetaldehyde as substrate
† Values calculated after ammonium sulfate purification

The activity of the resuspended ammonium sulfate fraction was measured before and after dialysis against the 100 mM glycine-NaOH. The total activity decreased by 15-20% at the end of dialysis. There was no precipitation of the ammonium sulfate fraction in the dialysis bag. Palmer (1962) reported a 25% loss of PAO activity after dialysis of ammonium sulfate fraction but he also demonstrated a protective effect of ammonium ions on PAO activity. The dialyzed ammonium sulfate fraction was used in the subsequent purification steps in this study.

Preliminary Purification Attempts

To further purify aldehyde oxidase after ammonium sulfate fractionation anion exchange chromatography, gel filtration, and preparative isoelectric focusing were attempted. However, none of these techniques resulted in a significant purification of aldehyde oxidase. The detailed procedures of these techniques are given in Appendix A.

After each step of purification when SDS-PAGE was used to evaluate protein purity, a considerable number of low-molecular weight protein bands were present. These bands were suspected to be due to the proteolytic action of proteases that survived the heat-treatment step. To prevent proteolysis, three protease inhibitors were evaluated. Phenylmethylsulfonylfluoride (serine protease inhibitor), pepstatin A (acid protease inhibitor) and cystatin (sulfhydryl protease inhibitor) were used. There was no effect of these protease inhibitors on low-molecular weight protein band profile when added separately or in a mixture to the ammonium sulfate fractions. Also, the intensity and the number of bands on SDS-PAGE did not change with the length of incubation. Therefore,

either the proteases were not active in the ammonium sulfate fraction or the protease inhibitors were not effective in controlling proteolysis. The details of this work is presented in Appendix A.

Affinity Chromatography

The most important purification step as shown in Table 1 was the affinity chromatography on Benzamidine-Sepharose 6B affinity ligand. On smaller affinity column the total yield of both aldehyde oxidase enzyme forms (PAO-I and PAO-II) was 40% of the ammonium sulfate fraction using 10 mM benzamidine as eluent. When affinity chromatography was scaled up to a total of 150-mL resin volume, the objective to get higher quantity of aldehyde oxidase was met, and a 35% total yield (Table 1) of porcine liver aldehyde oxidase (PAO) was achieved. There was no statistically significant difference ($p>0.05$) in the percentage yield of aldehyde oxidase activity recovery using smaller and larger affinity column. A 114-fold purification fold over the ammonium sulfate preparation is an indicator of the higher purity of the AOX preparation which was confirmed by SDS-PAGE (Figure 2).

The lower yield of aldehyde oxidase was due to desorption of the enzyme during washing (10 column volumes) with glycine-NaOH buffer. About 20% AOX activity was found in the unbound fraction. This suggested that the affinity adsorption of aldehyde oxidase to benzamidine Sepharose was not very specific. As shown on the SDS-PAGE (Figure 2), the aldehyde oxidase protein band was present in the unbound fraction (lanes 6 and 7), the wash step (lane 12), in the eluted fractions with 10 mM benzamidine (lanes 9

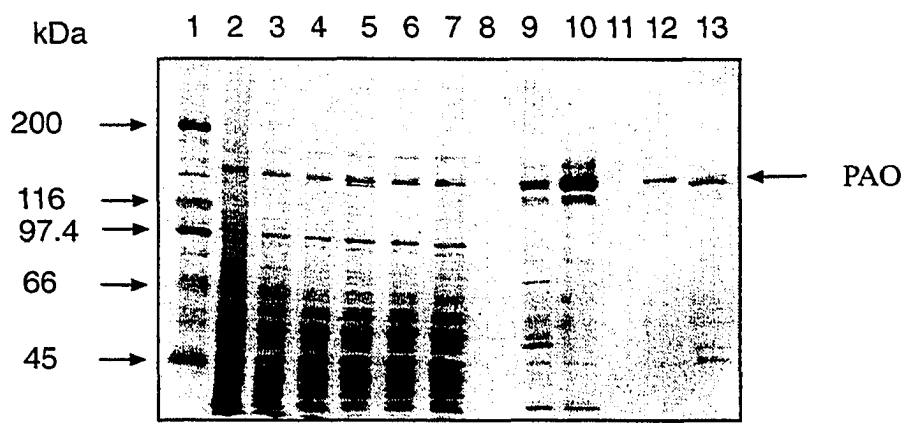

Figure 2. SDS-PAGE of porcine liver aldehyde oxidase purification. Lane 1:high molecular weight markers mix (1-μg) obtained from Bio-Rad, myosin (200 kDa) from rabbit skeltal muscle, β-galactosidase (116 kDa) from *E. coli*, phosphorylase B (97.4 kDa) from rabbit muscle, bovine serum albumin (66 kDa), and hen egg white albumin (45 kDa); lane 2: crude extract (1-μg); lane 3: heat treated extract (1-μg); lanes 4 and 5: ammonium sulfate fraction (1-μg); lanes 6 and 7: unbound (not retained) ammonium sulfate from affinity column (1-μg); lane 9 (0.5 μg) and 10: eluted PAO (0.5-μg); lane 12: desorbed protein (0.2 μg) during washing with glycine-NaOH buffer; and lane 13: desorbed protein (0.2-μg) from the colum after affinity elution. Proteins were stained using silver stain.

and 10) and in the column regeneration step after elution of PAO (lane 13). The desorbed protein (lane 13) after the elution of AOX with 10 mM benzamidine had a lower molecular weight (128 kDa band) than the eluted aldehyde oxidase (lanes 9 and 10) as well as desorbed protein (131 kDa) before affinity elution (lane 12). This difference in molecular weight could be due to two different proteins or enzyme forms of aldehyde oxidase, which will be discussed later in this chapter. Stell *et al.* (1989) obtained a 85% yield of affinity purified rabbit liver aldehyde oxidase with only three column volumes washing with buffer after the binding of AOX to Benzamidine Sepharose. In this study, even five column volumes were not adequate to produce pure AOX preparation as more protein bands were detected on SDS-PAGE of the affinity eluted PAO fraction. Ten-column-volume washing was identified as a method of choice for higher purity of PAO, but it also resulted in a lower yield of enzyme. Due to desorption of PAO in the unbound and wash fractions, it is evident that complete binding of aldehyde oxidase under the present conditions did not take place. Furthermore, it required a total of 72 h for PAO purification in the larger column due to flow rate and column fitting constraints.

The SDS-PAGE chromatography (Figure 2) of the affinity purified samples showed the presence of two bands close to 132 kDa (lanes 9 and 10). This molecular weight corresponds to the monomer of porcine liver aldehyde oxidase having 270 kDa molecular weight as suggested by Felsted *et al.* (1973). The two protein bands in this study (Figure 2) were suspected to be either two aldehyde oxidase enzyme forms or a breakdown product of AOX. To confirm this, native PAGE and enzyme activity staining were performed.

The results of activity staining are shown in Figure 3. The ammonium sulfate fraction and affinity chromatography purified fraction showed two enzyme activity bands when acetaldehyde was used as a substrate. This clearly suggested the presence of two enzyme forms of porcine liver aldehyde oxidase having different molecular weights and/or charges.

These two aldehyde oxidase enzyme forms were separated using a 0-25 mM affinity elution gradient of benzamidine. First enzyme form, PAO-I, eluted at 8 mM benzamidine concentration and the second enzyme form, PAO-II, at 25 mM benzamidine (Figure 4). Both PAO-I and PAO-II showed similar activity with acetaldehyde. However, the PAO-I had five times greater activity than PAO-II with n-pentanal as a substrate (Table 2) suggesting higher specific activity for longer aliphatic aldehydes. These results were confirmed by the activity staining of PAO-I and PAO-II with acetaldehyde and n-pentanal as substrates, respectively (Figure 5). The staining with n-pentanal as substrate showed only one enzyme form band on the native-PAGE gel (Figure 5), but with acetaldehyde two bands (Figure 5, bands not clearly visible in this figure) were visible when PAO-I and PAO-II were used in a mixture. Native PAGE of purified PAO-I and PAO-II showed only one band when stained with n-pentanal and acetaldehyde as substrates, respectively.

Analytical isoelectric focusing was performed to determine whether there was any difference in the pI of these two enzyme forms (Figure 6). There was only one major protein band at pI 5.8 of the isoelectrically focused AOX, indicating that the two pIs were very similar. The SDS-PAGE (gel not shown) of the extracted protein bands corresponding to

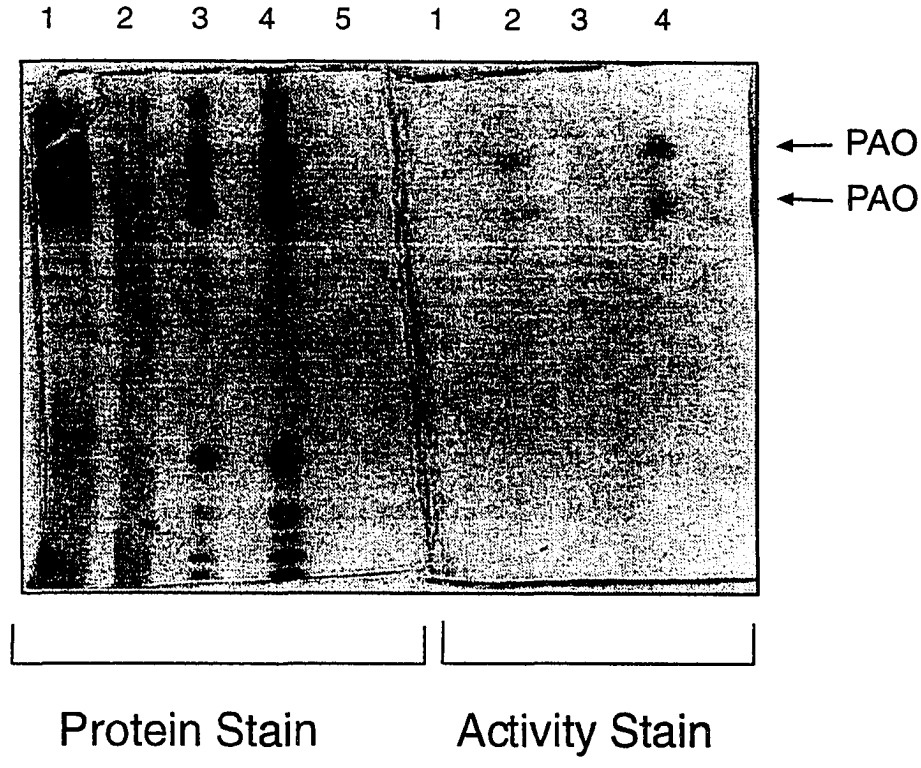

Figure 3. Native-PAGE and activity staining of porcine-liver aldehyde oxidase after elution with 10 mM benzamidine. Left half of the gel represents protein staining (Coomasie blue). Lane 1: High molecular weight markers (5-µg); lane 2: prestained high molecular weigh marker (20-µg); lanes 3 and 4: affinity purified PAO, 5 and 10-µg, respectively. Right half of the gel represents activity staining of PAO using acetaldehyde as substrate. Lanes 2 and 4: affinity purified PAO, 5 and 10-µg, respectively (Tasayco and Prestwich, 1990).

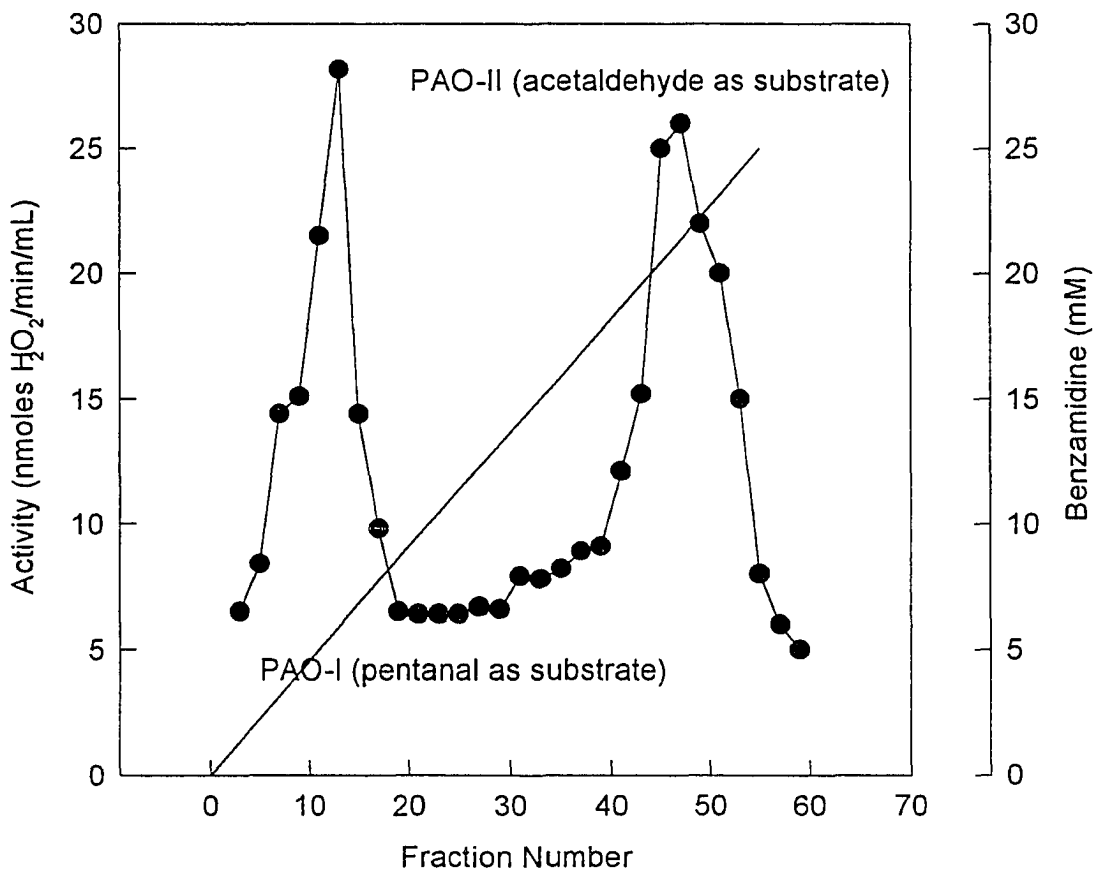

Figure 4. Elution of two aldehyde oxidase activity peaks using affinity chromatography. Peaks were eluted using 0-25 mM benzamidine gradient.

Table 2. Comparison of substrate specificities of two enzyme forms of aldehyde oxidase

Substrate (mM)	Specific activity of PAO-I (units/mg)	Relative† substrate specificity (%)	Specific. Activity of PAO-II (units/mg)	Relative† substrate specificity (%)
Acetaldehyde (33)	540.2	100	383.0	100
Propanal (33)	603.4	112	72.5	19
Butanal (33)	588.2	109	215.4	56
Pentanal (3.3)	1187.2	220	78.0	20
Hexanal** (0.33)	678.9	126	14.4	4

*one unit of aldehyde oxidase activity is defined as nmoles H_2O_2 produced/min/mL of enzyme.
**substrate concentration may not be saturating.
†relative activity with respect to acetaldehyde activity

PAO-I and PAO-II from IEF demonstrated the presence of two protein bands close to 128 to 131 kDa molecular weight. The SDS-PAGE (Figure 7) of the affinity gradient purification scheme (0-25 mM benzamidine gradient) which resulted in PAO-I peak (eluted with 8 mM benzamidine, lanes 6 and 7) and PAO-II peak (peak eluted at 25 mM concentration, lanes 9 and 10) showed the molecular weight of PAO-I to be 131 kDa (262 kDa) and 128 kDa (255 kDa dimer) for PAO-II. The separation of these two enzyme forms of PAO is clearly visible on the SDS-PAGE protein bands in Figure 7. A benzamidine gradient was necessary to separate PAO-I and PAO-II, otherwise PAO-I and PAO-II could not be separated when 10 mM benzamidine in glycine-NaOH buffer was used to elute the PAO activity as shown in lanes 9 and 10 of Figure 3.

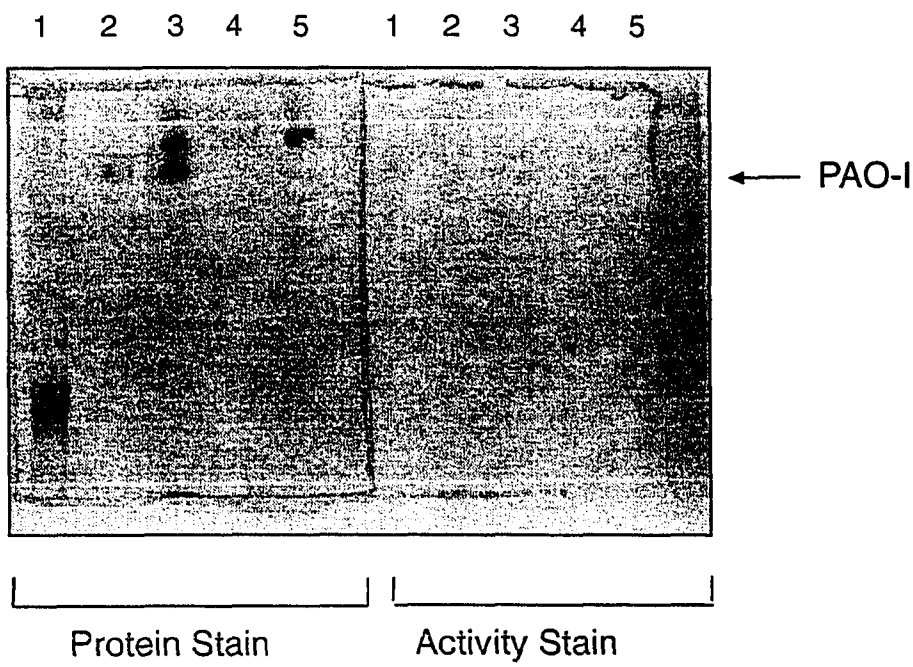

Figure 5. Native-PAGE and activity staining of porcine liver aldehyde oxidase after elution with 0-25 mM benzamidine gradient. Left half of the gel represents protein staining (Coomasie blue). Lane 1: high molecular weight prestained marker (5-μg); lane 2: PAO-I (5-μg); lane 3: PAO-I and PAO-II (10-μg each); lane 5: PAO-II (10-μg). Right half of the gel represents activity staining of PAO-I and PAO-II. Lane 1: PAO-I (5 μg) using n-pentanal as substrate; lanes 3 and 4: PAO-II (5 and 10 μg, respectively) did not stain.

