Characterization and Application of Porcine Liver Aldehyde Oxidase in the Off-Flavor Reduction of Soy Proteins

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Two enzyme forms of porcine liver aldehyde oxidase (PAO-I and PAO-II) (aldehyde:oxygen oxidoreductase, EC 1.2.3.1) were purified to homogeneity by using affinity chromatography. Both enzyme forms, PAO-I and PAO-II, have similar p*I* values of 5.8 and molecular masses of 262 000 and 25 000 Da, respectively. Compared with PAO-II, PAO-I showed greater affinity for the soy off-flavor-causing aldehydes *n*-pentanal and *n*-hexanal. Purified PAO-I was stable between pH 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 3-fold higher than the energy of activation (47 kJ/mol·K^{-1}). 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. The incubation of a soy protein extract and PAO-I reduced the pentanal and hexanal contents by as much as 90%. The sensory panelists perceived lower beany flavor ($p < 0.01$) of the enzyme-treated soy protein extract than the control.

Keywords: *Porcine liver aldehyde oxidase; purification; characterization; off-flavor; aldehydes; carboxylic acids*

INTRODUCTION

The off-flavor associated with soy proteins is a major technical impediment in the increased usage of soy proteins in human foods. The low cost (compared with sodium caseinate) and the high nutritional value of soy proteins have been motivating researchers to resolve the flavor problem. A number of methods have been proposed to reduce the off-flavor in soy proteins caused by aldehydes, ketones, furans, and alcohols (Sessa and Rakis, 1977; Kinsella and Damodaran, 1980). Medium-chain aldehydes, pentanal, hexanal, and heptanal, were found to be the major class of compounds contributing to the "beany" and "grassy" off-flavor of soy protein. These methods include heat treatment (Smith and Circle 1978), acid treatment (Man *et al.*, 1989, 1994), solvent azeotrope extraction (Eldridge *et al.*, 1971), and supercritical CO_2 technology (Eldridge *et al.*, 1986; Maheshwari *et al.*, 1995). These methods do significantly reduce off-flavor content but resulted in proteins with undesirable characteristics, including reduced solubilitt and/or prohibitive processing cost. Triple-lipoxygenase null mutants, developed to reduce off-flavor formation, had a lower crop yield and still contained beany and grassy off-flavor, which was generated by autoxidation after seed crushing (Kitamura, 1984).

Chiba *et al.* (1979a) used aldehyde dehydrogenase to successfully remove green and beany off-flavor from the aqueous suspension of soy protein isolate, but this approach would be impractical because NAD^+ is required as a cofactor. 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).

Purification and characterization of aldehyde oxidase (EC 1.2.3.1) from various sources, such as rabbit liver, pig liver, mouse liver, turkey liver, and drosophila, have been reported (Mahler *et al.,* 1955; Palmer, 1962; Rajagopalan and Handler, 1964). 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.

Because the effectiveness of porcine liver aldehyde oxidase (PAO) for off-flavor removal has not been investigated before, this study reports on the purification of two PAO forms to near homogeneity and the characterization of the PAO-I form. An application of PAO-I was attempted to reduce the off-flavor content of a model soy protein extract.

MATERIALS AND METHODS

Materials and Reagents. Porcine liver (Landrace × Yorkshire × Hampshire × Duroc hog of 180 days old) was obtained from the Iowa State University Meat Laboratory immediately post-mortem. Benzamidine−Sepharose 6B (Pharmacia, Uppsala, Sweden) was used as affinity packing. A Centriprep concentrator was purchased from Amicon Inc., (Beverly, MA). Benzamidine was purchased from Sigma Chemical Co. (St. Louis, MO). Biolytes (ampholytes) of pH 5.0−8.0 were obtained from Bio-Rad (Hercules, CA).

Acetaldehyde was purchased from Eastman Kodak Co. (Rochester, NY). Propanal, butanal, pentanal, hexanal, heptanal, and propionic, butyric, pentanoic, hexanoic, and heptanoic acids were purchased from Aldrich Chemical Co., Inc., (Milwaukee, WI). Air and nitrogen were obtained from R. R. Welding Supply Co. (Des Moines, IA). The enzymatic assay reagents, *o*-dianisidine dihydrochloride and horseradish peroxidase, were purchased from Sigma.

Protein and Activity Assays. The protein content was determined according to Bio-Rad protein microassay (Bio-Rad

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Laboratories) by using bovine serum albumin as the protein standard (Pierce, Rockford, IL). Absorbance was recorded at 595 nm.

