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UNIVERSITY OF ALBERTA

STEREOCHEMISTRY OF SHEEP SERUM AMINE OXIDASE REACTIONS

by

Young J. Kang (C)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF **Master of Science**

IN

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis

entitled **STEREOCHEMISTRY OF SHEEP SERUM AMINE OXIDASE REACTIONS**

submitted by **Young J. Kang**

in partial fulfillment of the requirements for the

degree of **Master of Science**

in **Food Chemistry**

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Date: Oct 11, 1990

DEDICATION

Dedicated to

My Mother, Father and Sisters

ABSTRACT

The stereochemical course of the oxidation of (1*R*)-[^{2}H]- and (1*S*)-[^{2}H]tyramine catalyzed by sheep serum amine oxidase has been investigated using ^{1}H NMR spectroscopy. Reactions were carried out in a coupled fashion such that the aldehydes produced by the amine oxidase were converted directly to alcohols with alcohol dehydrogenase. Both product alcohols obtained from incubations with the chiral amines and sheep serum amine oxidase contained deuterium, demonstrating that reaction occurred with net nonstereospecific proton abstraction from C-1. The enzyme also exhibited a solvent exchange pathway into C-2 of product. The magnitudes of kinetic isotope effects with different substrates were estimated in steady-state measurements by comparing the kinetic paramaters V_{max} and V_{max}/K_m obtained with fully protonated and dideuterated benzylamine and tyramine substrates. Benzylamine exhibited a kinetic isotope effect on V_{max} of 1.44 and V_{max}/K_m of 3.35, indicating that reoxidation of the enzyme is slow relative to the isotope sensitive step (i.e. C-H bond cleavage). Tyramine showed a kinetic isotope effect on V_{max} of 3.14 and V_{max}/K_m of 0.9, suggesting that reoxidation of the enzyme is rapid relative to the C-H bond cleavage step and the slow step must be at formation or dissociation of the intermediate E•S complex.

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Table of Contents

Chapter		Page
1	INTRODUCTION	1
2	EXPERIMENTAL PROCEDURES	6
	2.1 Isolation of Porcine Plasma Amine Oxidase	6
	2.1.1 Materials	6
	2.1.2 Methods	6
	2.1.2.1 Assay of enzyme activity	6
	2.1.2.2 Enzyme purification	7
	2.2 Isolation of Sheep Serum Amine Oxidase	10
	2.2.1 Materials	10
	2.2.2 Methods	11
	2.2.2.1 Assay of enzyme activity	11
	2.2.2.2 Enzyme purification	11
	2.3 Stereochemistry of Sheep Serum Amine Oxidase Reactions	15
	2.3.1 Materials	15
	2.3.2 Methods	15
	2.3.2.1 Oxidation of monodeuterated tyramines	15
	2.3.2.2 Solvent exchange into product	16
	2.4 Kinetic Isotope Effects	17
	2.4.1 Steady-state kinetics for benzylamine oxidation	17
	2.4.1.1 Materials	17
	2.4.1.2 Methods	17
	2.4.2 Steady-state kinetics for tyramine oxidation	18
	2.4.2.1 Materials	18
	2.4.2.2 Methods	18

Chapter Page

3 RESULTS AND DISCUSSION....19

3.1 Isolation of Porcine Plasma Amine Oxidase....19

3.2 Isolation of Sheep Serum Amine Oxidase....19

3.3 Stereochemistry of Sheep Serum Amine Oxidase Reactions....30

3.3.1 Oxidation of monodeuterated tyramines....30

3.3.2 Solvent exchange into product....38

3.4 Kinetic Isotope Effects....41

4 SUMMARY AND CONCLUSIONS....49

5 REFERENCES....51

List of Tables

Table		Page
3.1	Purification of porcine plasma amine oxidase	20
3.2	Partial purification of sheep serum amine oxidase	22
3.3	Further purification of sheep serum amine oxidase	25
3.4	Characteristics of ^{1}H NMR spectra of alcohols derived from coupled incubations of chiral amines with sheep serum amine oxidase	39
3.5	Kinetic parameters for the oxidation of benzylamine and tyramine	46

List of Figures

Figure — Page

1.1 Structure of amine oxidase cofactors 5

3.1 SDS polyacrylamide gel electrophoresis of eluates after different purification steps in the porcine plasma amine oxidase isolation 21

3.2 SDS-PAGE of low activity fraction from DE-52 of the sheep serum amine oxidase isolation 24

3.3 SDS-PAGE of Blue Sepharose eluates 26

3.4 Outline of sheep serum amine oxidase isolation 28

3.5 The proposed isolation scheme for SSAO 29

3.6 Partial 360 MHz ^1H NMR spectrum of (1*R*)-[^2H]tyramine derived from incubation of tyrosine decarboxylase with L-tyrosine in ^2H_2O 31