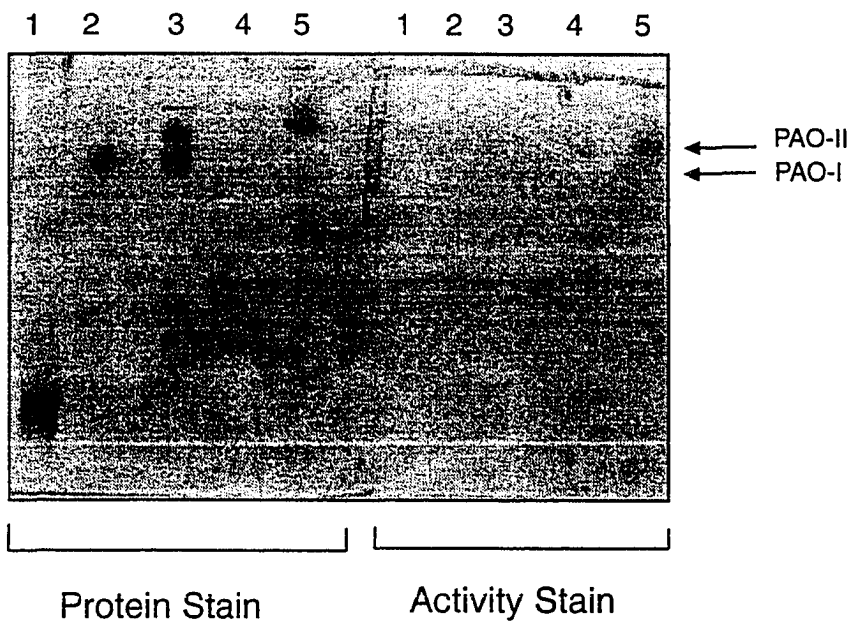

Figure 5. (continued). Left half of the gel represents protein staining (Coomasie blue). Lane 1: high molecular weight prestained marker (5-μg); lane 2: PAO-I (10-μg); lane 3: PAO-I and PAO-II (10-μg each); lane 5: PAO-II (10-μg). Right half of the gel represents activity staining of PAO-I and PAO-II. Lane 3: PAO-I (5 μg); lanes 4 and 5: PAO-II (5 and 10 μg, respectivly) using acetaldehyde as a substrate.

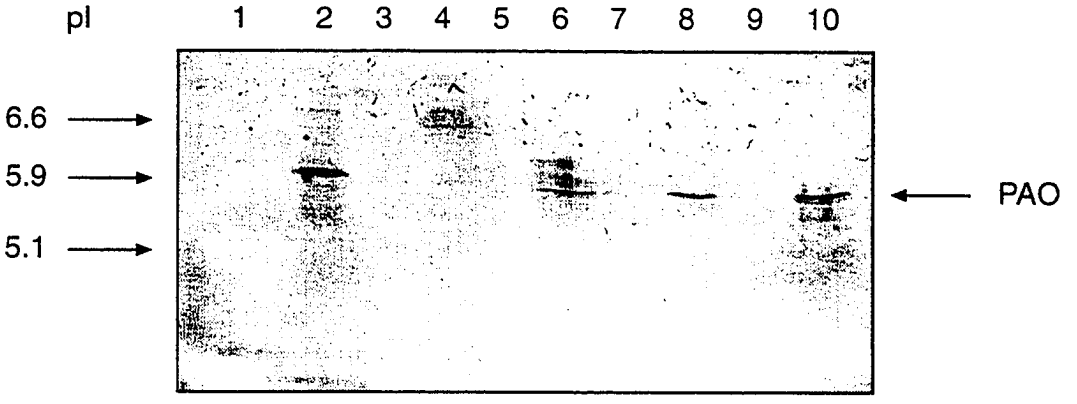

Figure 6. Isoelectric focusing of PAO-I and PAO-II. Lane 1: pI 5.1 marker (1 μg of bovine milk β-lactoglobulin); lane 2: pI 5.9 marker (1 μg of bovine erythrocyte carbonic anhydrase); lane 4: pI 6.6 marker (1 μg of human erythrocyte carbonic anhydrase); lane 6: PAO-I (1 μg); lane 8: PAO-II (1μg); lane 10: PAO-I and PAO-II (1 μg each).

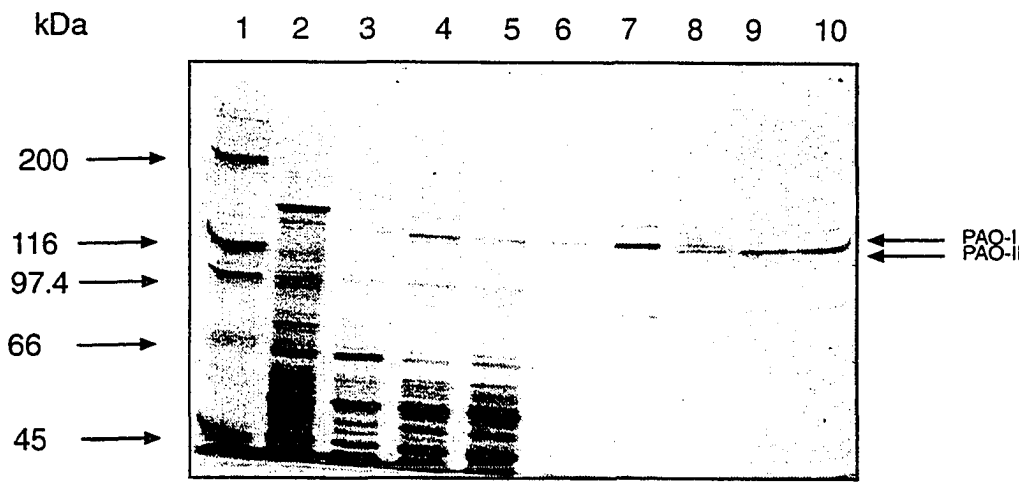

Figure 7. SDS-PAGE (7.5%) of four steps in PAO purification. Lane 1: high molecular weight markers as described in Figure 2 (1 μg); lane 2: crude homogenate (1.5 μg); lane 3: heat-treated extract(1 μg); lane 4: ammonium sulfate (55%) fraction (1 μg); lane 5: unbound ammonium sulfate (1 μg); lanes 6 and 7: affinity purified (0-25 mM benzamidine gradient, PAO-I eluted at 8 mM concentration) PAO-I (0.1 and 0.2 μg, respectively); lane 8: mixture of PAO-I and PAO-II (0.2 μg); lanes 9 and 10: affinity purified PAO-II (0.2 μg) eluted by 0-25 mM benzamidine gradient, eluted at 25 mM concentration. Proteins were stained by silver stain.

The results of SDS-PAGE analysis indicate that PAO exists in two enzyme forms with different substrate specificities (Table 2). PAO-I and PAO-II were purified to homogeneity with gradient affinity chromatography as shown in lanes 6 and 7 (PAO-I) and lanes 9 and 10 (PAO-II) of Figure 7, respectively. A few faint protein bands on SDS-PAGE could be the ghost bands due to silver staining technique in protein detection (Wray *et al.*, 1981). Table 2 showed that PAO-I had much higher substrate specificity for off-flavor-causing aldehydes such as pentanal and hexanal than PAO-II. Therefore, PAO-I was selected for further characterization studies.

Characterization of Porcine Liver Aldehyde Oxidase (PAO-I)

pH Stability

PAO-I was more stable at neutral and basic pHs than at acidic pHs. Less than 20% residual (ratio of PAO-I activity at time t to the original PAO-I activity at t=0) AOX activity was measured with pentanal as substrate at pH less than 4.9 and greater than 10.7 (Figure 8). Beyond this pH range, aldehyde oxidase was inactivated. PAO-I was most stable at pH 10. At this pH 96% of the original activity remained. Greater than 50 and 75% of the original activity remained after 60 minute of incubation in the pH range 5.9 to 6.6 and 7.1 to 10.7, respectively. There was a smaller loss in activity in the pH range of 7.6 to 10.0 than at other pH ranges. Therefore, this form of enzyme can withstand a broad pH fluctuation in the basic environment without substantially affecting protein stability. Although PAO-I is stable up to pH 10.7, its inactivation is more rapid beyond pH 10.7 than below the neutral pH values. Hence, it is important to monitor the pH at basic conditions as a slight increase in pH beyond

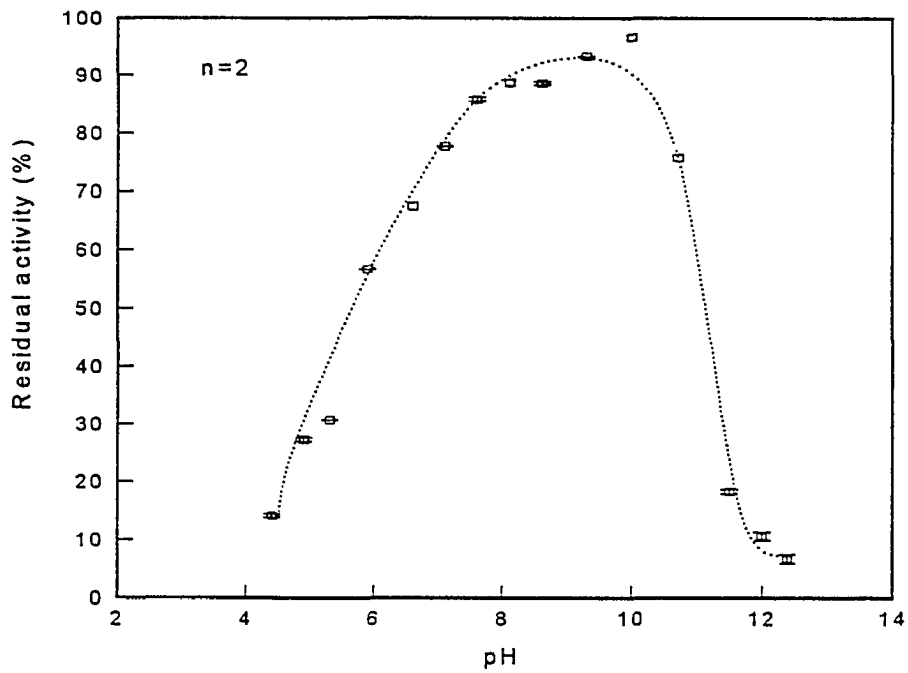

Figure 8. Effect of pH on the residual activity of PAO-I with pentanal as substrate after 60 minutes of incubation at 30 °C. Bars represent standard deviation and n represents number of replicates. Residual activity is defined as ratio of activity at time *t* to the original activity at *t*=0.

10.7 may result in rapid inactivation of PAO-I. The maximal stability of PAO-I at pH 10.0 agrees closely with Felsted *et al.* (1973) pH stability optima of 10.5 for hog-liver aldehyde oxidase.

The residual activities of PAO-I as a function of incubation pH are shown in Figure 9. The stability of PAO-I is inversely proportional to the steepness of the slope. The curves were more steep at neutral and acid pHs and at extreme basic conditions. The minimum inactivation constant (k_d) for this enzyme was between pH 9.3 and 10.0 (Figure 10 and Table B-1 in appendix). The k_d decreased from pH 4.4 to 8.6, remained constant at pH 9.3 and 10.0. The k_d values then increased again after pH 10.0 to 12.4. According to Palmer (1961) the aldehyde oxidase was most stable in the pH range between 9.0 and 9.5 with activity lower at neutral and acidic pH. The results of this study agrees very well with Palmer's study.

A broad pH stability of PAO-I follows the trend of pH stability of several other oxidases. Lipoxygenase from peas is stable between pH 4.5 to 8.0 at 25 °C (Chen and Whitaker, 1986). Catalase is stable between pH 3 and 10 (Whitaker, 1972) and horse radish peroxidase has pH stability between 3.5 to 12 (Maehly, 1955). The pH optima of PAO-I is similar to horse- and human-liver alcohol dehydrogenase which shows maximum stability between pH 10-11 (Lange *et al.*, 1976). Aldehyde dehydrogenase from different species has different pH optima. Human-, horse-, and rat-liver have a pH optima of 9.0. The pH optima for rabbit-liver is 7.0, whereas cow- and sheep-liver aldehyde dehydrogenases have a pH optima of 8.0. Therefore, PAO-I has the pH stability optima at alkaline pH like other oxidases from different species.

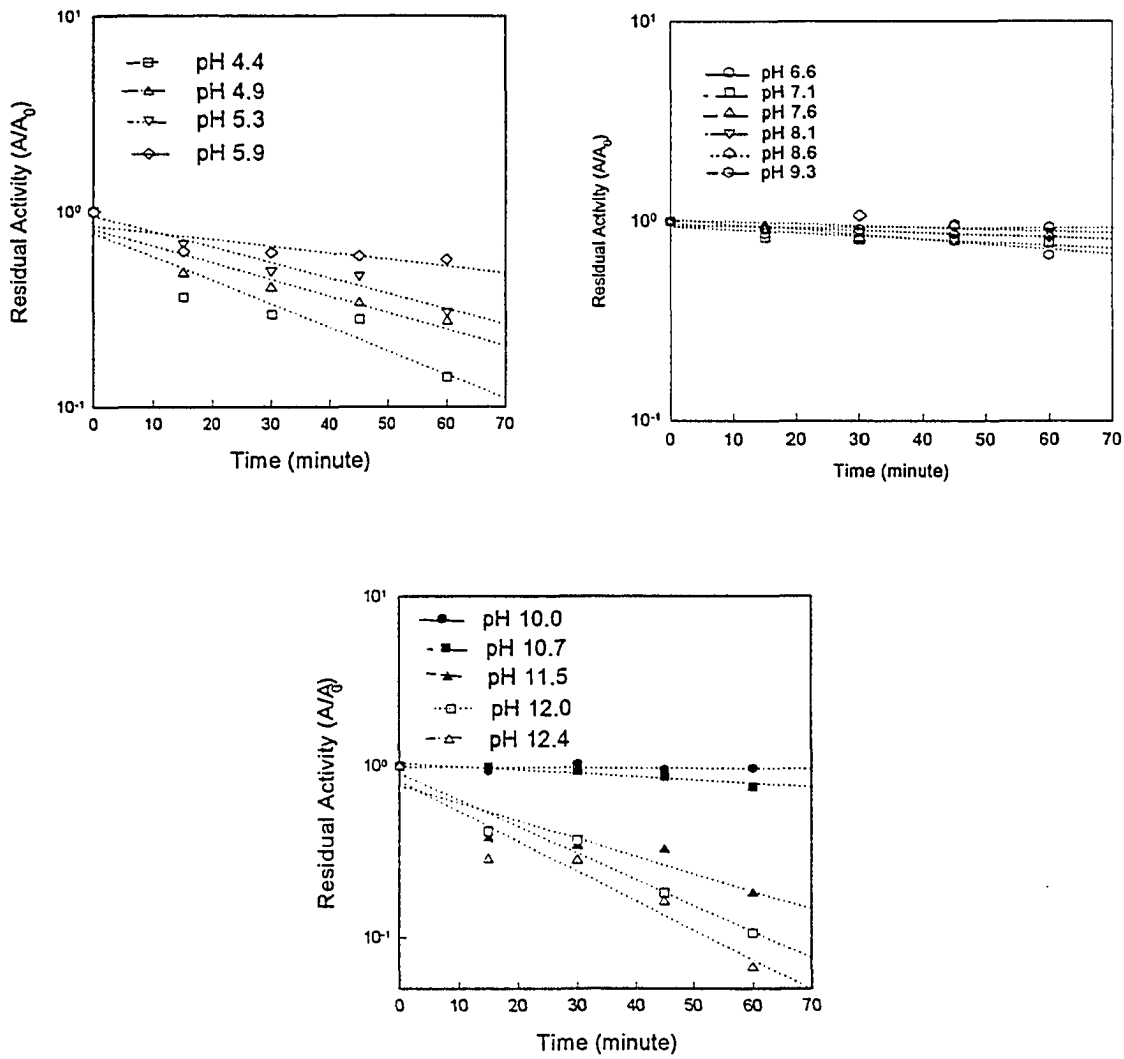

Figure 9. Residual activity of PAO-I at different pHs. Residual activity is defined as ratio of PAO-I activity at time t to the original PAO-I activity at t=0.

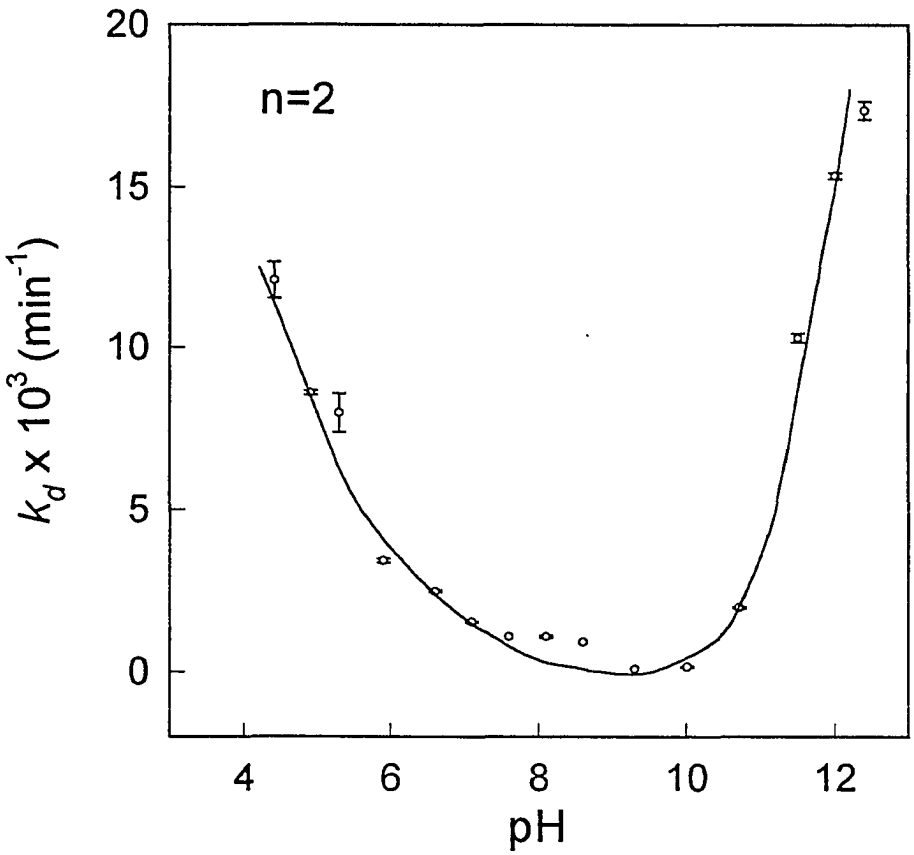

Figure 10. Effect of pH on the inactivation constant of PAO-I. Bars represent standard deviation and n represents the number of replicates.

Temperature Stability

The temperature stability study was carried out at pH 10.0 using 50 mM (I=0.15) sodium borate (borax) buffer. This pH 10.0 was chosen because PAO-I had the greatest stability at this pH. The borax buffer was selected because temperature has very little influence on the pH of the buffer compared with other amino-acid-based buffers (Robyt and White, 1987). The rate of inactivation of aldehyde oxidase increased with temperature. Figure 11 shows the percentage activity on PAO-I remaining after 60 min of incubation at pH 10.0. The effect of temperature of PAO-I stability at different times is shown in Figure B-1 in the appendix. Figure 12 and Table B-2 (for exact numbers see Appendix) show the effect of temperature on k_d values. The denaturation constant increased with temperature. The denaturation of aldehyde oxidase accelerated at temperatures above 45 °C. The k_d values increased from 1.94 x 10^{-3} min^{-1} at 45 °C to 7.5 x 10^{-3} min^{-1} at 50 °C and 27.75 x 10^{-3} min^{-1} at 60 °C. After 60 min of heat-treatment 75%, 40%, and 5% of PAO-I activity remained at 45, 50, and 55 °C, respectively. PAO-I was completely inactivated at 60 °C after 60 min. As expected, the highest temperature stability of PAO-I was at the lowest temperature studied (20 °C). Nonetheless, these results indicate that PAO-I can be used in soy processing up to 45 °C without significant loss of activity.