The activity assay was a modification of the aldehyde oxidase assay of Takahashi *et al.* (1979). The assay mixture consisted of 100 mM Tris-HCl, 0.3 mM *o*-dianisidine dihydrochloride, and 7.8 μg of horseradish peroxidase in a final volume of 1 mL and pH 7.5. Two different substrates, 33 mM acetaldehyde or 3.3 mM n-pentanal, were used. The activity of PAO was determined by measuring a change in absorbance due to oxidation of *o*-dianisidine dihydrochloride by the H_2O_2 produced during the oxidation of acetaldehyde or pentanal substrates. A change in absorbance at 445 nm and 30 °C was continuously recorded. A unit of aldehyde oxidase activity is defined as nanomoles of H_2O_2 produced per minute per milliliter of enzyme. Incremental amounts of enzyme were used to determine the maximum concentration of the enzyme required to be in the linear range of the reaction rate for the coupled assay.

A single-point assay was used in the energy of activation and the pH effect studies. The reaction was carried out at a preselected temperature in a 3-mL buffer containing PAO-I and the substrate. A 200-μL aliquot of reaction mixture was withdrawn at various time intervals and boiled to stop the reaction. The color was developed by adding the boiled reaction mixture in a 400-μL assay buffer of pH 7.5 which contained *o*-dianisidine and peroxidase. The change in absorbance was measured at 445 nm.

Enzyme Purification. All purification steps were carried out at 4 °C. A 150–200-g porcine liver was sliced into small pieces and homogenized in the extraction buffer (7.8) containing 1.15% KCl and 0.1 mM EDTA (Takahashi *et al.*, 1979) with a tissue homogenizer (Tekmar, Cincinnati, OH). The pH of the extraction buffer during homogenization was continuously adjusted with 1 M NaOH. The homogenized sample was centrifuged at 30000*g* for 30 min, and the clarified crude extract was used for aldehyde oxidase purification.

The crude extract was divided into several 250-mL Erlenmeyer flasks, containing 100 mL of extract, which were placed in an 80 °C bath (Fisher Scientific, Pittsburgh, PA) and continuously stirred. 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 reduce the temperature to 5 °C and centrifuged at 30000*g* for 30 min, and the pellet was discarded. The supernatant was collected and subjected to ammonium sulfate fractionation. A stepwise 35–55% ammonium sulfate cut had the optimal combination of the enzyme yield and specific activity. The ammonium sulfate pellet was centrifuged at 30000*g* for 45 min and stored at −18 °C. A portion of the frozen ammonium sulfate pellet was solubilized in a glycine–NaOH buffer (pH 9.0) containing 100 mM glycine, 100 mM NaCl, 0.1 mM EDTA, and 2 mM L-cysteine (Sigma Chemical Co.) and dialyzed by using dialysis tubing with 15 000 Da molecular mass cutoff (Spectrum, Houston, TX) against the same buffer using three 2-L changes.

Two hundred fifty milliliters of benzamidine–Sepharose 6B (Pharmacia) resin was packed in a 50 cm × 2.5 cm chromatography column (Bio-Rad). The dialyzed ammonium sulfate fraction was diluted to a protein concentration of 10 mg/mL, and 200 mg of protein was loaded on the column at a flow rate of 5 mL/h by using a peristaltic pump (Rainin Instrument Co., Inc., Woburn, MA). The column was washed with 10 column volumes of glycine–NaOH (pH 9.0) buffer at a flow rate of 30 mL/h. 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 at 20 mL/h. Each PAO peak was pooled and concentrated in a Centriprep concentrator to a final volume of 2 mL, yielding approximately 250 and 350 μg of PAO-I and PAO-II, respectively. The concentrated pools were dialyzed against glycine–NaOH buffer.

Nondenaturing PAGE and Activity Staining. A nondenaturing polyacrylamide gel electrophoresis PAGE (Maizel, 1971) was performed on 7.5% acrylamide gels at 150 V for 95 min with a Mini-Protean II electrophoresis system (Bio-Rad). Crude, heat-treated, ammonium sulfate precipitated, and affinity-purified aldehyde oxidase preparations were loaded at levels between 25 and 280 μg of protein. Bovine serum albumin, β-amylase, β-galactosidase (Sigma Chemical Co.) and a high molecular mass prestained standard (Bio-Rad) were used as molecular weight markers. At the end of the electrophoresis, the gel was cut in two. Half was stained with Coomassie blue (Sigma Chemical Co.) to identify the protein bands. The other half was used for an aldehyde oxidase activity stain. The staining solution consisted of 100 mM Tris, 0.8 mg of 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 30–60 min.