3.7 Partial 360 MHz ^1H NMR spectrum of (1*S*)-[^2H]tyramine derived from incubation of tyrosine decarboxylase with DL-[2-^2H]tyrosine in H_2O 32

3.8 The ^1H NMR spectrum of alcohol derived from coupled incubation of (1*S*)-[^2H]tyramine and sheep serum amine oxidase with NADH and alcohol dehydrogenase 34

3.9 The ^1H NMR spectrum of alcohol derived from coupled incubation of (1*R*)-[^2H]tyramine and sheep serum amine oxidase with NADH and alcohol dehydrogenase 35

3.10 The ^1H NMR spectrum of alcohol derived from incubation of sheep serum amine oxidase and tyramine in ^2H_2O 40

3.11 Double-reciprocal plot of initial rate data for the oxidation of benzylamine by sheep serum amine oxidase (0.012 units) in 100 mM potassium phosphate buffer, pH 7.0, at 25°C 47

3.12 Double-reciprocal plot of initial rate data for the oxidation of tyramine by sheep serum amine oxidase (0.04 units) in a coupled system containing alcohol dehydrogenase and NADH in 100 mM potassium phosphate buffer, pH 7.0, at 25°C 48

List of Abbreviations

ADH	alcohol dehydrogenase
AO	amine oxidase
BPAO	bovine plasma amine oxidase
Con A	concanavalin A
DNA	deoxyribonucleic acid
E•S complex	enzyme•substrate complex
EC	Enzyme Commission
HMW	high molecular weight
^{D}k	intrinsic isotope effect
$^{D}k_{eq}$	isotope effect on equilibrium constant
$^{D}V_{max}$	isotope effect on V_{max}
$^{D}V_{max}/K_m$	isotope effect on V_{max}/K_m
KIE	kinetic isotope effect
V_{max}	maximal velocity
K_m	Michaelis constant
NADH	nicotinamide adenine dinucleotide (reduced form)
ppm	parts per million
PAGE	polyacrylamide gel electrophoresis
PPAO	porcine plasma amine oxidase
^{1}H NMR	proton nuclear magnetic resonance
R_f	relative mobility
RNA	ribonucleic acid
SSAO	sheep serum amine oxidase
SDS	sodium dodecyl sulfate
TLC	thin-layer chromatography
$[E_t]$	total enzyme concentration
TDC	tyrosine decarboxylase

1. INTRODUCTION

Amine oxidases catalyze the oxidative deamination of amines (mono-, di- and polyamines) with the stoichiometric formation of aldehyde as follows:

$$RCH_2NH_2 + O_2 + H_2O \longrightarrow RCHO + NH_3 + H_2O_2 \qquad (1\text{-}1)$$

These enzymes can be separated into three classes based on differences in cofactor, in substrate specificities and inhibitor sensitivities. The three classes are the flavin-containing monoamine oxidases (EC 1.4.3.4), the copper-containing amine oxidases (EC 1.4.3.6), and the semicarbazide-sensitive amine oxidases, which have not yet been given an Enzyme Commission designation (Mondovi, 1985).

Amine oxidases are ubiquitous, they are found in microorganisms as well as in higher forms of life. They are of great biological interest since many of their substrates are biogenic amines which are formed *in vivo* in various metabolic processes. Many of these biogenic amines appear to be involved in regulatory enzyme mechanisms, vital to higher mammals. For instance, polyamines such as spermine and spermidine seem to influence the synthesis of DNA and RNA (Kapeller-Adler, 1970 and Feuerstein & Marton, 1989).

The physiological role in neurotransmitter metabolism of the first class of enzymes, the flavin-containing monoamine oxidases, has made them the subject of intensive investigation, notably as targets for antidepressant drugs (Kapeller-Adler, 1970). The semicarbazide-sensitive amine oxidases found in vascular tissue and fat have not been well studied. At present, the nature of the prosthetic group is unknown, however, kinetically and stereochemically they appear to be distinct from the other two classes of amine oxidases (Callingham and Barrand, 1987).

The copper amine oxidases, which are the subject of this thesis, are widespread in nature — they are found in microorganisms, plant seedlings, serum, kidney, intestinal mucosa, liver and lung. In mammals they were first described as spermidine oxidase activity found in the serum of sheep (Hirsh, 1953) and oxen (Tabor *et al.*, 1953).

The first report of a copper amine oxidase in plants was made in 1948 by Werle and coworkers. They reported the occurrence of an enzyme that catalyzes the degradation of 1,4-diaminobutane, 1,5-diaminopentane, and histamine in extracts of some leguminous plants, sage and lavender.