The temperature stability of PAO-I is similar to lipoxygenase from English pea lipoxygenase. Chen and Whitaker (1986) observed a 5 and 40% loss in activity when lipoxygenase was incubated at 35 °C and 50 °C for 30 min, respectively. However, lipoxygenase was completely inactivated after 4 min at 65 °C and after 30 s at 80 °C. PAO-I showed similar temperature stability compared to lipoxygenase.

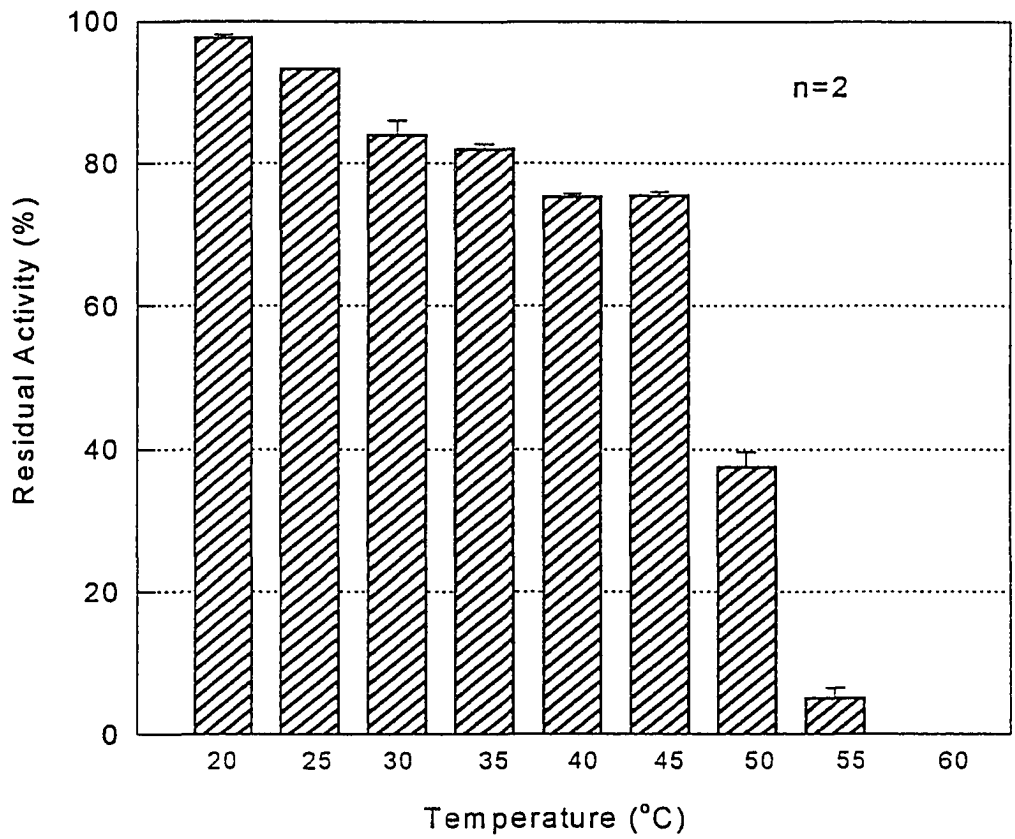

Figure 11. Effect of temperature on PAO-I activity after 60 minutes of incubation at pH 10.0. Bars represent standard deviation and n represents the number of replicates. Residual activity is defined as the ratio of PAO-I activity at time *t* and at *t*=0.

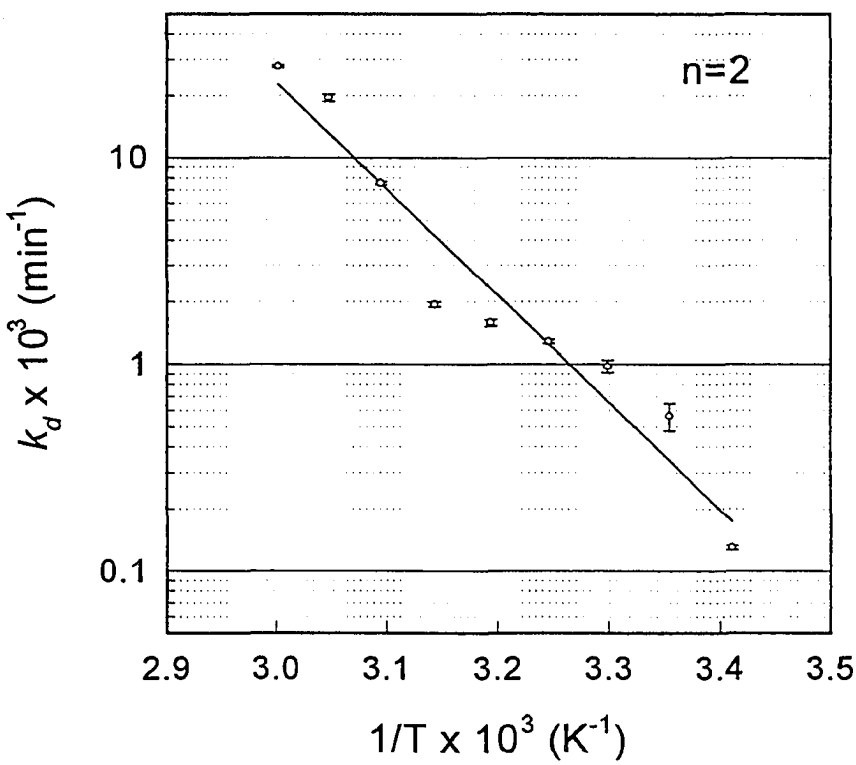

Figure 12. Effect of temperature on the inactivation constant (k_d) of PAO-I at pH 10.0. Bars represent standard deviation and n represents the number of replicates.

Energy of Activation and Denaturation of PAO-I

The energy of activation was determined by measuring the initial rates as close to time zero as possible in temperature range between 20 and 55 °C (Figure 13). The energy of denaturation was calculated from the slope of the log of denaturation constant (k_d) versus the inverse of absolute temperature (greater than 45 °C) (Figure 12). The energy of denaturation (158.1 kJ/mol · K) was more than three times higher than the energy of activation (47 kJ/mol · K). The energy of activation and denaturation values fall in the intermediate range when compared with other enzymatic reactions (Whitaker, 1972). Higher the energy of activation and denaturation, greater is the effect of temperature on reaction rates. The specific reaction rate constant ($\Delta H^{\ddagger}$) for the denaturation of PAO-I and for the conversion of n-pentanal to n-pentanoic acid was calculated to be 158.1 kJ/mol and 44.5 kJ/mol (Figure B-2 in Appendix). Since 20.9 kJ/mole energy is required to break one non-covalent bond during denaturation (Whitaker, 1972), there were eight non-covalent bonds broken during the denaturation of PAO-I at pH 10.0. This value of $\Delta H^{\ddagger}$ is only valid for pH 10.0 as $\Delta H^{\ddagger}$ is a function of pH because the number of electrostatic bonds which help maintain structure of a protein change with pH (Whitaker, 1972).

Lipoxygenase from immature green peas had 18.9 kJ/mol · K^{-1} energy of activation and 103.8 kJ/mol · K^{-1} energy of denaturation (Chen and Whitaker, 1986). Tappel *et al.* (1953) reported a value of 18 kJ/mol · K^{-1} for the activation of the oxidation of linoleic acid catalyzed by soybean lipoxygenase. A 47 kJ/mol · K^{-1} energy of activation and temperature stability data suggest that PAO-I can be used at a higher temperature to achieve faster rates without significantly impairing protein stability. Higher energy of activation for the

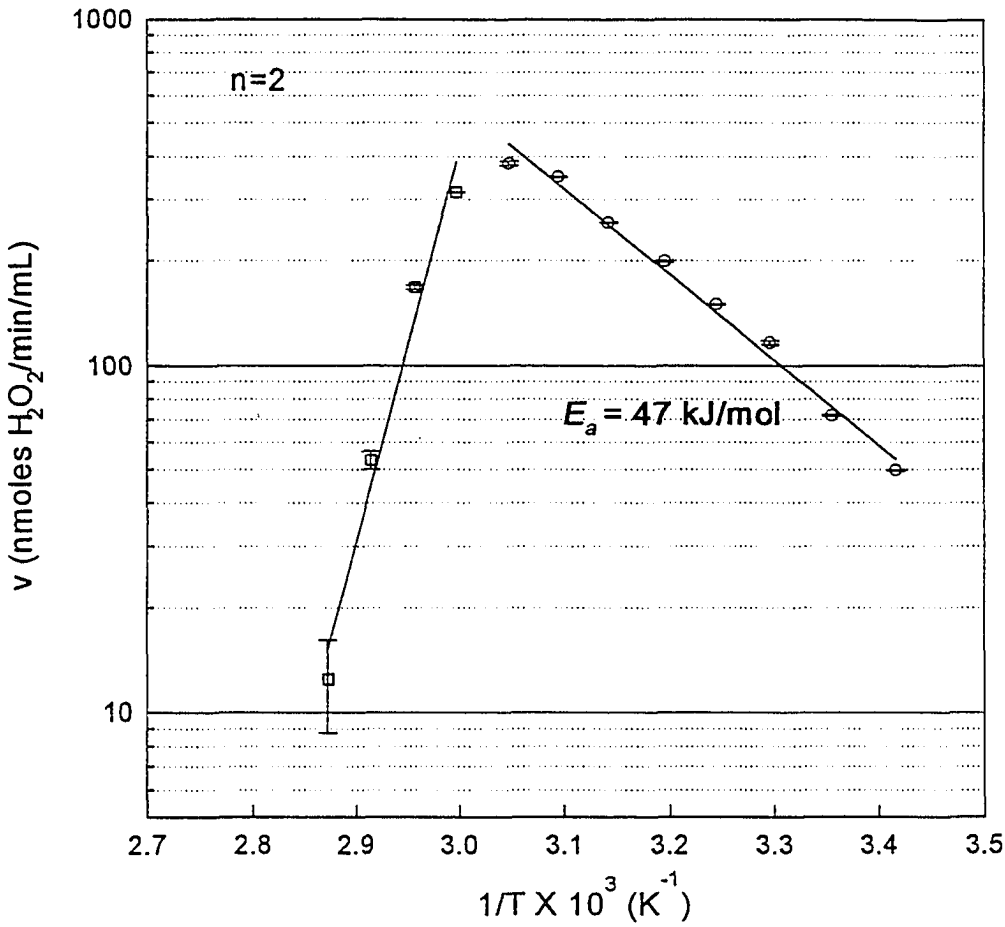

Figure 13. Energy of activation, E_a (◦) of PAO-I with pentanal as the substrate. Bars represent standard deviation and n represents the number of replicates. (□) represents the initial rate of PAO-I when PAO-I started to denature.

conversion of aldehydes to carboxylic acids by PAO-I compared with the peroxidation of linoleic acid by lipoxygenase may prove helpful in removing the off-flavor faster than what lipoxygenase can produce at a given temperature.

Storage Stability of PAO-I

The storage stability of PAO-I at 4 °C and pH 9.0 (100 mM of 0.2 ionic strength glycine-NaOH buffer) and at pH 7.0 (100 mM of 0.5 ionic strength of sodium phosphate buffer) was determined for a period of 5 months. The percentages of residual activity at different time intervals are shown in Figure 14. The PAO-I was more stable at pH 9.0 than at pH 7.0. More than 40% activity was lost at pH 7.0 after one month of storage compared to only 13% loss in activity at pH 9.0. PAO-I had only 11% activity after 3 months of storage at pH 7.0 (phosphate buffer) with a completely loss of activity afterwards. The trend at pH 9.0 was irregular with the decrease in aldehyde oxidase activity at the end of three months followed by an increase. The results of PAO-I activity increase after three months cannot be explained by this study and needs further investigation. Nonetheless, storage stability data confirmed that pH 9 to 10 should be the optimal storage pH at refrigerated conditions (Figures 9 and 14). Cabre and Canela (1987) also found that pH value of 8.2 was effective in maintaining 100% activity of bovine-liver aldehyde oxidase at 4 °C for 19 days. The storage pH of 9.3 used be Takahashi *et al.* (1979) for bovine-liver aldehyde oxidase suggested that basic pH maximized activity retention. Therefore, like bovine- and rabbit-liver aldehyde oxidase, porcine-liver aldehyde oxidase have a pH stability optimum between pH 9 and 10.

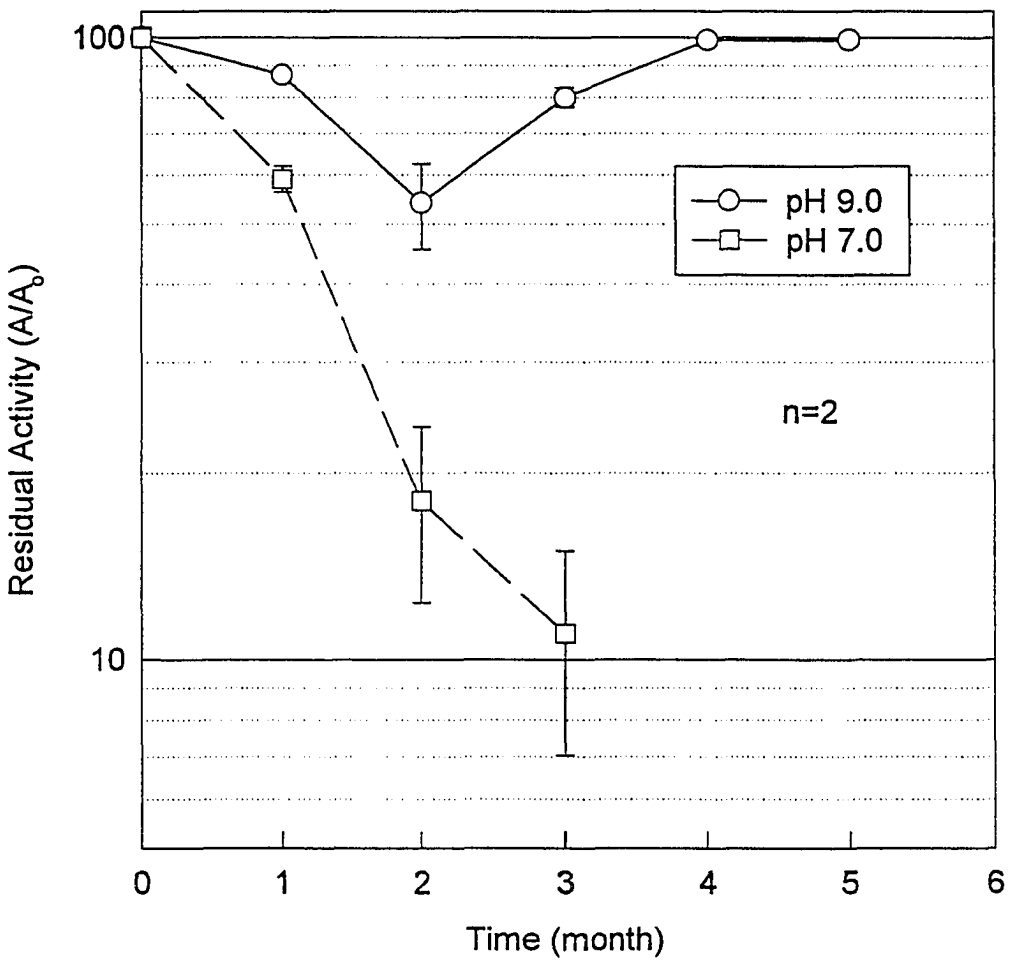

Figure 14. Change in residual activity with time at pH 7.0 and 9.0 at 4 °C. Bars represent standard deviation and n represents the number of replicates. Residual activity is defined as the ratio of PAO-I activity at time *t* and at *t*=0.

Freeze-Thaw Stability of PAO-I.

PAO-I was subjected to eleven freeze-thaw cycles and the activity was measured each time when the PAO-I was thawed. Freeze-thaw cycle did not result in appreciable loss in activity (Figure 15). Only a 15% loss in the PAO-I activity resulted after the eleventh freeze-thaw cycle.

Lyophilization

Lyophilization of PAO-I did not cause significant loss in activity. Up to 96% activity was recovered upon reconstituting the freeze-dried PAO-I with glycine-NaOH buffer. This freezing study indicate that aldehyde oxidase can be stored at -18 °C for an extended time.

Effect of pH on the Rate of Reaction

Since oxygen and aldehydes are two substrates (bimolecular reaction) in the conversion of aldehydes to carboxylic acids, an assay was performed to determine O_2 limitation. Using five different oxygen concentrations (0.13, 1.21, 2.13, 2.84 and 4.52 mM), a K_m for oxygen was found to be 1.04 mM at a constant n-pentanal concentration of 3.3 mM (Figure 16). The K_m and V_m were calculated using the Wilkinson (1961) fit. The assay buffer at 30 °C had a 10.5% oxygen content which corresponds to about 1.35 mM oxygen content. The K_m for oxygen was at least three times higher than the K_m for n-pentanal at various pHs (Figure 16 and Table B-3 in appendix). Therefore, it was concluded that the oxygen concentration would remain saturating in determining K_m and V_m in the oxidation of pentanal to pentanoic acid by PAO-I.

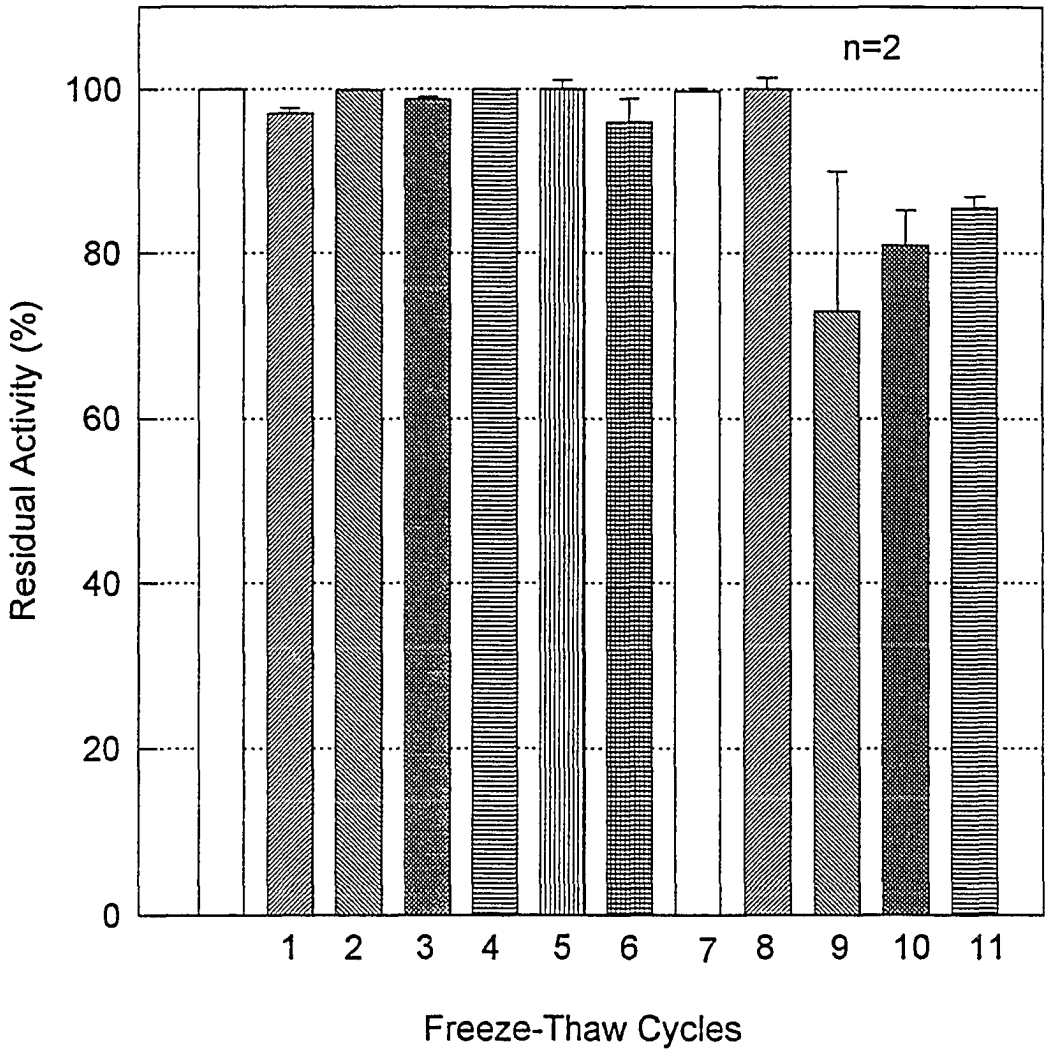

Figure 15. Effect of freeze-thaw cyles on PAO-I activity. Bars represent standard deviation and n represents the number of replicates. Residual activity is defined as in Figure 14.