SDS–PAGE. A 7.5% gel was used in SDS–PAGE (Laemmli, 1970) by following the Bio-Rad procedure for the Mini-Protein electrophoresis system (Bio-Rad). High molecular mass protein standards, myosin (200 kDa) from rabbit skeletal muscle, β-galactosidase (116 kDa) from *Escherichia coli*, phosphorylase B (97.4 kDa) from rabbit muscle, bovine serum albumin (66 kDa), and hen egg white albumin (45 kDa), were purchased from Bio-Rad and used at 1 μg per lane. Similarly, the other lanes were loaded with 1 μg of PAO fractions, unless stated otherwise. The electrophoresis was performed at a constant voltage of 175 V for 1 h or until the dye traveled out of the gel. The gels were stained according to 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 by using the Bio-Rad protocol. The gels were stained with silver staining.

pH and Temperature Stability. PAO-I was incubated for up to 60 min at 30 °C in a 5-mL buffer ranging from pH 4.4 to 12.4. The pH of the buffers was measured after the enzyme was added. After the incubation, a 100-μL aliquot of PAO-I was adjusted to the assay pH by adding 1 mL of the assay buffer, and then the residual activity was determined. The denaturation constant (k_d) was determined by assuming first-order kinetics (Dixon and Webb, 1979)

$$k_d = 2.3/t \log(A_o/A) \quad (1)$$

where A_o is the original activity and A is the residual activity.

For temperature stability, PAO-I was incubated between 20 and 65 °C at increments of 5 °C in 5 mL of 50 mM sodium borate–NaOH buffer at pH 10.0. After the incubation, the temperature of the PAO-I sample was adjusted to 30 °C (assay temperature). The denaturation constants (k_d) were estimated by using eq 1, and the energies of activation and denaturation were estimated by the Arrhenius equation (Stauffer, 1989).

Effect of Oxygen Concentration on the Enzyme Assay. 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). Oxygen-saturated and oxygen-free buffers were mixed at 30 °C in different ratios to obtain 35, 22, 16, 9.4, and 1% oxygen concentrations, which corresponded to 4.52, 2.84, 2.13, 1.21, and 0.13 mM oxygen, respectively. The oxygen-saturated buffer was obtained by bubbling air and the oxygen-free buffer by bubbling nitrogen for 30 min.

Effect of pH on the Rate Constants. The effect of pH on V_m and K_m was determined by measuring the initial rate of reaction by using 8–10 different concentrations of *n*-pentanal. A 100-fold difference between the lowest and highest experimental concentrations of *n*-pentanal was maintained (Whitaker, 1972). Concentrations of *n*-pentanal >9 mM were not used because substrate inhibition of PAO-I was observed above that concentration. The oxidation reaction of *n*-pentanl obeyed Michaelis–Menten kinetics. The Michaelis–Menten constants (V_m and K_m) were determined by using the method of Wilkinson (1961).

Off-Flavor Removal from Soy Extract. A high-speed flour mill (Magic Mill III Plus, Salt Lake City, UT) was used to grind normal food-grade variety (XLRB) soybeans (Nichii, Inc., Jefferson, IA) to produced soy flour. The defatting of

Table 1. Purification of Pancreatic Aldehyde Oxidase

fraction	vol (mL)	total act.[a] (units)	total protein (mg)	spec act. (units/mg)	purifn fold	yield (%)
ammonium sulfate fractionation (35−55% cut)	2	790	200	3.9	1	100
affinity chromatography (0−25 mM benzamidine gradient)						
PAO-I pool	10	119	0.25	470	119	15
PAO-II pool	10	158	0.36	439	111	20

[a] Activity determined with acetaldehyde as substrate.

XLRB soy flour (500 g) was performed at 25 °C to minimize protein denaturation during the defatting. The flour was stored at 4 °C. The soy extract was prepared by mixing 10 g of defatted soy flour with 100 mL of distilled water. The mixture was stirred at 200 rpm for 2 h at room temperature and then centrifuged at 30000*g*. The pH of the supernatant was adjusted to pH 9.0 with 1 M NaOH. Reaction mixtures (5 mL) containing either 1:200 or 1:1000 protein weight ratio of PAO-I to total soy protein were incubated at 35 °C and pH 9.0 for 0, 15, 30, 45, and 60 min. At the end of each incubation period, 1-mL aliquots were transferred into 50-mL GC bottles and the reaction was stopped by adding 5 M HCl. The headspace analysis is described in the next section.