An oxidative degradation of spermidine and spermine by bacterial preparations was first observed by Silverman and Evans in 1944, who used whole cells or lyophilized cell extracts of *Pseudomonas pyocyaneae (aeruginosa).*

Most copper amine oxidases lack strict substrate specificity. For instance, the classical serum amine oxidase catalyzes oxidative deamination of aromatic amines (such as tyramine and benzylamine) and peptidyl lysine, in addition to polyamines. Blaschko and coworkers (1959), who were some of the first investigators studying mammalian plasma amine oxidases, reported that ruminant enzyme acted on spermine and spermidine, while these were not significantly attacked by the non-ruminant plasma amine oxidase. The non-ruminant enzyme acted relatively rapidly on benzylamine and mescaline, but more slowly on tyramine and many monoamines. Blaschko also reported that the amine oxidases of mammalian plasma were inhibited by carbonyl reagents.

One of the best-characterized copper enzymes, bovine plasma amine oxidase, has a mol. wt. of about 170,000 and apparently consists of two identical subunits (Mondovi, 1985). Porcine plasma amine oxidase, which has a mol. wt. of approximately 195,000, is also a dimer (Falk *et al.*, 1983). A concentrated solution of either enzyme is pink in color.

When Hirsh (1953) first found spermine oxidase activity in sheep serum, it was a crude preparation, derived only from clotting and centrifugation of the blood. Sheep serum amine oxidase (SSAO) was isolated and purified by Rucker and Goettlich-Riemann (1972) through a series of steps involving ammonium sulfate fractionation, extraction and calcium adsorption. Though its purity (0.032 units/mg) was an improvement over previous preparations, it was considerably lower than those reported for bovine (0.36 units/mg) and

porcine (0.1 units/mg) plasma amine oxidases. Therefore, one objective of the research described in this thesis was to isolate and further purify SSAO.

Mammalian copper amine oxidases have long been recognized to play key roles in cellular processes. They catalyze the oxidative removal of biogenic amines from blood plasma, the crosslinking of collagen and elastin in connective tissue biogenesis, and the regulation of intracellular spermine and spermidine levels (Hartmann and Klinman, 1988).

The role of copper in these enzymes is not clear and mechanistic studies of these enzyme systems have been seriously hampered by the uncertainty as to the exact nature of the covalently bound cofactor. It was first believed to be pyridoxal phosphate (Mondovi *et al.*, 1967), and then thought to be pyrroloquinoline quinone (PQQ) (Moog *et al.*, 1986). More recently, bovine plasma amine oxidase has been shown to contain 3,4,6-trihydroxyphenylalanine (Topa). Topa has been found in the pentapeptide at the active site:

Leu - Asn - X - Asp - Tyr

where X is Topa

Topa formation might represent a new posttranslational modification involving oxidation of an active site tyrosine (Janes *et al.*, 1990) (Figure 1.1).

Copper amine oxidases are characterized by unusual stereochemical patterns in their reactions. Dopamine and tyramine are oxidized with abstraction of the pro-*R* hydrogen at C-1 by the porcine plasma amine oxidase, the pro-*S* hydrogen by pea seedling amine oxidase, and a net nonstereospecific proton abstraction by the bovine plasma enzyme (Coleman *et al.*, 1989), while benzylamine oxidation catalyzed by the same enzyme (BPAO) is stereospecific with abstraction of the pro-*S* hydrogen at C-1. The pro-*R* (or pro-*S*) hydrogen refers to one of the chemically identical hydrogens at a prochiral centre i.e. the methylene carbon of benzylamine or C-1 of tyramine. If one of the methylene hydrogens at this centre is replaced by a deuterium, then a chiral centre is produced. The designation pro-*R* is used for the deuterium substitution that generates a chiral centre with an *R* configuration as defined by

Cahn- Ingold- Prelog system. The designation pro-*S* is used for the deuterium substitution that generates a chiral centre with an *S* configuration. For a review of stereochemical nomenclature used in this thesis see Walsh (1979). The copper amine oxidases provide the first example in which a reaction catalyzed by enzymes in the same formal class occurs by all three possible stereochemical routes. Solvent exchange profiles are consistent within each stereochemical class of enzyme: the pro-*R* and nonstereospecific enzymes exchange solvent into C-2 of product aldehydes, the pro-*S* enzymes do not.

In this study, the stereochemical course of oxidation of tyramine was established for another copper amine oxidase isolated from sheep serum (SSAO). The deuterium isotope effects with benzylamine and tyramine substrates were also estimated in steady-state kinetic experiments. In addition to these kinetic and stereochemical studies of SSAO, porcine plasma amine oxidase was isolated for use in other stereochemical studies and to gain experience in enzyme isolation methodologies.