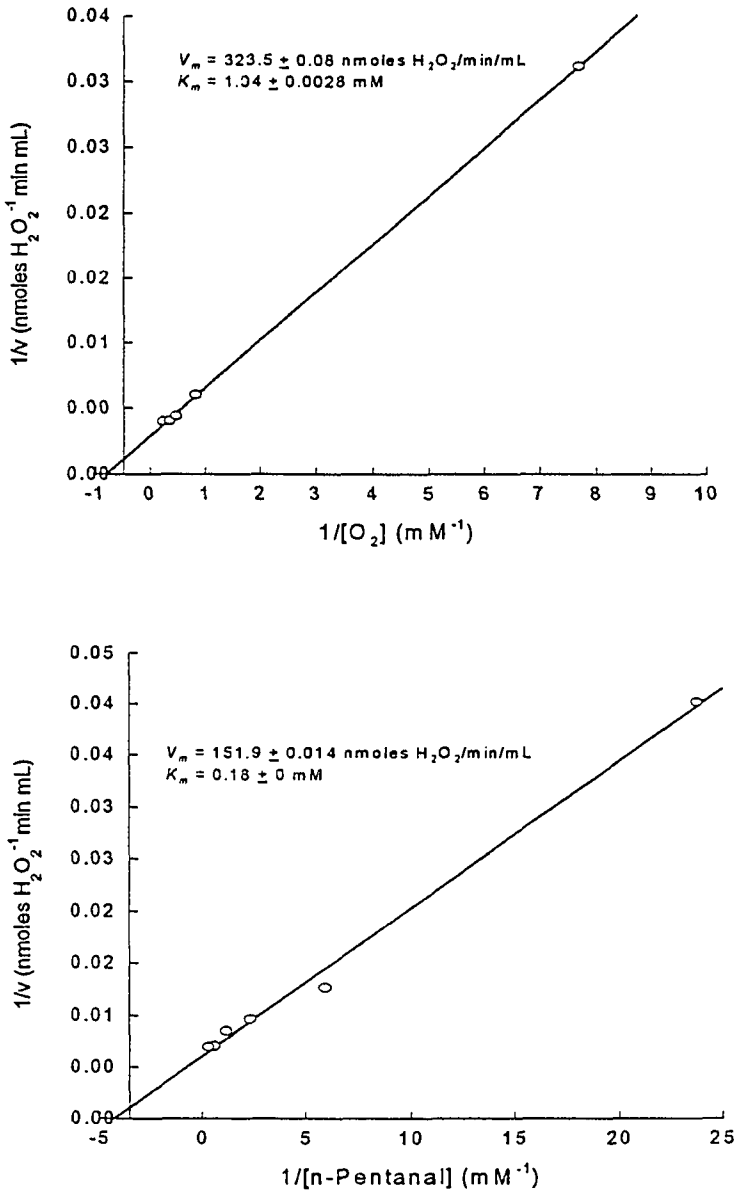

Figure 16. Lineweaver-Burk plots for determining K_m for O_2 (fixed n-pentanal concentration), and for n-pentanal (fixed O_2 concentration) at pH 7.5.

The effect of pH on V_m and K_m was determined by measuring the initial rate of reaction by using 8-10 concentrations of n-pentanal. The concentrations were varied so that at least a 100-fold difference in the lowest and highest concentration existed. The oxygen depletion as well as n-pentanal oxidation followed Michaelis-Menten kinetics (M-M), thus K_m is M-M constant and the V_m is the maximal rate as defined by M-M. The V_m and K_m were determined using Wilkinson program rewritten in BASIC language (Wilkinson, 1961). The results of V_m and K_m are summarized in Table B-3 in the Appendix. The V_m had the greatest value (707.6 units) with the lowest K_m (18 μM) at pH 9.0 (Figure 17). There was a moderate increase in V_m from pH 5.0 to 7.05. There was a greater than four times increase in V_m at pH 8 and seven times increase at pH 9 compared with pH 7.05. This could be due to the ionization of active site amino acid residues at that pH. After a maxima at pH 9, the value of V_m decreased by one-half at pH 10 and decrease was even greater at pH 11 and 12. The values for K_m showed an opposite trend of V_m with increasing pHs, i.e., the K_m was the smallest at pH 9.0 and increased at pHs lower or higher than 9.0. The small standard error in the measurement of V_m and K_m indicates that the Wilkinson method was better in the determination of these parameters than the Lineweaver-Burk method (Table B-3 in the Appendix). The trend of increasing V_m with a decreasing K_m follows the trend of many enzymes (Dixon and Webb, 1979). The small K_m and high V_{max} indicate that n-pentanal is a very good substrate for PAO-I at pH 9.0.

Figure 18 shows the optimal pH for stability and activity of PAO-I. The pH stability curve is skewed towards higher pHs. The residual activity seems to plateau in the pH range of 7.6 to 8.6 with a slight increase of 5 and 8% in residual activity at pH 9.3 and 10.0, which

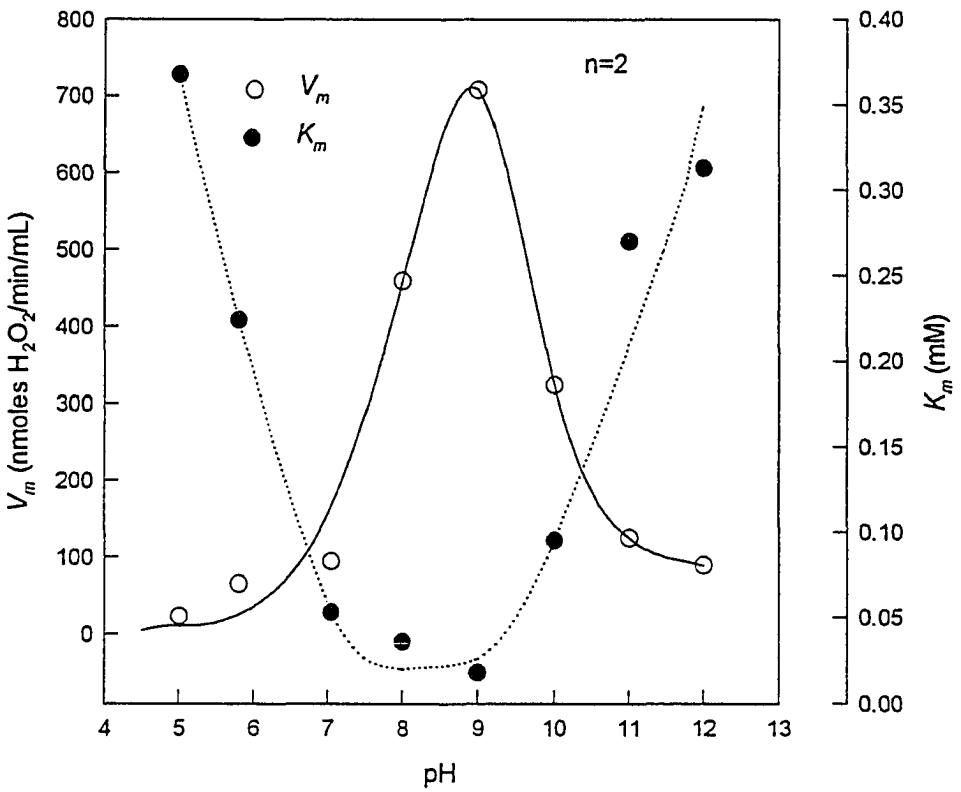

Figure 17. Effect of pH on the $V^{H+}{}_m$ and $K^{H+}{}_m$. The average standard deviations for $V^{H+}{}_m$ and $K^{H+}{}_m$ were 0.99 and 0.021, respectively; n, represents the number of replicates.

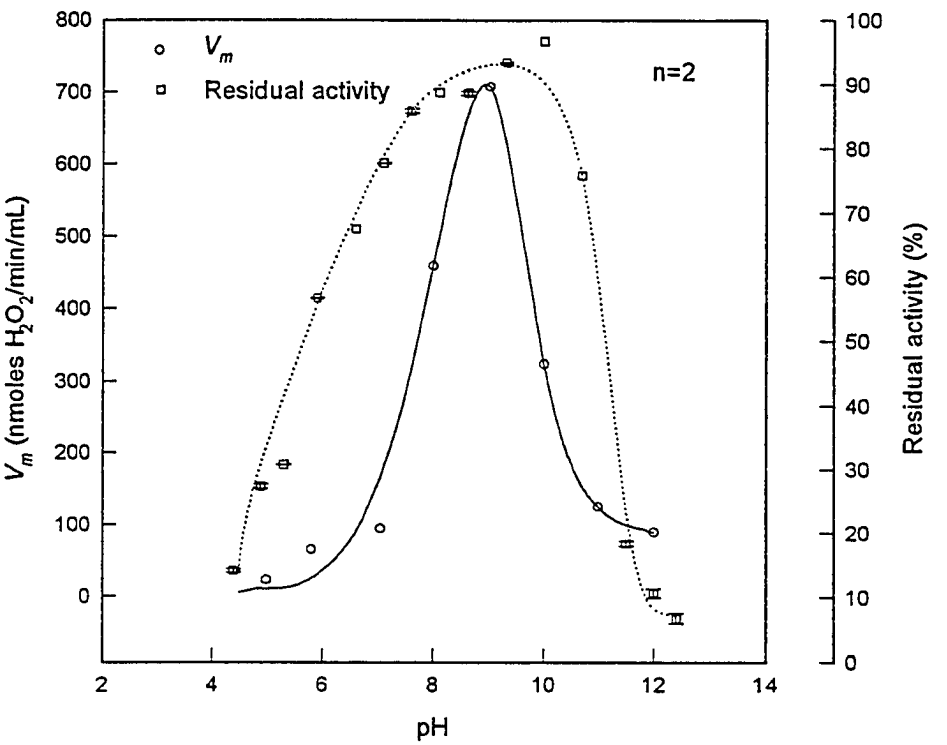

Figure 18. Effect of pH on $V^{H+}{}_m$ and residual activity of PAO-I after 60 min. The average standard deviation for $V^{H+}{}_m$ estimation was 0.99; bars represent standard deviation for residual activity; and n, represents the number of replicates. Residual activity is defined in Figure 14.

was not statistically significant ($p>0.05$). The maximal rate curve shows a single activity maxima at pH 9.0. All these studies were performed at 30 °C, where 82% PAO-I activity remained after 60 min. The optimum conditions for the oxidation of n-pentanal should be pH 9 and 30 °C. The temperature can be increased to 35 °C as Figure 11 demonstrates similar temperature stability of PAO-I at 30 and 35 °C.

Lineweaver-Burk plots for the various pHs also show very good linearity (Figure 19). However, the Lineweaver-Burk method resulted in larger standard error in the measurement of V_m and K_m (Table B-3 in Appendix). Therefore, this method was not used in determining these parameters; instead Wilkinson fit was used in the calculation of V_m and K_m.

The initial rates as a function of pH and substrate (n-pentanal) concentrations are plotted in Figure 20. There is a steep increase in the initial rate at a lower substrate concentration. After reaching the V_m, the reaction rate plateaus. We did not use n-pentanal concentration higher than 9 mM, as at that level the PAO-I was inhibited and a lower initial rate was obtained. Palmer (1962) also observed an inhibition of aldehyde oxidase activity at higher acetaldehyde concentrations. According to his study, there are two thiol groups present in the active center, where one of the sulfhydryl group binds to the excess aldehyde, which blocks the enzyme for aldehyde-carboxylic acid catalysis.

Interpretation of Amino Acids Involved in the Active Site of PAO-I

The V_m and K_m data gathered at various pHs were used to elucidate which amino acids involved in the active site of the PAO-I. The pKa of amino acids are involved the in breakdown of the ES complex (substrate to product conversion), i.e., rate-limiting step

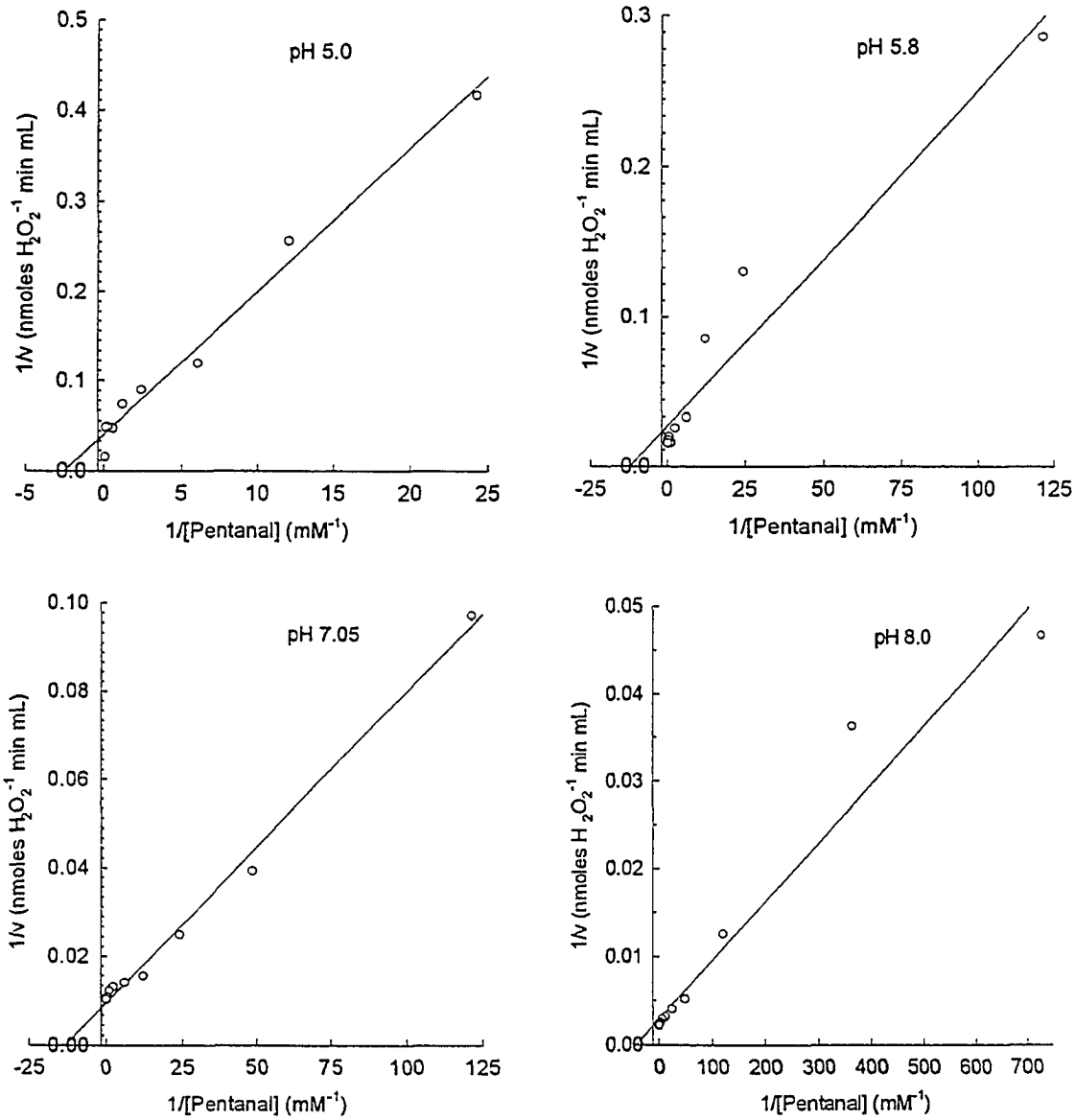

Figure 19. Lineweaver-Burk plots for the oxidation of n-pentanal by PAO-I at various pHs. Two replicates were used for each S, v pair. The V_m and K_m were calculated using the Wilkinson method (Table B-3 in the appendix).