Gas Chromatography (GC) and Headspace Analysis. The equilibrium headspace of the control and PAO-I-treated water extracts of soy flour was analyzed by using a Varian 3400 (Sunnyvale, CA) gas chromatograph equipped with a flame ionization detector (FID). Two different GC columns were used in this work: a 30 m long DB-Wax fused-silica capillary column with 0.25-mm i.d. and 0.25-μm 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, Bellefonte, PA). The DB-Wax column temperature was maintained at 40 °C for 10 min, and then the temperature was changed to 200 °C at 10 °C/min and held at 200 °C for 10 min (Maheshwari *et al.*, 1995). An incremental temperature program was used for the NUKOL column. The initial temperature of the column was set at 40 °C; the temperature was then 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 and 12.5 °C/min to reach 175 and 200 °C, respectively. The column was held at 200 °C for 2 min. The total running time for both columns was 36 min. For a detailed carrier gas, makeup gas, and time−temperature protocol, please refer to Maheshwari *et al.* (1995).

To measure the residual aldehydes and synthesized carboxylic acids, the pH of the samples in the 50-mL GC bottles was adjusted to pH 2.0 with 5 M HCl and the GC bottles were sealed and then equilibrated for 2 h at 35 °C before the headspace analysis. Three injections per incubation time interval were made for each reaction. A 5.0-mL gastight syringe was used to inject between 25 μL and 2.5 mL of headspace aliquots at a rate of 2.0 mL/min. The temperature program was initiated after 2 min of cryofocusing (Maheshwari *et al.*, 1995). The decrease in the amount of aldehydes (C_2−C_7) and the concomitant increase in their corresponding carboxylic acids was followed by GC. The volatile compounds were identified by using acetaldehyde, propanal, *n*-butanal, *n*-pentanal, *n*-hexanal, heptanal, and acetic, propionic, butyric, pentanoic (valeric), hexanoic (caproic), and heptanoic acid as external standards.

Sensory Analysis. A paired comparison test (O'Mahony, 1986) was conducted twice to determine the difference in the beany flavor in the control and PAO-I-treated water extract of defatted soybean flour. Control samples contained denatured PAO-I to account for possible off-flavor binding or release by the enzyme. Fifteen sensory panelists were chosen on the basis of their ability to identify the beany flavor. The samples were presented to panelists in 50-mL GC bottles at room temperature. A sensory panel room with white fluorescent light was used for the sensory evaluation. 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.

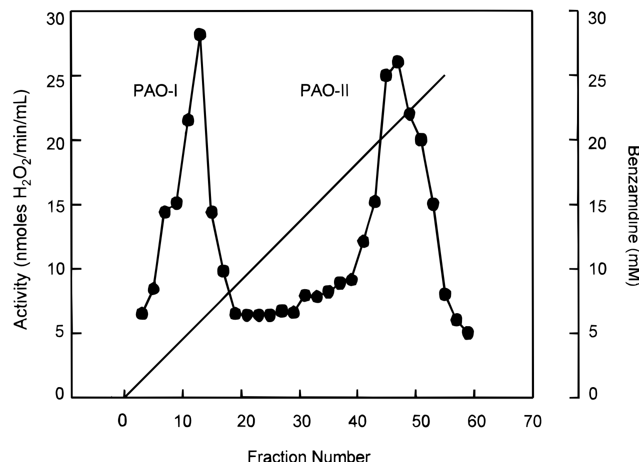

Figure 1. Elution of aldehyde oxidase activities from benzamidine−Sepharose column by using 0−25 mM benzamidine gradient. PAO-I and PAO-II activities were assayed with pentanal and acetaldehyde as substrate, respectively.

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 of PAO. Table 1 presents the purification scheme for PAO. Catalase was present in the crude and heat-treated fractions, and a true estimate of PAO activity could not be made in the crude homogenate, heat-treated extract, and ammonium sulfate fraction because of catalase contamination. The attempts to inactivate catalase in the crude and heat-treated homogenate by freeze−thaw cycling as recommended by Worthington (1988) were unsuccessful; therefore, the ammonium sulfate fractionation is listed as the first step in the purification scheme given in Table 1. Similar to Palmer (1962a), we measured a loss of 15−20% of PAO activity during the dialysis of the ammonium sulfate pellet.

PAO activity was eluted as two peaks (PAO-I and PAO-II) from the benzamidine−Sepharose 6B column by using a 0−25 mM benzamidine gradient. PAO-I was eluted at 8 mM and PAO-II at 25 mM benzamidine concentration (Figure 1). Highly purified PAO fractions were obtained at the expense of the yield. A combination of extensive (10-column buffer) washing and incomplete desorption of PAO resulted in a total of 35% PAO activity yield. The other washing and desorption combinations gave inferior purity of the PAO fractions.