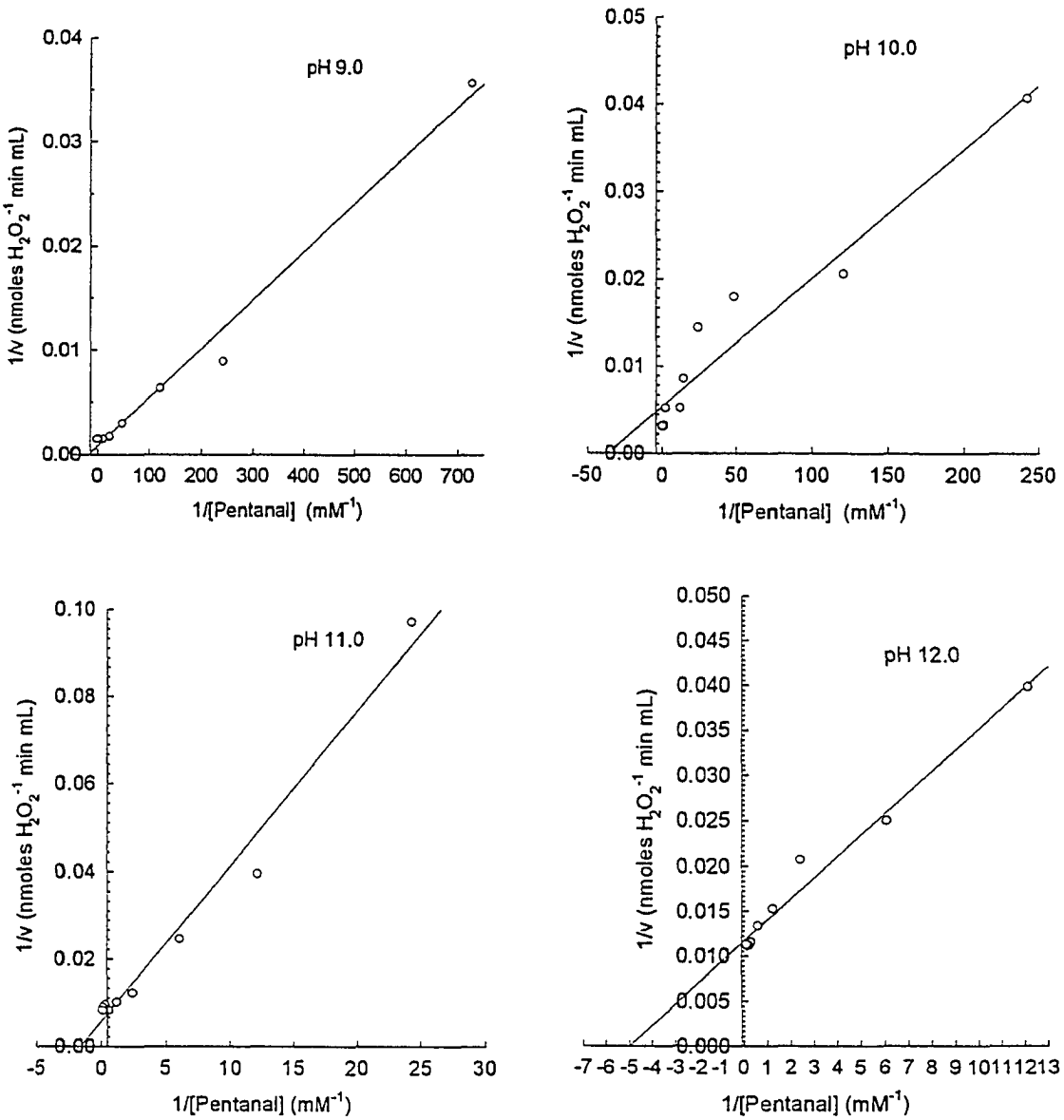

Figure 19. (continued)

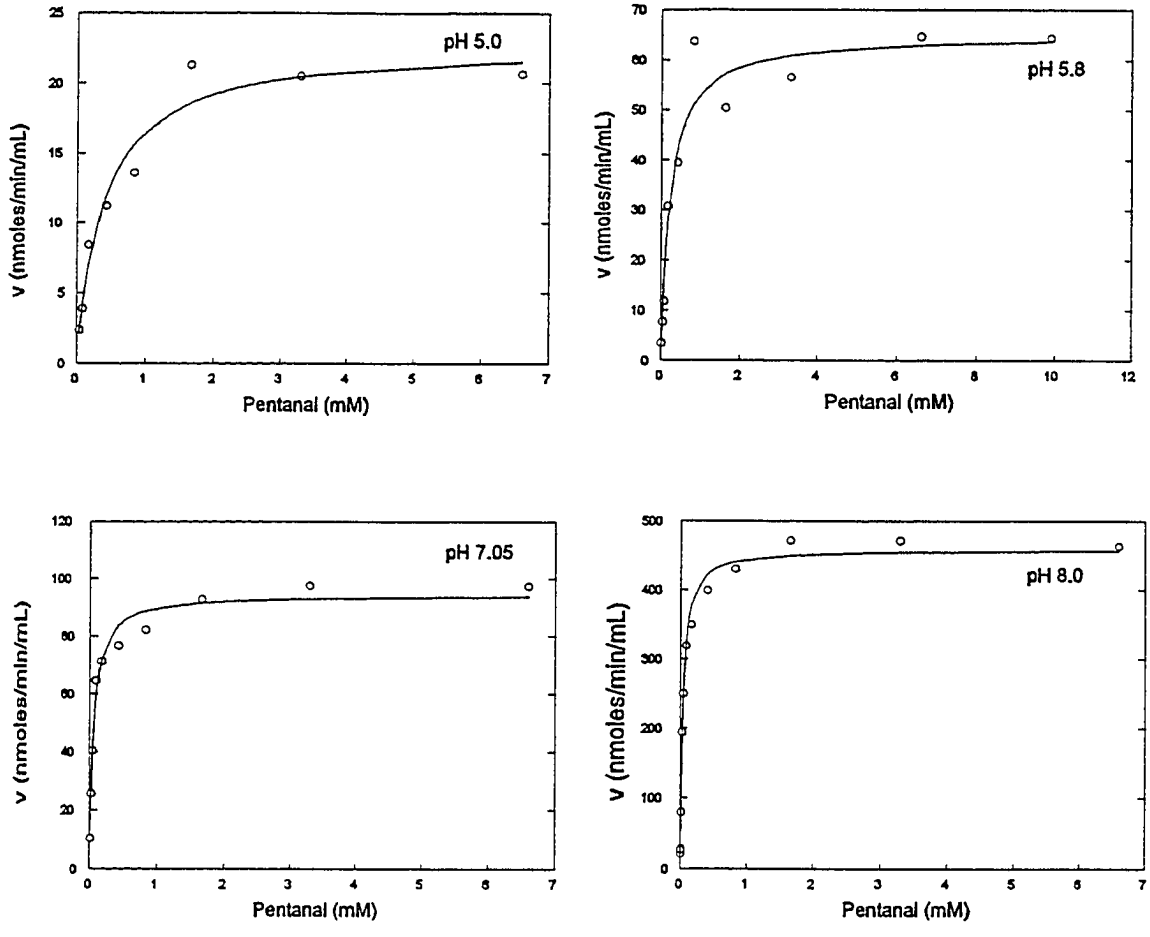

Figure 20. Effect of pH and substrate concentration on the initial rate (v) of n-pentanal oxidation by PAO-I.

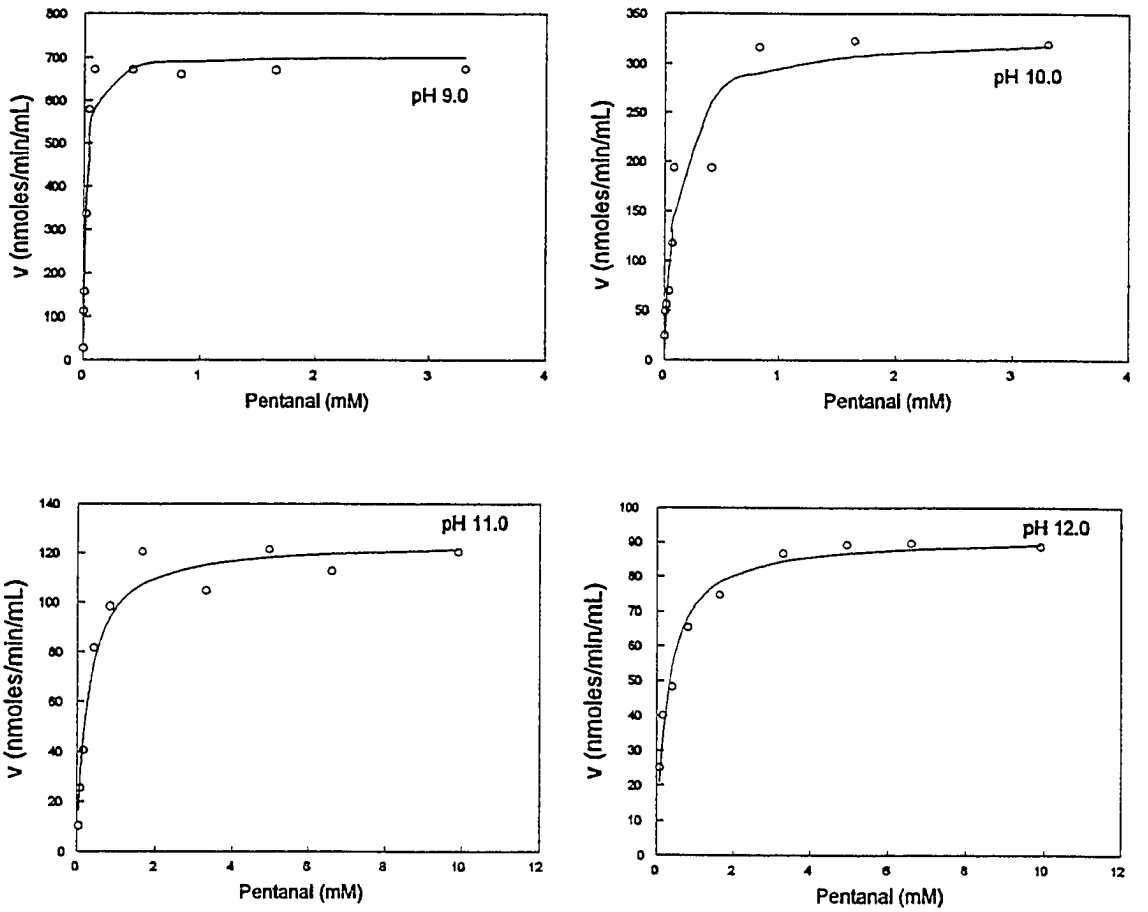

Figure 20. (continued)

was obtained using the Bell program (Stauffer, 1989) because the log ($V^{H+}{}_m$) versus pH plot resulted in a bell-shaped curve (curve drawn based on the pK estimates from the Bell program) as shown in Figure 21. The two pKs, pK_1^{ES} and pK_2^{ES}, were estimated to be 6.2 and 11.3, respectively (Table 3). Table 4 shows the pK of various prototropic groups involved in the enzyme catalysis. According to this table, imidazole group (histidine) and a guanidinium group (arginine) could be involved in the rate-limiting step, i.e. breakdown of ES complex.

Table 3. pK of amino acids involved in the active site of PAO-I.

PAO-I-pentanal complex			Binding of pentanal to PAO-I		
	pK	Amino acids		pK	Amino acids
pK_1^{ES}	6.2	Histidine	pK_1^{E}	7.5	Lysine, cysteine
pK_2^{ES}	11.3	Arginine	pK_2^{E}	9.9	Lysine, tyrosine

Table 4. pK of prototropic groups in proteins

Group	pK*a*
Carboxyl	3.0-3.2; 3.0-4.7
Imidazolium (Histidine)	5.6-7.0
Sulfhydryl	8.0-8.5
Ammonium (Lysylamino)	7.6-8.4; 9.4-10.6
Phenolic hydroxyl	9.8-10.4
Guanidinium	11.6-12.6

(Whitaker, 1972; and Stryer, 1988).

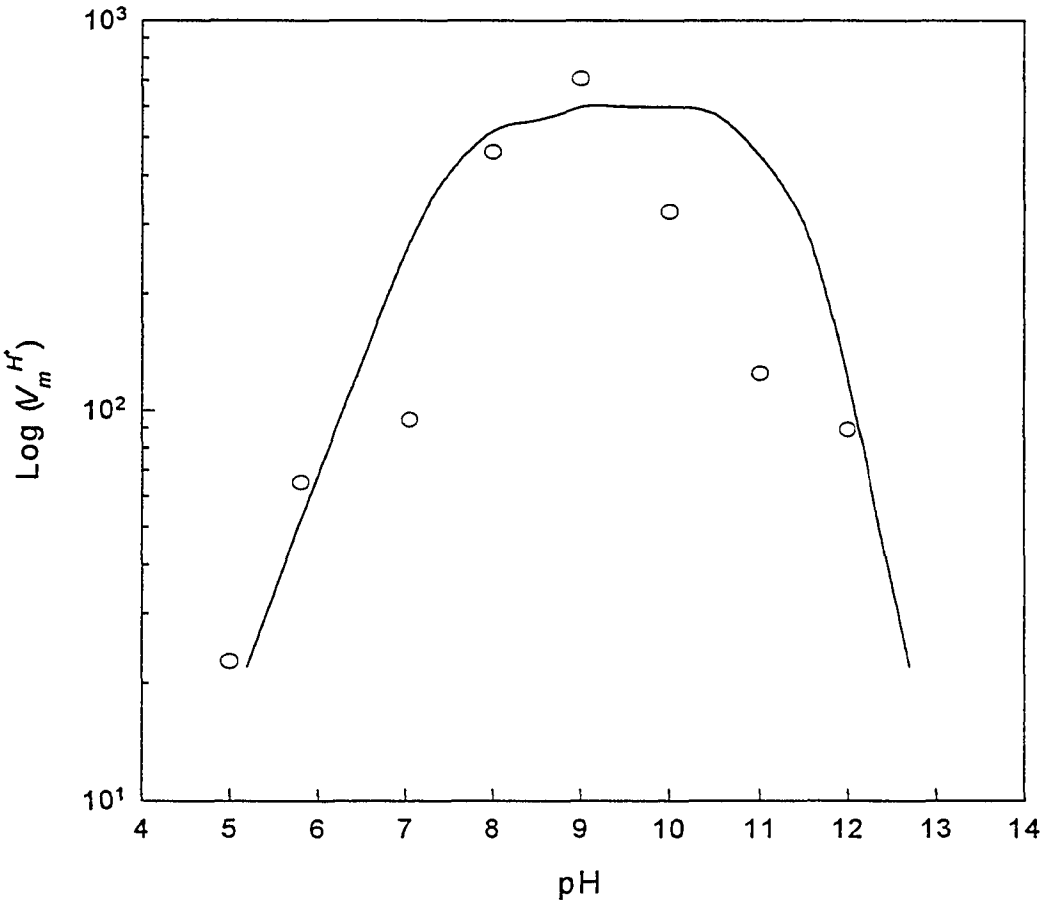

Figure 21. Effect of pH on the rate-limiting step (dissociation of ES complex). The pK_1^{ES} and pK_2^{ES} from experimental data resulted in 6.2 and 11.3, respectively using the Bell program.

To determine the pKs of the amino acid residues involved in the binding of substrate to the free enzyme, $\log(V^{H+}{}_m/K^{H+}{}_m)$ was plotted against pH (Figure 22). The bell-shaped curve (curve drawn based on the pK estimates from the Bell program) for the free enzyme was not as broad as that for the breakdown of ES complex (Figure 21). The apparent pK_1^E and pK_2^E values that were initially estimated by using Bell (Basic language) program were 7.4 and 10.0, respectively. According to Alberty and Massey (1954) when the estimated pK_1 and pK_2 are separated by less than 3 pH units, an error in the calculation with the Bell program has been observed. To correct for this error, a modified Bell program was used based on Albert and Massey's equations. The true pK_1^E and pK_2^E were calculated and their values of 7.5 and 9.9 fell within the range described by Albert and Massey. They observed an error in pK*a* of 0.07 for the two pKs separated by 2.3 units. Although the 0.1 pH difference in measurement of true pK_1^E and pK_2^E in this study did not cause significant error in this case, the correction of the apparent pKs could become important if the difference between pK_1 and pK_2 becomes less than 2 pH units. The pK_1^E value of 7.5 did not fall in the range of pKs for reported amino acids in the Table 4. However, the closest values are those of the lysylamino group (7.6) and of sulfhydryl group (8.0). Rajagopalan *et al.* (1964) and Coughlan (1977) reported that a presence of cysteine (sulfhydryl) group in the active site of aldehyde oxidase was important in the substrate binding and the electron transfer mechanism during oxidation of purines and many aldehydes by rabbit-liver aldehyde oxidase. In addition, the cynolysis (SCN) of aldehyde oxidase resulted in the inactivation of the enzyme (Coughlan, 1977). Based on these two observations, one can speculate that there is a SH group in the active site of PAO.

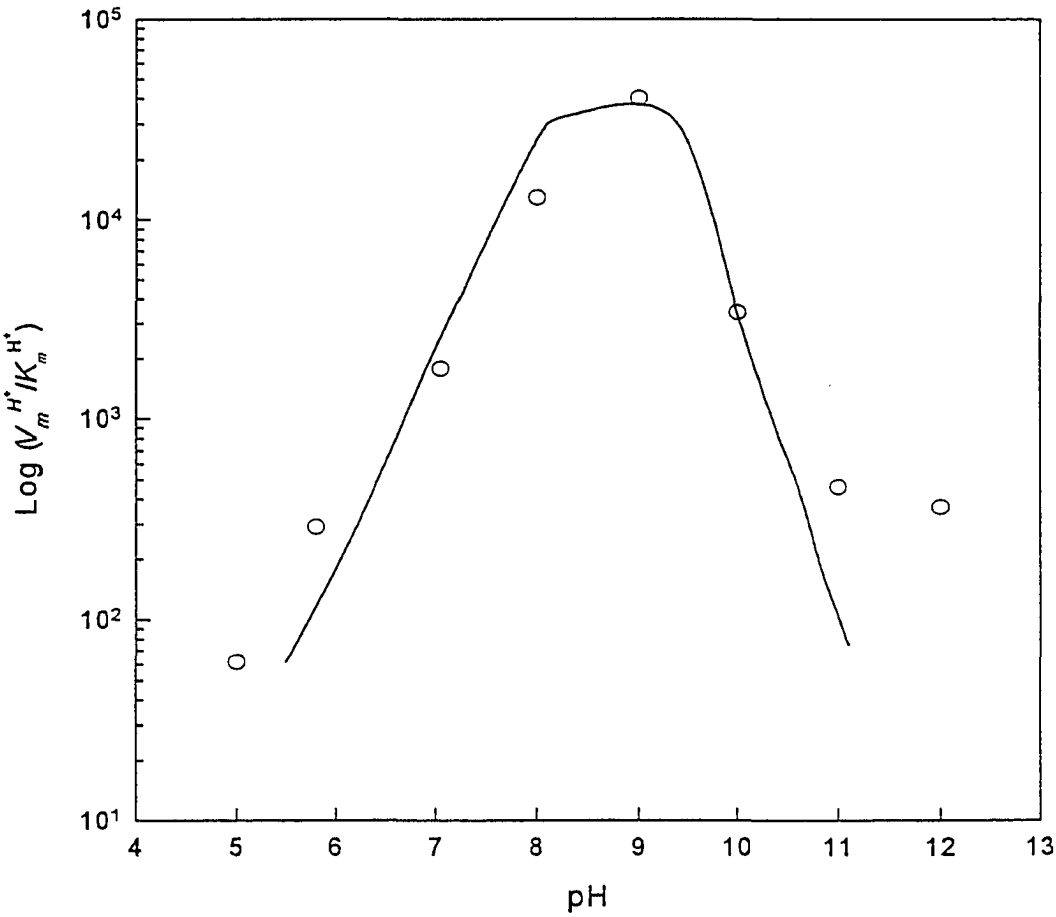

Figure 22. Effect of pH on the substrate binding (free enzyme). pK_1^E and pK_2^E from experimental data were in 7.5 and 9.9, respectively, using the modified Bell program.

The pK_2^E of 9.9 may correspond to a protonated tyrosyl or lysine amino acid residue in the active site. Rajagopalan (1964) found the presence of arginine and lysine groups in the binding of substrate to the free aldehyde oxidase from rabbit-liver.

Although these preliminary data regarding the identification of amino acids involved in the breakdown of ES complex and binding of substrate to enzyme may agree with the literature, any conclusion about their identity should be made with caution. Solheim and Fromm (1980) conducted pH kinetic studies of bovine-brain hexokinase and concluded that the determination of the amino acids in the active site that are involved in the substrate binding and in the rate-limiting step cannot be made with certainty unless ΔH_{ion} was determined. Further work is needed to obtain ΔH_{ion} values by performing experiments with at least two more temperatures. This work was not performed in this study as our objective was to get preliminary information about the amino acids in the active site of PAO-I. Any shift in pK upon the addition of an organic solvent, such as dioxane, in the presence of imidazole buffer would further confirm the interpretation of prototropic groups in the active site of PAO-I. The presence of a histidine group (estimated from Dixon-Webb plots) can be confirmed by adding dioxane in the reaction medium. The pK of histidyl group should not change with the inclusion of organic solvent in the imidazole buffer, but will change drastically in the presence of a carboxylic buffer. X-ray crystallography and chemical modification of aldehyde oxidase will also help considerably in determining active site amino-acid residues (Whitaker, 1972).

Gas Chromatographic Analysis

Extract Preparation

The water extract of defatted soy bean flour (XLRB variety) was prepared in 1:20 and 1:10, soybean to water ratio, to determine the amount of soy protein needed for the detection of off-flavor compounds from the headspace analysis by gas chromatography. A 5-mL aliquot of 1:20 soy-extract did not release sufficient amount of off-flavor-causing aldehydes to be detected by headspace gas chromatography by using either DBWAX or NUKOL column. A 1:10 water-extract of soybeans turned out to be a better choice in the detection of these volatiles.

Effect of pH on Headspace Volatiles

Initially, the effect of pH on the released volatiles from the water extract of soy flour in the headspace at 35 °C was determined. The initial pH of the water extract (6.6) was adjusted to pH 7.0 and 9.0. The pH 7.0 was chosen as a neutral pH value and pH 9.0 was selected because PAO-I showed the maximal rate and high stability. At lower pH, a change in Schiff-base equilibrium (Cheftel *et al.*, 1985) is expected to occur. Therefore, more aldehydes are expected to be released at a lower pH, whereas at higher pH, more binding between aldehyde and amino group occurs. This trend was observed in this work (Figure 23). The total volatiles released at pH 7.0 were 35% higher than those present at pH 9.0. When the pH 9.0 soy extract was adjusted to bring a final pH to 7.0, the total volatiles increased again. The increase in total volatiles in the pH adjusted soy-extract was significantly ($p<0.05$) higher than at pH 9.0. Although, the pH readjusted samples had

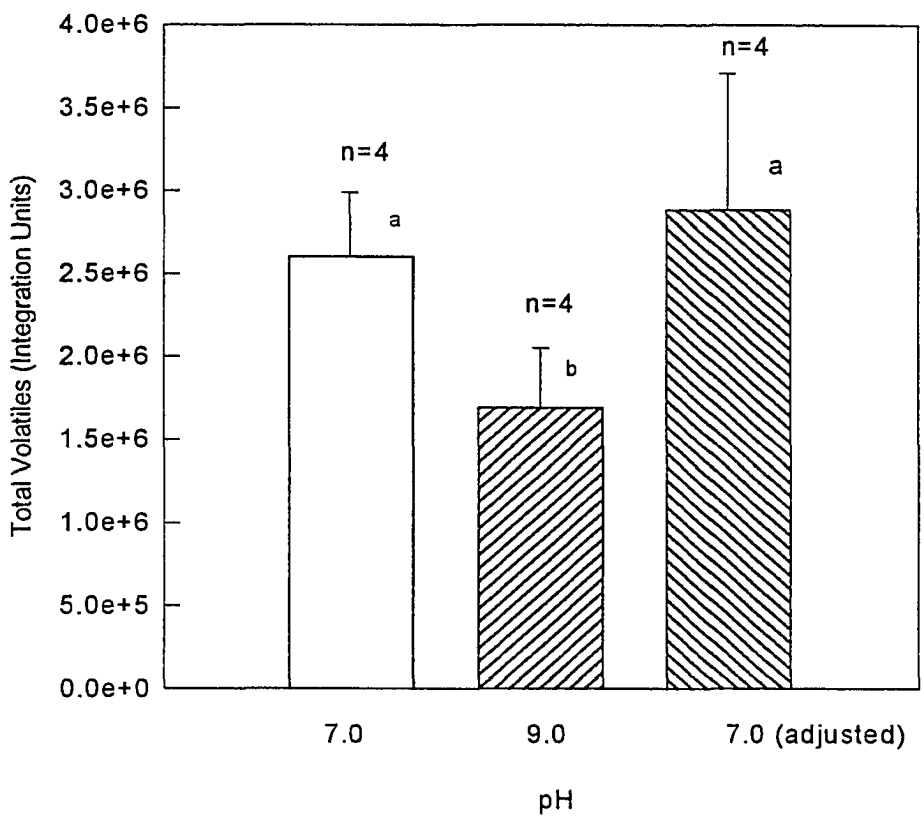

Figure 23. Effect of pH on the total volatiles in the headspace of water extract of soybean. Bars represent standard deviation and n represents the number of replicates. Means with same letter (a and b) are not statistically significant ($p>0.05$). Adjusted pH 7.0 was achieved by adding HCl to the soy extract of pH 9.0.

higher volatiles (10%) than the volatiles at pH 7.0, it was not statistically significant ($p>0.05$).

In spite of 35% more volatiles (free volatiles) in the headspace at pH 7.0 compared with that at pH 9.0, pH 9.0 was selected to study the application of PAO-I in the off-flavor reduction from water extract of soybean because the PAO-I was 17% more stable at pH 9.3 than at pH 7.1 (Figures 8 or 18). Furthermore, as described earlier, the V_m of PAO-I was sevenfold higher at pH 9 compared to pH 7.05 (Figure 18).

Cooray (1992) and Takahashi *et al.*(1979) have reported that off-flavor compounds are bound to soy proteins both in the free as well as in the bound form. We expected that the conversion of free aldehydes to carboxylic acids would shift the equilibrium of aldehyde binding to soy proteins from bound to free aldehydes. Aldehydes would therefore be available for a more efficient removal of off-flavors by PAO-I.

Headspace Analysis of PAO-I Treated Soy-extract

The retention times of aldehydes and their corresponding carboxylic acids on the NUKOL column are given in Table 5. The carboxylic acid could not be separated well using a DBWAX column. Therefore, DBWAX column was not used in the detection of carboxylic acids. The separation of the aldehyde (C_2 to C_5) peaks was not very good on the NUKOL column. However, the separation of their corresponding carboxylic acid separation was very good. The GC chromatogram did not resolve the aldehyde peaks satisfactorily at an attenuation of 8 and a detector sensitivity of 10^{-12} amps/sec, but the peaks were identified by comparing their retention times to the external standard retention

Table 5. Average retention time[a] of aldehydes and carboxylic acids on NUKOL column.

Aldehyde	Retention Time[a] (min)	Carboxylic acid	Retention Time[a] (min)
Acetaldehyde	2.53 ± 0.01[b]	Acetic acid	5.23 ± 0.01
n-Propanal	2.57 ± 0.01	Propionic acid	6.23 ± 0.04
n-Butanal	2.65 ± 0.03	Butyric acid	7.36 ± 0.03
n-Pentanal	2.80 ± 0.02	Pentanoic acid	9.25 ± 0.06
n-Hexanal	3.02 ± 0.01	Hexanoic acid	10.97 ± 0.05
n-Heptanal	3.31 ± 0.01	Heptanoic acid	12.99 ± 0.03

[a]n=3
[b]standard deviation

times and the integration units were used to get the quantitative estimation of volatiles.

Before performing headspace analysis, an activity assay was performed with water extract of soybean as a substrate (native aldehydes) using 0.3 wt% PAO-I (compared to total soy protein content). The rate of enzyme reaction with native aldehydes was similar to that of externally added pentanal. Therefore, a water extract of soybeans incubated with different amount of PAO-I was further tested to determine the change in headspace volatiles. Initially, a 1000:1 soy protein to PAO-I ratio was used to determine the increase in carboxylic-acid GC peak area with a decrease in aldehyde GC peak areas. Four incubation periods of 30, 60, 120 and 240 min were chosen. Sasaki *et al.*(1982) showed that a 5-h incubation of soy protein extract with aldehyde dehydrogenase was necessary to significantly reduce beany-flavor from soy proteins. The reduction in n-butanal,

n-pentanal, and n-hexanal amount in headspace slowed after 60 min (Table 6). Up to 60% n-pentanal and n-hexanal were reduced from the PAO-I treated soy extract headspace after 4 h of incubation and greater than 100% increase was detected for pentanoic and hexanoic acid after 2 h incubation. In case of acetaldehyde, propanal and heptanal there was an increase in aldehydes after 60 min. This could be due to the release of bound aldehydes. The PAO-I might have started to loose its activity (denature) or aldehydes (native aldehydes in soybean) might start depleting after 60 min. Therefore, the maximum time of incubation of soy protein extract with PAO-I was set at 60 min. Furthermore, a shorter reaction time is desired for a process to be economical.

The data in Table 6 and Figure 24 shows a sigmoidal behavior of change in headspace aldehydes between 0 and 60 min. The decrease in aldehyde concentrations occurred early in the treatment. Up to 73% of the n-pentanal and 40% of n-hexanal were reduced after 45 and 60 min, respectively. There was no effect of PAO-I in reducing the headspace n-pentanal content after 45 min. The increase in butanoic, valeric (pentanoic acid) and hexanoic acid was detected after 15 min. There was a significant difference ($p<0.05$) in headspace aldehydes and their corresponding carboxylic acids at different times (Figure 24). The acetaldehyde, n-butanal, n-pentanal, n-hexanal, and n-heptanal were significantly reduced after 60, 45, 30, 60, and 15 min, respectively compared with the control (at zero time). The reduction in headspace volatiles of propanal was not statistically significant ($p>0.05$) using the Student's t-test. Increases in acetic, butanoic, pentanoic, hexanoic and heptanoic acid were significant ($p<0.05$) after 60, 15, 30, 45 and 60 min, respectively. The propionic acid peak was not detected at any time, which agrees with no

Table 6. Effect of amount of PAO-I and incubation time on headspace volatiles

Time (min)	Protein :PAO-I	% Aldehyde reduction						% Carboxylic-acid increase					
		C2	C3	C4	C5	C6	C7	C2	C3	C4	C5	C6	C7
0		-	-	-	-	-	-	-	*	*	-	-	*
30		45.6	78.5	56.2	44.1	17.6	50.9	0	*	*	370.5	27.0	*
60	1000:1	11.2	87.6	56.6	71.4	27.5	32.6	0	*	*	263.1	199.4	*
120		27.5	71.0	67.7	73.2	48.9	7.1	0	Det†	*	327.4	172.4	Det†
240		0	97.1	0	61.2	59.2	0	0	*	*	217.1	61.6	*
0		-	-	-	-	-	-	-	*	-	-	-	*
15	1000:1	54.6	15.4	25.0	50.0	1.1	72.2	0	*	104.0	14.8	66.0	*
30		52.8	8.0	45.3	58.2	24.9	77.6	7.0	*	536.2	38.7	70.1	*
45		61.3	48.3	44.8	72.8	36.3	81.5	121.8	*	429.9	35.8	170.9	*
60		74.4	57.4	66.5	53.4	40.0	80.0	154.5	*	641.4	138.7	198.6	*

Table 6. (continued)

Time (min)	Protein :PAO-I	% Aldehyde reduction						% Carboxylic-acid increase					
		C2	C3	C4	C5	C6	C7	C2	C3	C4	C5	C6	C7
0		0	0	0	0	0	0	0	*	*	0	*	*
15		42.3	21.7	35.6	75.4	61.5	0	12.2	Det	Det	20.3	Det	Det
30	200:1	64.5	75.1	53.3	84.4	77.1	2.08	22.7	Det	Det	65.4	Det	Det
45		67.1	95.5	56.3	86.6	82.7	24.1	77.4	*	*	80.2	Det	*
60		87.0	95.3	70.2	90.8	81.9	35.4	97.3	*	*	526.2	Det	*

*Peaks were not detected (at 2000 integration units cut-off)
†Peaks were detected, but percentage increase could not be calculated as no peak was detected at t=0.
Aldehyde carbon numbers, C2, C3, C4, C5, C6, and C7 correspond to acetaldehyde, propanal, n-butanal, n-pentanal, n-hexanal, and n-heptanal, respectively.
Carboxylic acid carbon numbers, C2, C3, C4, C5, C6, and C7 correspond to acetic, propionic, butanoic, pentanoic, hexanoic, and heptanoic acid, respectively.

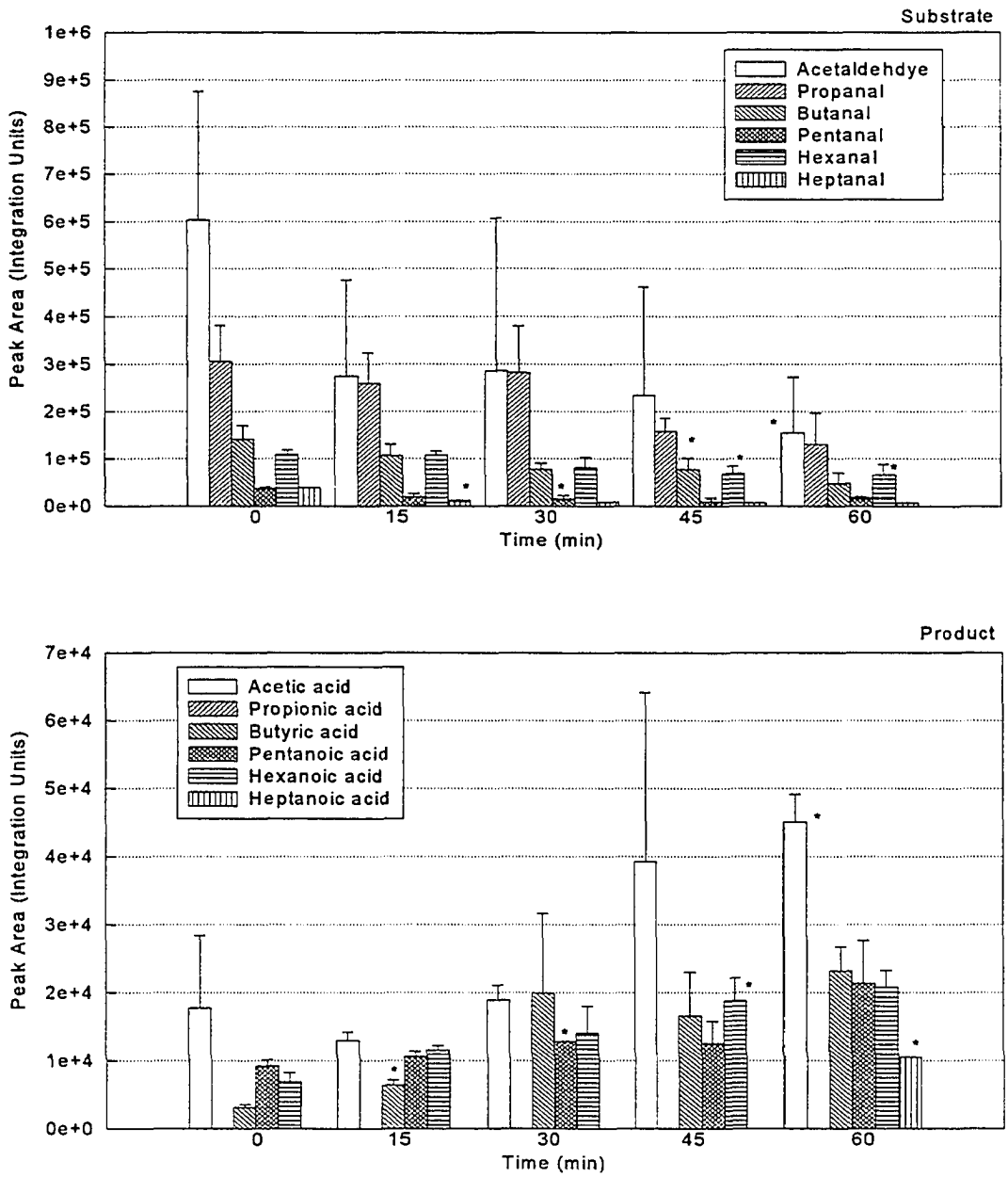

Figure 24. Headspace analysis of PAO-I-treated water extract of soybean at a 1000:1 total protein ration of soy protein to PAO-I. The bars represent standard deviation, n, the number of replicates (n=3) and (*) indicates that the peak areas were significantly different ($p<0.05$) compared with untreated soy extract.

significant reduction of propanal by PAO-I. The increase in carboxylic-acid headspace volatiles was more difficult to detect as the vapor pressures of the carboxylic acids are 1% times of their corresponding aldehydes (Table 7). The high concentration of organic acids in headspace indicate an excellent bioconversion of aldehydes to carboxylic acids by PAO-I. Although the reduction in aldehydes and the increase in carboxylic acids were significantly different ($p<0.05$) than the control, the time required to obtain a significant change was still long, and the percentage reduction was only 70 and 40% for n-pentanal and n-hexanal, respectively after 60 min. Therefore, higher concentration of PAO-I compared with soy protein was attempted to achieve faster reaction rates and a higher bioconversion of substrates to products.

A five-time greater amount (200:1 soy protein to enzyme ratio) of PAO-I (Figure 25 and Table 6) resulted in a much faster reaction rate and greater n-pentanal and n-hexanal reduction compared with a 1000:1 soy protein to PAO-I treatment. Up to 91% pentanal and 82% hexanal could be reduced from soy extract with PAO-I after 60 min. Therefore, a total reduction of n-pentanal and n-hexanal was 20 to 40% more by using five times more PAO-I. The rates of n-pentanal and n-hexanal reduction were high during the first 15 min and then they plateaued. This was probably because the amounts of native n-pentanal and n-hexanal in the reaction mixture were started to deplete after 15 min. This agrees well with the substrate specificity results (Table 2) that PAO-I oxidized n-pentanal and n-hexanal faster than acetaldehyde, propanal, and n-butanal. The n-hexanal and n-heptanal were significantly reduced ($p<0.05$) after 15 min of incubation. Butanal (64% reduction) and n-pentanal (84% reduction) headspace volatiles were significantly lower ($p<0.05$) than

Table 7. Vapor pressure data and moles present in 2.5 mL headspace at 35 °C

n-Aldehydes				Carboxylic acid			
Carbon chain	Vapor Pressure (mm Hg)	Initial moles (control soy-extract)	Final moles (PAO-I treated soy-extract)	Carbon Chain	Vapor Pressure (mm Hg)	Initial moles (control soy-extract)	Final moles (PAO-I treated soy-extract)
C2	1226.4	6.1e-8	7.9e-9	C2	27.15	1.1e-10	2.2e-10
C3	471.3	2.0e-10	9.3e-12	C3	7.84	*	1.8e-14
C4	166.1	3.3e-10	9.7e-11	C4	2.23	*	4.9e-14
C5	56.9	5.5e-10	5.0e-11	C5	0.61	2.4e-13	1.5e-12
C6	27.2	8.8e-13	1.6e-13	C6	0.09	*	1.1e-13
C7	6.1	1.3e-12	8.7e-13	C7	0.05	*	1.7e-13

*Not detected by GC at 2000 IU cutoff.
Aldehyde carbon numbers, C2, C3, C4, C5, C6, and C7 correspond to acetaldehyde, propanal, n-butanal, n-pentanal, n-hexanal, and n-heptanal, respectively.
Carboxylic acid carbon numbers, C2, C3, C4, C5, C6, and C7 correspond to acetic, propionic, butanoic, pentanoic, hexanoic, and heptanoic acid, respectively.