The molecular masses of PAO monomers estimated as 131 kDa for PAO-I and 128 kDa for PAO-II (Figure 2) correspond in size to the monomers of porcine liver aldehyde oxidase (Felsted *et al.*, 1973). To determine whether the two enzyme fractions are different forms

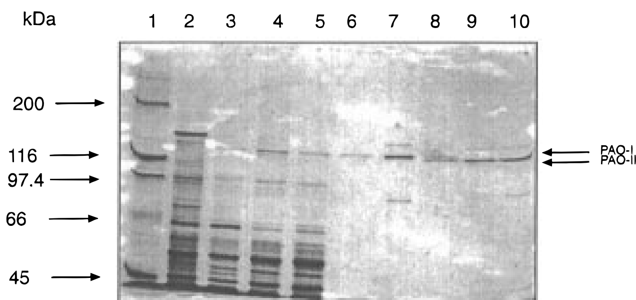

Figure 2. SDS−PAGE of the samples from the four-step purification PAO: (lane 1) high molecular weight markers as (1 μg); (lane 2) crude extract (1.5 μg); (lane 3) heat-treated extract (1 μg); (lane 4) ammonium sulfate fraction (1 μg); (lane 5) flow-through ammonium sulfate fraction (1 μg); (lanes 6 and 7) PAO-I (0.1 and 0.2 μg, respectively) eluted at 8 mM benzamidine PAO-I; (lane 8) mixture of PAO-I and PAO-II (0.2 μg); (lanes 9 and 10) affinity-purified PAO-II (0.2 μg) eluted at 25 mM benzamidine. Protein bands were stained by silver stain.

Table 2. Comparison of Substrate Specificities of the Two Enzyme Forms of Pancreatic Aldehyde Oxidase

substrate (mM)	spec act. of PAO-I (units/mg)	rel[a] substr specificity (%)	spec act. of PAO-II (units/mg)	rel[a] substr specificity (%)
acetaldehyde (33)	540	100	383	100
propanal (33)	603	112	72.5	19
butanal (33)	588	109	215	56
pentanal (3.3)	1187	220	78.0	20
hexanal[b] (0.33)	679	126	14.4	4

[a] Relative activity with respect to acetaldehyde activity. [b] Substrate concentration may not be saturating.

or PAO-I is a degradation product of PAO-II, several experiments were performed. Comparison of substrate specificities of purified PAO-I and PAO-II revealed that, except for acetaldehyde, the specific activity of PAO-I was significantly greater than that of PAO-II (Table 2). Because PAO-I had activity similar to that of PAO-II when incubated with acetaldhyde and 5 times greater than that of PAO-II with *n*-pentanal, these two substrates were used for gel activity staining. The mixture of PAO-I and PAO-II stained with *n*-pentanal as substrate showed one band on the native PAGE gel, and when stained with acetaldehyde, there were two bands (stained gels not shown). Protein staining of the native PAGE confirmed the presence of two distinct bands of PAO-I and PAO-II. Native PAGE of purified PAO-I and PAO-II showed only one band when stained with *n*-pentanal and acetaldehyde as substrates, respectively. The analytical isoelectric focusing showed that both enzyme fractions have a p*I* of 5.8. On the basis of the presented evidence we have concluded that PAO has two enzyme forms with slightly different molecular masses and significantly different specificities for longer (>C2) aliphatic aldehydes (Table 2). Because PAO-I had much greater substrate specificity for off-flavor-causing aldehydes (pentanal and hexanal) than PAO-II, the PAO-I form was selected for further characterization.

Characterization of PAO-I. *pH Stability.* The values of denaturation constants (k_d) of PAO-I as a function of incubation pH are plotted in Figure 3. Less than 20% PAO activity was measured with pentanal as a substrate at a pH <4.9 and >10.7; beyond this pH range PAO was inactive. PAO-I was most stable at pH 10, at which 96% of the original activity was measured after 1 h of incubation. The maximal stability of PAO-I at pH 10.0 agrees with the pH values reported for other

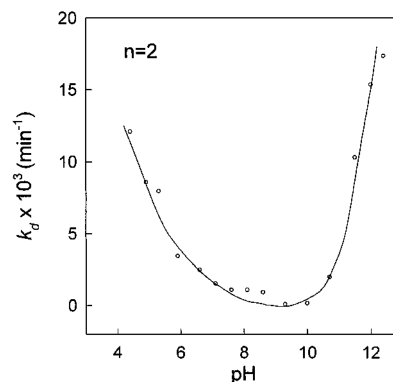

Figure 3. Effect of pH on the denaturation constant (k_d) of PAO-I; *n* is the number of replicates.