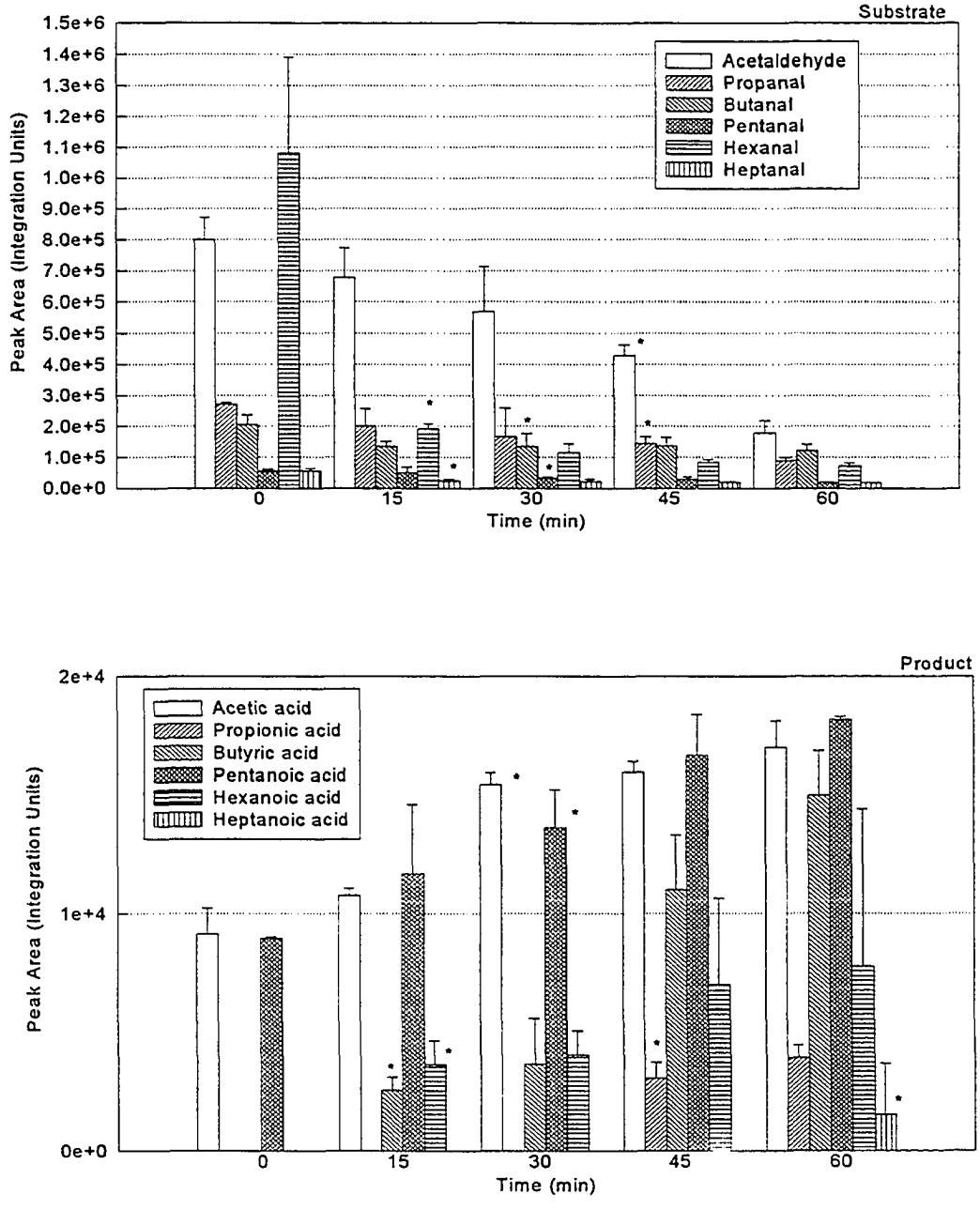

Figure 25. Headspace analysis of PAO-I treated water extract of soybean at a 200:1 total protein ratio of soy protein to PAO-I. The bars represent standard deviation, n, the number of replicates (n=3), and (*) indicates that the peak areas were significantly different ($p<0.05$) compared with untreated soy extract.

control after 30 min, whereas, 45 min incubation of soy extract with PAO-I was required to get a significant reduction of acetaldehyde (67% reduction) and propanal (95% reduction). A significant increase ($p<0.05$) in pentanoic acid (65% increase) after 30 min was observed while hexanoic acid was increased significantly after 15 min. There was a 500% increase in pentanoic acid level after 60 min treatment. The percentage increase of hexanoic could not be calculated because no hexanoic acid was detected in the control.

The headspace volatile content of all other aldehydes studied was also reduced with increase in the corresponding organic acids. Acetaldehyde headspace was reduced by PAO-I to 87%, propanal 90%, n-butanal 70%, and n-heptanal 35% after 60 min (Table 6). The acetic acid was increased by PAO-I significantly in the soy extract headspace after 30 min, propanal after 45 min, and butyric acid in 15 min (Figure 25). The percentage increase of these carboxylic acids could not be measured because at peak cut-off value of 2,000 integration, the propionic, butyric, hexanoic, and heptanoic acid peaks were not detected at 0 min by using 10^{-12} amps/sec FID detector sensitivity.

The reduction of headspace aldehydes and the increase in carboxylic acid were significantly different than control at a faster rate for the treatment of 200 part of soy extract with one part of PAO-I (total protein basis) than the 1000:1 ratio of soy extract to PAO-I (Figures 24 and 25). Therefore, a 200:1 ratio of soy protein to PAO-I of mixture was selected for the sensory analysis study (discussed later in this chapter).

The bovine aldehyde oxidase removed only 23% of the hexanal after 20 min from the a model mixture as analyzed by gas chromatography (Takahashi *et al.*, 1979). The complete oxidation of externally added 1.0 mM n-hexanal required the presence of catalase

in the reaction mixture as oxygen was becoming limiting. In our study, 77% of the n-hexanal was removed in 30 min (Table 6) and there was no requirement of catalase. The purified PAO-I from affinity chromatography did not show any catalase activity, whereas Takahashi *et al.* Octyl-sepharose purified AOX from bovine liver was catalase positive. Therefore PAO-I is a better source of enzyme than bovine-liver aldehyde oxidase.

The number of moles of aldehydes and carboxylic acids were calculated from the vapor pressure data (Daubert and Danner, 1993 and Montgomery, 1952) assuming ideal gas behavior. Table 7 shows the vapor pressure of aldehydes and carboxylic acid analyzed in this study by GC at 35 °C. Using a standard curve (vapor pressure versus moles), the peak areas of volatiles were correlated with the moles of these compounds in the control as well as treated samples. The decrease in number of moles in aldehydes paralleled the increase in carboxylic acid. Since most of the carboxylic acid were not detected at a 2000 integration unit (IU) cutoff, a stoichiometric comparison was difficult. The lower vapor pressure of carboxylic acids than aldehydes (Table 7) might have resulted in lower carboxylic acid volatiles in headspace than those actually generated as a result of aldehydes oxidation by PAO-I. For example, an eleven fold decrease in pentanal (vapor pressure = 56.9 mm Hg) resulted only in a sevenfold increase in pentanoic acid (vapor pressure = 0.61 mm Hg).

Sensory Evaluation

The sensory analysis was performed using a one-tailed paired comparison test (Mahony, 1986). Fifteen panelists were trained to identify the beany-flavor associated with soy proteins. The panelists were given a soy extract incubated with a 1/200th part of

PAO-I (protein basis) for 30 min at 35 °C and an untreated control. The panelists were asked to identify the sample with the lower beany flavor. All of the fifteen sensory panelists identified the untreated soy-extract as having more beany flavor than the treated control ($p<0.01$). The statistical significance was determined using the tables provided by Meilgaard (1991). Two out of fifteen panelists preferred the beany flavor of the control over the treated sample with very low beany-flavor. These panelists were from Asia where beany flavor is preferred in soy products. The low beany flavor sensory data agreed well with the gas chromatography analysis. Figure 25 shows that the beany-flavor-causing aldehydes were significantly reduced from the headspace of soy extract after 30 min of incubation with PAO-I. The similar time of incubation was adequate for the sensory panelists to perceive lower beany flavor significantly. Our sensory analysis data agree with the observation of Takahashi *et al.* (1979) about the reduction of beany flavor of soybean. These authors emphasized a need of catalase in the reaction mixture to prevent oxygen limitation. Catalase oxidized the H_2O_2 and produced oxygen. In this study, catalase was not essential because additional oxygen (from the oxidation of hydrogen peroxide) was not required in the oxidation of aldehydes to their corresponding carboxylic acids.

Although the carboxylic acid (pentanoic and hexanoic) are known to have objectionable flavor, the flavor threshold of these acids (1-10 ppm) is much higher than their corresponding aldehydes (Stahl, 1973; Forss, 1973; Hammond, 1989). The flavor threshold for propanal, butanal, and hexanal were 170, 70, and 30 ppb, respectively (Wick, 1965). The results of sensory analysis indicated that a 1/200th amount of PAO-I compared

with soy extract (15 mg/mL) caused significant reductions in off-flavor aldehydes content in 30 min and that the formation of carboxylic acids did not cause any objectionable flavor.

CONCLUSIONS AND RECOMMENDATIONS

The affinity chromatography on Benzamidine-Sepharose 6B column with a benzamidine gradient was an essential step in achieving homogenous preparation of the two enzyme forms of porcine liver aldehyde oxidase, PAO-I and PAO-II. The molecular weights of PAO-I and PAO-II were estimated to be 265 and 255 kDa, respectively. The isoelectric points of the two enzyme forms were similar (pI 5.8). Ion exchange chromatography on DEAE and PEI resins, size exclusion chromatography on Sephadex and Sephacryl resins, and isoelectric focussing were ineffective in purifying aldehyde oxidase from the ammonium sulfate fraction.

PAO-I had a greater substrate specificity for medium chain aldehydes (pentanal and hexanal) compared with PAO-II. The pH optima for activity and stability was pH 9 and 10, respectively. PAO-I was more stable at basic pH than at neutral and acidic pH. Less than 20% loss in activity of PAO-I at 35 °C and a moderate energy of activation (47 kJ/mol) suggest that PAO-I can be commercially used in the removal of off-flavors from the soy protein extract. Five amino acids residues, histidine, lysine, cysteine, tyrosine, and arginine, appeared to be involved in the catalytic step and binding of substrate to PAO-I. More work is required to confirm the participation of these amino acid residues in the catalysis.

The gas chromatography and sensory analysis data agree with each other. An incubation time of 30 min was adequate in oxidizing off-flavor aldehydes to carboxylic acids, resulting in significantly reduced beany flavor of soy-extract model system.

The storage stability of PAO-I was higher at pH 9 than at pH 7. PAO-I was stable for up to eight freeze-thaw cycles. Lyophilization of purified enzyme did not impair PAO-I activity. Therefore, there is a potential for application of PAO-I in soybean processing to improve the flavor profile of soy proteins.

Future Work

An affinity column of a larger diameter is desired to achieve higher flow rate to cut down the process time. There is also a need for further optimization of binding and elution conditions to maximize the AOX yield.

The application of PAO-I in more food products should be studied. For example, tofu should be prepared with PAO-I-treated water extract of soybean. The sensory characteristics of tofu should be studied as a function of time to monitor any beany flavor regeneration. Due to the requirement of basic pH for the PAO-I activity, the desired point of application of PAO-I will be during the preparation of soy protein isolate, as alkali is used to extract the protein in the isolate preparation. After the alkali extraction of soy proteins, the extract can be treated with PAO-I by bringing the pH close to 9.0 before the final acid precipitation.

The low yield of purified PAO-I could limit its commercial success unless further yield optimization is achieved. The ammonium sulfate fraction provides higher yield of PAO-I, but this fraction of porcine-liver aldehyde oxidase would not obtain Kosher approval. The acceptance of PAO-I in the population sensitive to religious beliefs and animal rights groups would also be limited. Therefore, it will be desirable to clone the

gene for PAO-I in a GRAS (generally recognized as safe) microorganism. If a cheaper microbial preparation of PAO-I can be obtained through fermentation, then the enzyme should be used in a free form, otherwise, a suitable immobilization method is required for this technology to be economically viable. Further work on the operation and storage stability will also be required for the immobilized enzyme.

Alternate Technologies for Flavor Improvement of Soy Proteins

The removal of the lipoxygenase gene was shown to reduce the off-flavor from soy proteins. But the off-flavor generated due to the auto-oxidation of soybean lipids still poses a major problem. The acceptance of the lipoxygenase null soybean variety by farmers and its yield has not been tested. Soy proteins have hydrophobic pockets that irreversibly bind the off-flavor compounds by hydrophobic interaction. Future work could focus on changing the hydrophobicity of soy proteins to reduce the binding of off-flavor compounds. Because, this could change the functionality of soy proteins, one has to evaluate other relevant characteristics of the modified proteins such as emulsification, foaming properties, water holding capacity, gelation, and cohesion.

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APPENDIX A

PURIFICATION OF ALDEHYDE OXIDASE (OTHER STUDIES)

Ion-Exchange Chromatography

An anion exchange chromatography on DEAE-Sephadex and DEAE-Toyopearl resins was performed to purify dialyzed ammonium sulfate fraction. The DEAE Sephadex anion-exchange resin (Pharmacia, Inc., Piscataway, NJ) and DEAE-Toyopearl resin (Supelco, Inc., Bellefonte, PA were used. A 50-mL DEAE-Sephadex column (30 x 1.5 cm) was equilibrated with 20 mM Tris-HCl buffer containing 0.1 mM DTT and 0.1 mM EDTA at pH 8.3. Dialyzed ammonium sulfate fraction (25 mg total protein) was loaded on the column at a rate of 20 mL/hr. The column was washed with 500-mL buffer (ten column volumes) at a rate of 1 mL/min. PAO was eluted in 3-mL fractions with a 0.1-0.5 M NaCl gradient at a flow rate of 20 mL/hr.

The purification results (Table A-1) shows that only twofold purification was achieved by DEAE-Sephadex chromatography after ammonium sulfate fractionation. However, SDS-PAGE of the ion-exchange purified PAO did not show (gel not shown) any increase in purification over the ammonium sulfate fraction. Therefore, this ion-exchange method was not very satisfactory in PAO purification.

A DEAE Toyopearl 650S resin was also tested for purification of PAO. An AllTech chromatography column of (19 x 1.5 cm) was packed with 34 mL DEAE-Toyopearl gel. The protein loading and washing conditions were similar to the DEAE-Sephadex run. Three elution gradient 0-0.2, 0-0.35 and 0-0.5 M NaCL were examined in three separate runs in

Table A-1. Purification of aldehyde oxidase on DEAE-Sephadex.

Fraction	Volume (mL)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification fold	% Yield
Crude extract	1000	700	21960	0.03	1	100
Heat-treated	830	743	10650	0.07	2	106
Ammonium sulfate	46	23276	4464.3	5.2	173.8 (1*)	3325 (100*)
Ion-exchange	75	9675	916.5	10.5	351.8 (2*)	1381 (42*)

*Purification fold and activity yield calculated after ammonium sulfate fractionation.

an effort to optimize purification conditions. The 0-0.2 and 0-0.35 mM NaCl elution gradients resulted in a very broad protein peaks and poor resolution. No measurable aldehyde oxidase purification was achieved. The 0-0.5 M NaCl gradient resulted in a two-fold purification similar to that with DEAE-Sephadex (Table A-1). The SDS-PAGE gel of the purification of PAO on DEAE-Toyopearl is shown in Figure A-1. There was no change in PAO protein bands profile from DEAE-Toyopearl purified aldehyde oxidase compared with heat-treated and ammonium sulfate fraction. The unbound and eluted protein fractions did not have any difference in band profile. In conclusion, anion exchange chromatography on DEAE-resins was not effective in purifying aldehyde oxidase.

Weak anion exchange chromatography using polyethyleneimine-Acti disk with phosphate buffer (pH 7.2) and Tris-HCl buffer (pH 8.7) was performed. Both ammonium

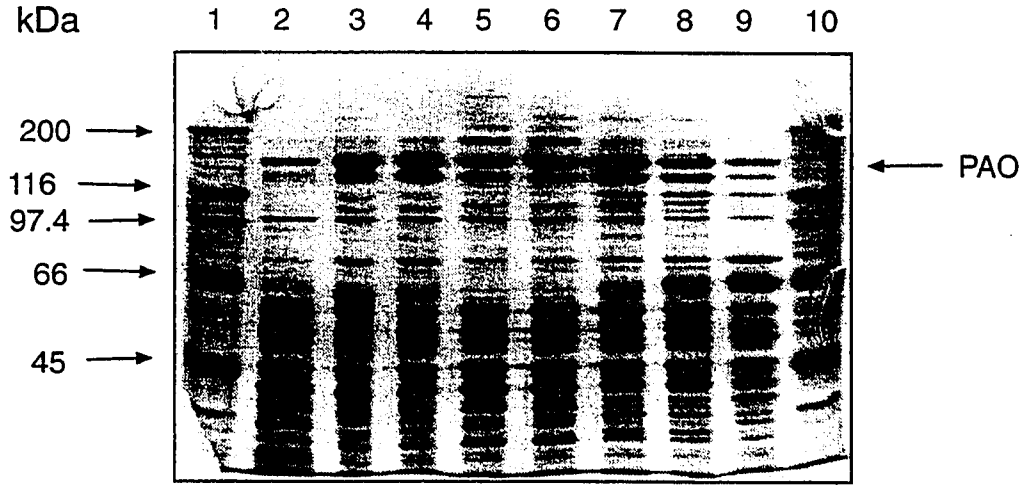

Figure A-1. SDS-PAGE of four steps in PAO purification. Lanes 1 and 10: high-molecular-wight markers obtained from BioRad, myosin (200 kDa) from rabbit skeletal muscle, β-galactosidase (116 kDa) from *E. coli*, phosphorylase B (97.4 kDa) from rabbit muscle, bovine serum albumin (66 kDa), and hen egg white albumin (45 kDa); lane 2: crude homogenate; lane 3: heat-treated extract; lane 4: ammonium sulfate fraction; lanes 5-7: DEAE-Toyopearl purified aldehyde oxidase; lanes 8 and 9: unbound (not retained) ammonium sulfate fraction. Each lane has total 1 μg total protein load.

sulfate fraction (50 mg) and gel filtration purified (1.8 mg) sample (see next section) were evaluated. The elution was carried out with 0-0.2, 0-0.5 and 0-1 M NaCl gradients. None of these gradients resulted in a significantly increased purification of aldehyde oxidase (data not shown).

Gel Filtration Chromatography

A descending size-exclusion chromatography of the ammonium sulfate fraction was performed on a 100 x 1 cm Pharmacia column packed with a Sephadex-200 gel. A 5-mL sample containing 50 mg total protein was loaded and eluted with Tris-HCl buffer (pH 8.3) at a flow rate of 15 mL/h. The protein elution was monitored by continuously measuring the absorbance at 280 nm with ISCO (Lincoln, NE) detector. The Sephadex-200 gel collapsed during the run and the protein separation was very poor.

A Sephacryl-300 HR was chosen as an alternate gel filtration because it can sustain greater pressure drops than Sephadex-200 (manufacturer's recommendation). Despite using a low flow rate (20 mL/h), this method did not result in satisfactory AOX purification (Table A-2). The majority of aldehyde oxidase activity was present in the first 50 mL of the elution (first protein peak, lane 5 to 10 in Figure A-2). A second protein peak was eluted immediately after first peak. The second peaks was eluted in a total volume of 30-mL. Although the aldehyde activity was very low in the second peak, a molecular weight band corresponding to PAO marker (Lane 11 and 12) on SDS-PAGE gel (Figure A-2) was observed. The presence of aldehyde oxidase in both protein peaks could be due to the polymerization of PAO during size-exclusion. Polymerization of PAO could have been due

Table A-2. Purification of aldehyde oxidase on a Sephacryl-300 HR.