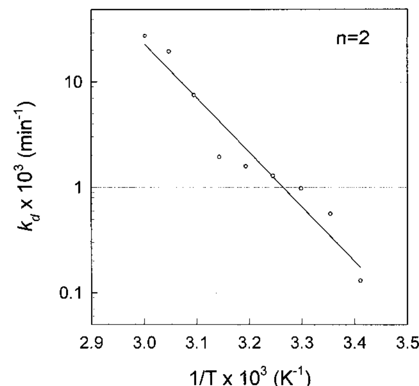

Figure 4. Effect of temperature on the denaturation constant (k_d) of PAO-I at pH 10.0; *n* is the number of replicates.

PAOs by Felsted *et al.* (1973) and Palmer (1962a,b). A relatively broad pH stability has also been observed with several other oxidoreductases such as pea lipoxygenase (Chen and Whitaker, 1986), catalase (Whitaker, 1972), and horseradish peroxidase (Maehly, 1955).

Temperature Stability. The temperature stability study was carried out at pH 10.0 with 50 mM ($I = 0.15$) sodium borate (borax) buffer, which was selected because temperature variation has very little influence on the pH of the buffer (Robyt and White, 1987). The rate of denaturation of PAO-I with temperature followed first-order kinetics (Figure 4). The denaturation of PAO-I accelerated at temperatures above 45 °C. The k_d values increased from 1.94×10^{-3} min^{-1} at 45 °C to 7.5×10^{-3} min^{-1} at 50 °C and 27.75×10^{-3} min^{-1} at 60 °C. After 60 min of heat treatment at 45, 50, and 55 °C, the residual PAO-I activity was 75, 40, and 5%, respectively. PAO-I was completely inactivated after 60 min at 60 °C. The temperature stability of PAO-I was similar to that of the English pea lipoxygenase (Chen and Whitaker, 1986). As expected, the highest temperature stability of PAO-I was at 20 °C, the lowest studied temperature. The temperature stability data indicate that PAO-I can be used in the off-flavor removal without significant loss of activity at or below 45 °C.

Energy of Activation and Denaturation of PAO-I. The energy of activation (47 kJ/mol·K) was determined by measuring the initial rates at temperatures between 20 and 55 °C. The energy of denaturation of 158.1 kJ/mol·K was calculated from the slope of the plot shown in Figure 4. The specific reaction rate constant ($\Delta H^{\ddagger}$) for the denaturation of PAO-I, calculated as 158.1 kJ/mol, is only valid for pH 10.0 because $\Delta H^{\ddagger}$ is a function of pH. Assuming that 20.9 kJ/mol is required to break a noncovalent bond during denaturation (Whi-

taker, 1972), we estimated that eight noncovalent bonds were broken during the denaturation of PAO-I at pH 10.0.

The energy of denaturation for PAO-I was more than 3 times higher than the energy of activation. Compared with other enzymatic reactions (Whitaker, 1972), the estimated energies of activation and denaturation of PAO-I are of intermediate magnitude as those of lipoxygenase. For example, a lipoxygenase from immature green peas had an energy of activation of 18.9 kJ/mol·K^{-1} and an energy of denaturation of 103.8 kJ/mol·K^{-1} (Chen and Whitaker, 1986), whereas Tappel *et al.* (1953) reported an activation energy value of 18 kJ/mol·K^{-1} for the oxidation of linoleic acid catalyzed by soybean lipoxygenase. The higher energy of activation for the conversion of aldehydes to carboxylic acids (47 kJ/mol) by PAO-I compared with the peroxidation of linoleic acid (18 kJ/mol) by lipoxygenase suggests that the removal of off-flavor would be faster than the off-flavor generated by lipoxygenase at a given temperature.

Storage Stability of PAO-I. The storage stability of PAO-I at 4 °C and pH 9.0 (100 mM glycine–NaOH buffer) and at pH 7.0 (100 mM phosphate buffer) was also investigated. PAO-I was more stable at pH 9.0 than at pH 7.0. Greater than 40% of the initial activity was lost after 1 month of storage at pH 7.0 compared with 13% loss in activity at pH 9.0

PAO-I was subjected to 11 freeze–thaw cycles that did not appreciably affect the enzyme activity. Only a 15% loss in PAO-I activity was measured after the 11th freeze–thaw cycle. Freeze-drying of PAO-I samples also did not cause significant loss in activity. More than 95% of the initial activity was recovered when the freeze-dried PAO-I was reconstituted in 100 mM glycine–NaOH buffer.

Effect of pH on the Rate of Reaction. Because O_2 and aliphatic aldehydes are substrates in the conversion of aldehydes to carboxylic acids, we have checked whether O_2 is a limiting substrate. Under the assay conditions (30 °C) the oxygen content in the buffer was 1.35 mM. The K_m for O_2 determined at a constant *n*-pentanal concentration (3.3 mM) was 1.04 mM. The latter value was at least 3 times greater than the K_m values for *n*-pentanal at various pH values; therefore, the O_2 concentration would remain in excess during the oxidation of aldehydes to corresponding acids by PAO-I.

The *n*-pentanal oxidation followed Michaelis–Menten kinetics; estimated V_m and K_m values as a function of pH are plotted in Figure 5. The maximum V_m and the minimum K_m values were obtained at pH 9.0. There was a moderate increase in V_m from pH 5.0 to 7.0. We measured >4 times increase at pH 8.0 and 7 times at pH 9.0 compared with that at pH 7.0. The steep increase of V_m between pH 7.0 and 9.0 may be related to the ionization of the amino acid residues in the active site. The K_m values showed the opposite trend of V_m with increasing pH, a trend that has been observed with many enzymes (Dixon and Webb, 1979).

Off-Flavor Reduction of Enzyme-Treated Soy Protein Extracts. To determine the optimum pH of soy protein extracts for off-flavor removal, we investigated the effect of pH on total volatiles present in the water extract 35 °C (Figure 6). The initial pH of the soybean extract (6.6) was adjusted to pH 7.0 and 9.0; pH 7.0 was chosen as a neutral pH value and pH 9.0 because it was the optimal pH for activity and stability

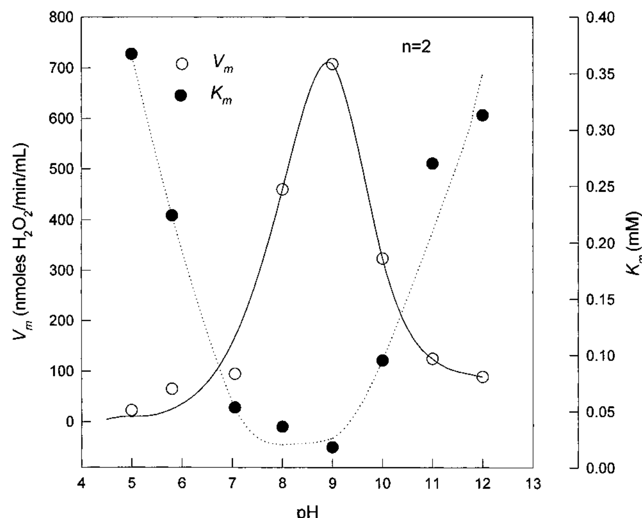

Figure 5. Effect of pH on the V_m and K_m values. The average standard deviations for V_m and K_m were 0.99 and 0.021, respectively; *n* represents the number of replicates.

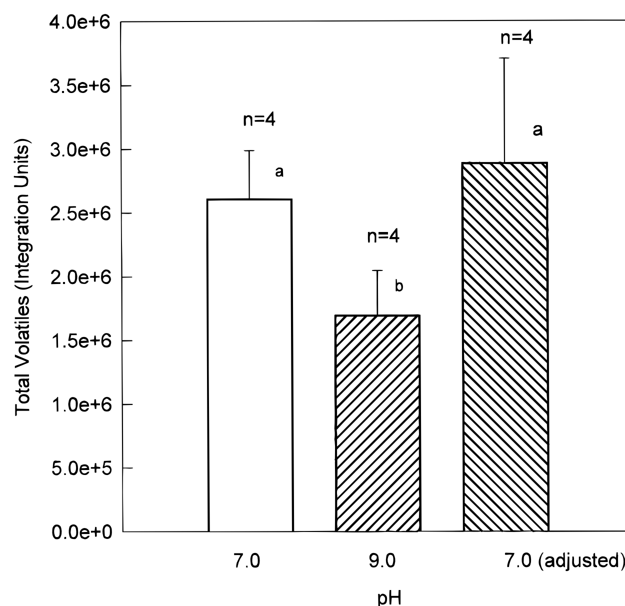

Figure 6. Effects of pH on the total volatiles in the headspace of the water extract of soybeans. Bars represent standard deviation, and *n* represents the number of replicates. Means with the same letter (a and b) are not statistically significant ($p > 0.05$). Adjusted pH 7.0 was achieved by adding HCl to the soy extract of pH 9.0.

of PAO-I. The change in the Schiff base equilibrium of aldehydes caused by the pH shift (Cheftel *et al.*, 1985) resulted in 35% greater concentration of volatiles in the extract at pH 7.0 than at pH 9.0 (Figure 6). When the pH 9.0 of the soy extract was readjusted to pH 7.0, the total volatiles increased again. In spite of the greater free volatiles at pH 7.0, we chose to investigate the off-flavor removal by PAO-I at pH 9.0 for two reasons: (i) PAO-I is 20% more stable at pH 9.0 than at pH 7.0 and (ii) V_m for *n*-pentanal is 7 times greater at pH 9.0 than at pH 7.0.