Fraction	Volume (mL)	Total Activity (units)	Total Protein (mg)	Specific Activity (units/mg)	Purification Fold	% Yield
Crude	348	449	8683	0.05	1	100
Heat-Treated	300	1545	3549	0.43	8	344
Ammonium Sulfate	14	11718	1417	8.28	159 (1*)	2600 (100*)
Size-exclusion	8	1051	108	9.73	187 (1.2*)	234 (9*)

*Values calculated after ammonium sulfate fractionation.

to lower concentration of thiol reagent (0.1 mM DTT). Earlier work of Felsted *et al.* (1971) showed that polymerization of pig liver aldehyde oxidase occurred during column chromatography. The gel electrophoresis results of their work showed the presence of several polymers of aldehyde oxidase with varying catalytic properties. The catalytic property of polymerized forms of AOX was lower than the native dimer. These authors also showed the presence of aggregates when PAO was stored at -18 °C for two weeks. The aggregation was prevented by adding 5 mM cysteine in the running as well as storage buffer. Mahler *et al.*(1954) also showed the polymerization of pig liver aldehyde oxidase, but there was no difference in the activities in the polymerized and the native dimer of aldehyde oxidase. We recovered 9% of the total activity present in the ammonium sulfate fraction by using size-exclusion chromatography. This lower activity yield could be due to the lower activity of the polymerized aldehyde oxidase as suggested by Felsted *et al.* (1971).

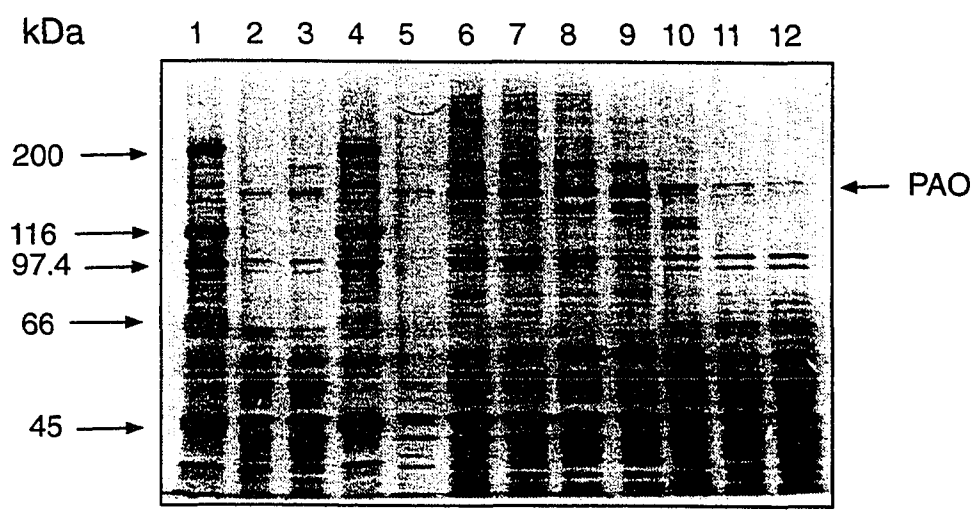

Figure A-2. SDS-PAGE of three steps in PAO purification. Lanes 1 and 4: high-molecular-wight markers obtained from BioRad, myosin (200 kDa) from rabbit skeletal muscle, β-galactosidase (116 kDa) from *E. coli*, phosphorylase B (97.4 kDa) from rabbit muscle, bovine serum albumin (66 kDa), and hen egg white albumin (45 kDa); lane 2: heat-treated extract; lane 3: ammonium sulfate fraction; lanes 5-10: first peak fractions eluted from the gel filtration column; lanes 11 and 12: second peak fractions eluted from the gel filtration column. Each lane except lane 5 (0.5 μg) has 1 μg total protein load.

Aldehyde oxidase fraction from the DEAE-Toyopearl was also loaded on the gel filtration column. However, no further significant purification of aldehyde oxidase was observed.

Preparative Isoelectric Focusing (IEF)

A preparative isoelectric focussing was carried out in a Rotofor (Bio-Rad, Hercules, CA). About 250 mg of dialyzed ammonium sulfate preparation in 5-mL Tris-HCl buffer (without salt) containing 10% (w/v) glycerol (to prevent precipitation of protein) was applied to the Rotofor cell containing 50-mL of prefocussed 2% Biolytes of pH range between 3 and 10. The focussing was initiated at a constant power (for maximum voltage) of 12 W and continued until the voltage plateaued with a total run time of 6 h. At the end of the isoelectric focussing, the samples were harvested (drained) in twenty tubes. The pH, protein content (UV_{280}) and the aldehyde oxidase activity in the tubes were measured.

The aldehyde oxidase activity was focussed in three peaks at pH 5.3 , 6.4 and 7.2 (Figure A-3). The maximal activity of isoelectric focussed aldehyde oxidase was measured at pH 6.4. The activities at pH 5.3 and 7.2 were similar. This suggests that the aldehyde oxidase could be present in two to three enzyme forms between pH ranges of 5 to 5.8, 6.4 to 6.8, and 6.8 to 7.5. The SDS-PAGE (gel not shown) of all the twenty fractions illustrated the separation of various protein bands by isoelectric focussing. The molecular weight band corresponding to aldehyde oxidase marker was present in the pH range between 5.0 and 7.5; however, the lower molecular weight protein bands were still present in all of the fractions. The removal of ampholytes by dialysis against Tris-HCl buffer (pH 8.3) was necessary to

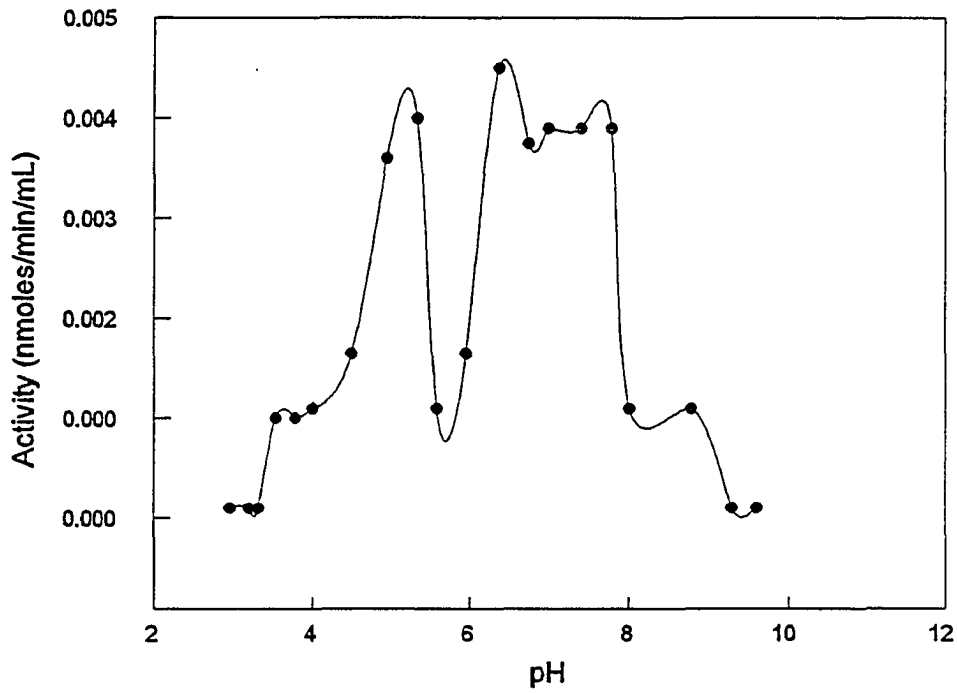

Figure A-3. Separation of PAO activity by preparative isoelectric focussing.

adjust the pH for PAO storage. The dialysis of the focussed samples resulted in the 90% loss of PAO activity.

In spite of using glycerol (10% w/v) as stabilizer, the precipitation of the proteins could not be prevented during isoelectric focussing. To reduce protein precipitation, another run with 25 mg of total protein obtained after the gel filtration was carried out. Although, the protein precipitation was substantially reduced, no measurable PAO activity was detected after isoelectric focussing. The activity measurement was difficult because initial activity present in the gel filtration fraction was only 20% that of ammonium sulfate fraction. Furthermore, a tenfold dilution of isoelectric focussed sample occurred as 5-mL of gel-filtration preparation was added in 45-mL solution of ampholyte during IEF.

In conclusion, the isoelectric focussing was detrimental to the PAO activity. It was also ineffective in separating lower molecular weight proteins from the aldehyde oxidase containing fractions.

Effect of Protease Inhibitors on the Activity of Aldehyde Oxidase

Because the presence of low-molecular-weight proteins on the SDS-PAGE gel (Figures A-1 and A-2) after ion-exchange, gel filtration and preparative isoelectric focussing, a study to determine the effect of protease inhibitors in preventing the possible proteolysis was initiated. Fresh pig liver was homogenized in the extraction buffer as described in materials and methods section. The pellet was discarded and the supernatant was subjected to a second centrifugation to obtain a clearer supernatant. The pellet was discarded and the supernatant was subjected to heat treatment at 54 °C for 4 min. The heat-treated supernatant

(control) was centrifuged and supernatant was divided into six aliquots. To each aliquot ammonium sulfate crystals were added to get a final concentration of 35%. Protease inhibitors were also added in four out of six aliquots. The samples were labeled as AS1, control; AS2, boiled control; AS3, control with 1 mM PMSF (serine protease inhibitor); AS4, control with 10 μg cystatin (sulfhydryl protease inhibitor), AS5, with 50 μg pepstatin (carboxylic protease inhibitor); and AS6, a mixture of equal proportions of AS3, AS4 and AS5. Each mixtures was stirred for 3 h at 4 °C. The mixtures were centrifuged at 17,000 x g for 30 min. The pellets were discarded and the supernatants of AS3, AS4, AS5 and AS6 were again subjected to a similar level of protease inhibitors and the ammonium sulfate concentration was then raised to 55%. The mixtures were centrifuged as before and stirred for 3 hours at 4 °C. The supernatant was discarded and the pellets were incubated at 4 °C for 12, 36, 56, and 226 h, respectively. At the end of each incubation period, the PAO activity (Table A-4) of each pellet was measured by dissolving a fraction of the pellet in standard buffer. The pellet was then frozen at -18 °C. The frozen samples were thawed and SDS-PAGE (gel not shown) was performed at the end of a 226 h incubation period.

The specific activity and the relative activity were decreased with an increase in incubation time (Table A-3). There was no significant difference between the specific activities of all ammonium sulfate fractions with or without protease inhibitor treatments. The relative activity of the ammonium sulfate fraction containing equal proportions of protease inhibitor mix had higher relative activity than the control ammonium-sulfate fraction. The higher relative activity of AS6 sample was measured after each incubation period. Therefore, the effect of protease inhibitor was not significantly different than

control. The SDS-PAGE gel (not shown) showed that the addition of protease inhibitors in the ammonium sulfate fractions did not reduce the presence of low-molecular-weight protein band pattern at any incubation period. These results indicate that the proteolysis may not be occurring in the ammonium sulfate fraction during the incubation period studied and the use of protease inhibitors is not necessary.

Table A-3. Effect of protease inhibitors on enzyme activity in the ammonium sulfate precipitate.

Fraction	Time (hrs)							
	12		36		56		226	
	Sp. act. units/ mg	Rel. act. (%)	Sp. act. units/ mg	Rel. act. (%)	Sp. act. units/ mg	Rel. act. (%)	Sp. act. units/ mg	Rel. act. (%)
AS1 control	0.414	100	0.315	100	0.28	100	0.287	100
AS2 + boiled	ND**							
AS3 + PMSF	0.452	110	0.287	91	0.266	95	0.273	95
AS4 + cystatin	0.326	78	0.301	96	0.294	105	0.294	105
AS5 + pepstatin	0.398	97	0.329	104	0.301	108	0.301	115
AS6 + protease inhibitor mix	0.767	187	0.413	131	0.3501	125	0.336	117

**Not detected

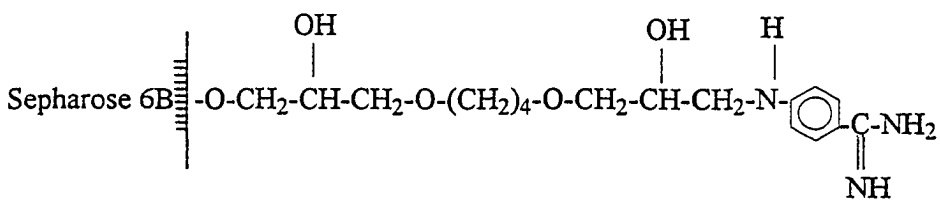

Figure A-4. Partial structure of Benzamidine Sepharose 6B

APPENDIX B

CHARACTERIZATION STUDIES

Table B-1. Effect of pH on the inactivation constant

pH	k_d x 10^3
4.4	12.10
4.9	8.54
5.3	7.94
5.9	3.43
6.6	2.48
7.1	1.54
7.6	1.09
8.1	1.09
8.6	0.92
9.3	0.10
10.0	0.17
10.7	1.98
11.5	10.29
12.0	15.36
12.4	17.33

Table B-2. Effect of temperature on denaturation constant

Temperature (°C)	k_d x 10^3
20	0.13
25	0.54
30	0.97
35	1.28
40	1.58
45	1.94
50	7.50
55	19.56
60	27.75

n=2

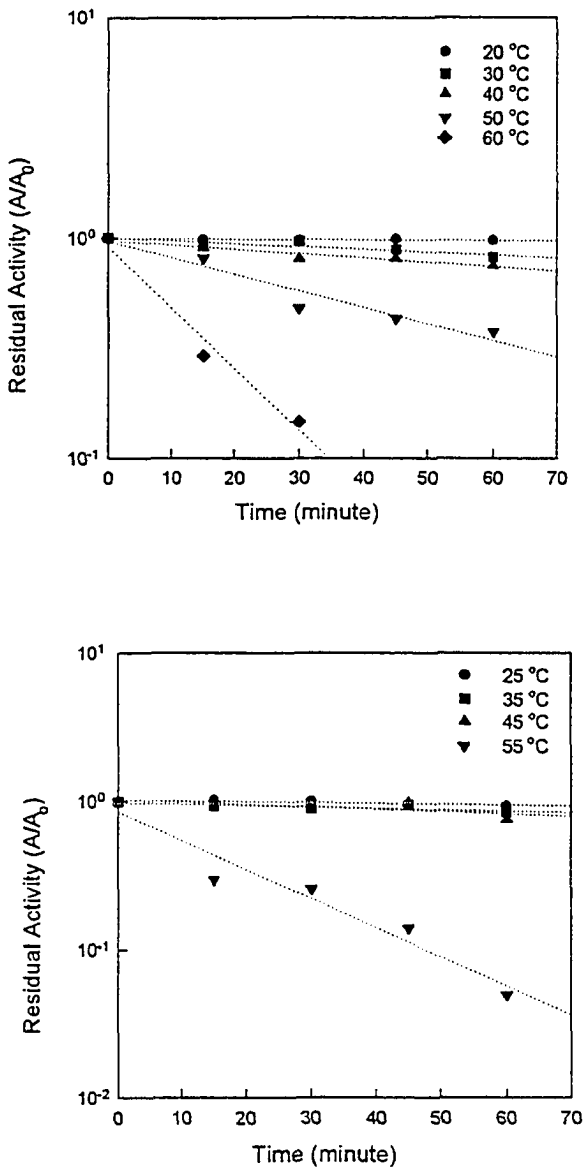

Figure B-1. Residual activity of PAO-I at different temperatures at pH 10.0. Residual activity is defined as the ratio of PAO-I activity at time t and at t=0.

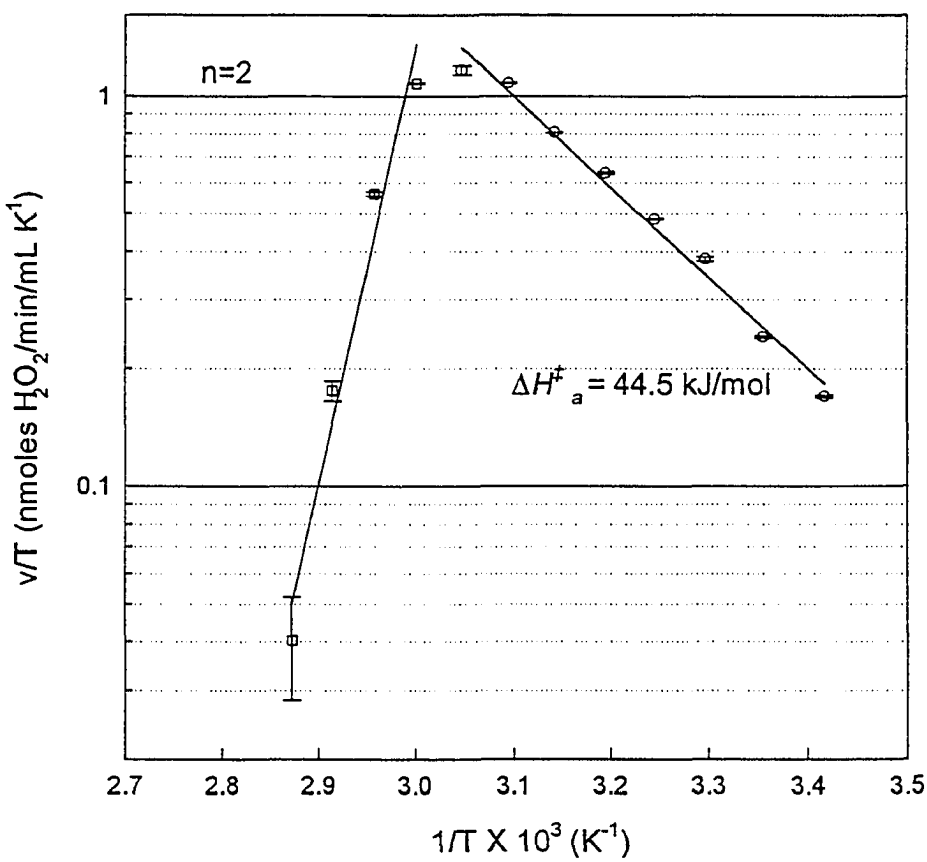

Figure B-2. Effect of temperature on specific rate of reaction constant ($\Delta H^{\ddagger}$). Negative slope represents rate of reaction for the conversion of pentanal to pentanoic acid by PAO-I (◦) and the positive slope represent activity of PAO-I denaturation (□) of PAO-I started at pH 10.0 (refer Fig 12 for k_d values). Bars represent standard deviation and n represents the number of replicates.

Table B-3. Effect of pH on the V_m and K_m.

pH	V_m (nmoles H_2O_2/min/mL)		K_m (μM)	
	Wilkinson	Lineweaver-Burk	Wilkinson	Lineweaver-Burk
5.0	22.71 ± 0.023	21.92 ± 1.52	367.5 ± 1.8	320 ± 55.5
5.8	65.02 ± 0.014	37.51 ± 18.74	224.0 ± 0.1	83.0 ± 25.0
7.05	94.52 ± 0.007	105.90 ± 8.52	53.0 ± 0	62.0 ± 11.5
8.0	459.40 ± 0.004	347.20 ± 77.78	36.1 ± 0	34.6 ± 14.9
9.0	707.60 ±0.009	1201.18 ± 311.18	18.1 ± 0	17.0 ± 6.4
10.0	323.80 ± 0.020	188.73 ± 81.87	95.0 ± 0	68.3 ± 16.6
11.0	124.70 ± 0.009	28.80 ± 68.11	270.2 ± 0.1	154.0 ± 68.9
12.0	89.11 ± 0.010	85.62 ± 5.62	312.9 ± 0.1	200.0 ± 59.1

n=2