Preliminary screening experiments established that a PAO-I dosage of 1:200 (1 part of PAO-I to 200 parts of soy protein) was optimal for the efficient reduction of off-flavor-causing aldehydes at pH 9.0 (Figure7). Most of the *n*-pentanal and *n*-hexanal reduction occurred during the first 15 min of incubation. Up to 91% of the initial pentanal and 82% of the initial hexanal content were converted to corresponding acids in 60 min.

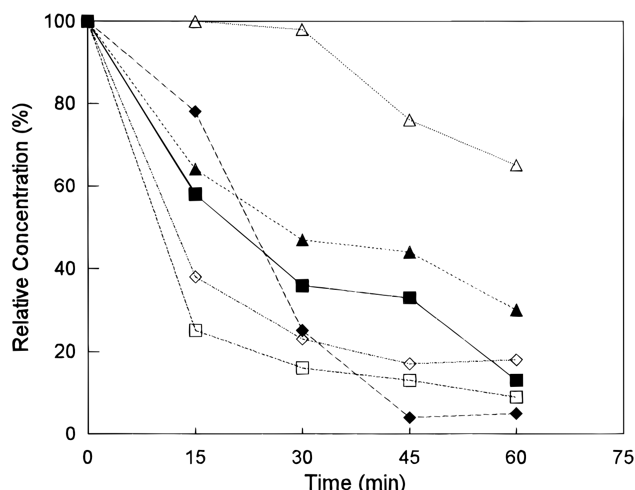

Figure 7. Kinetics of aldehyde reduction in PAO-I-treated soybean extracts at pH 9.0: (■) acetaldehyde; (◆) *n*-propanal; (▲) *n*-butanal; (□) *n*-pentanal; (◇) *n*-hexanal; (△) *n*-heptanal.

The other aldehydes were also significantly reduced after 60 min of incubation with PAO-I. The presence of corresponding carboxylic acids was detected at the end of the reaction, confirming that the reduction of aldehyde content in the extract occurred because of the oxidation of aldehydes to acids.

The sensory analysis was performed by using a one-tailed paired comparison test (O'Mahony, 1986). Fifteen panelists were trained to identify the beany flavor associated with soy proteins. The panelists were given a soy extract incubated with PAO-I (1:200) for 30 min at 35 °C and an untreated extract (control) and were asked to identify the sample with the lower beany flavor. All 15 sensory panelists identified the untreated soy extract as having more beany flavor than the enzyme-treated control ($p < 0.01$). The sensory data agreed with the headspace analysis of the soy extract incubated for 30 min with PAO-I (see Figure 7). In other words, the panelists as well as the gas chromatographic analysis indicated that the medium-chain aldehyde level in the headspace was significantly reduced compared with untreated control.

Although the carboxylic acids (pentanoic and hexanoic) are known to have objectionable flavor, the flavor threshold of these acids (1−10 ppm) is much higher (at least 2 orders of magnitude) than their corresponding aldehydes (Stahl, 1973; Forss, 1973; Hammond, 1989). The results of sensory analysis indicated that PAO-I-treated soy protein extracts had significantly lower off-flavor content and that the formation of carboxylic acids did not cause any objectionable flavor.

CONCLUSIONS

The affinity chromatography on benzamidine−Sepharose 6B column with a benzamidine gradient was an essential step in achieving homogeneous preparation of the two enzyme forms of porcine liver aldehyde oxidase, PAO-I and PAO-II. The molecular masses of PAO-I and PAO-II were estimated as 265 and 255 kDa, respectively.

PAO-I had a greater substrate specificity for medium-chain aldehydes (pentanal and hexanal) compared with PAO-II. Maximum enzyme activity (V_m) was obtained at pH 9.0. PAO-I was more stable at basic pH than at neutral and acidic pH; greatest enzyme stability was measured at pH 10.

The headspace and sensory analyses of PAO-I-treated soy extracts indicated that the aldehyde level in the headspace was significantly reduced compared with the untreated control. An incubation time of 30 min was adequate to oxidize off-flavor aldehydes to carboxylic acids, resulting in a significantly reduced beany flavor of the soy extract.

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.

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