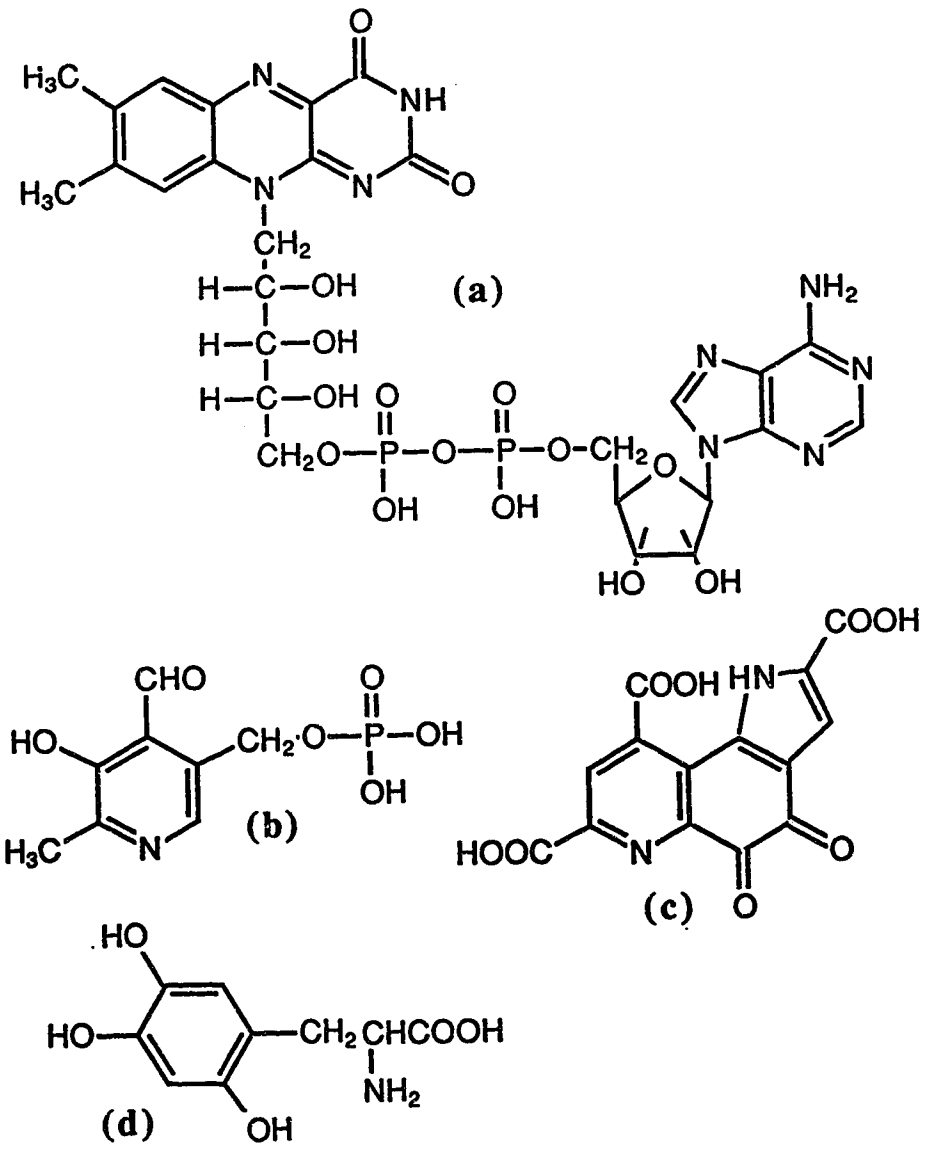


Figure 1.1. Structure of amine oxidase cofactors.
(a) Flavin adenine dinucleotide (FAD)
(b) Pyridoxal-5'-phosphate (PLF)
(c) Pyrroloquinoline quinone (PQQ)
(d) 3,4,6-Trihydroxyphenylalanine (Topa)

2. EXPERIMENTAL PROCEDURES

2.1 Isolation of Porcine Plasma Amine Oxidase (PPAO)

The enzyme was isolated from pig blood by the method of Falk *et al.* (1983) and Coleman *et al.* (1989) with some modifications. A new step (AH-Sepharose chromatography) was introduced to try to improve the preparation.

2.1.1 Materials

Fresh pig blood was obtained from a local slaughterhouse (Gainers, Edmonton, AB). Benzylamine was purchased from Sigma Chemical Company (St. Louis, MO). Hydroxyapatite (Bio-Gel HTP), Bio-Gel A 1.5 m and electrophoresis reagents were obtained from Bio-Rad Laboratories (Mississauga, ON). Con A-Sepharose was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), DE-52 cellulose was from Whatman (Maidstone, England), and AH-Sepharose was prepared by Ms. C. Scaman in our laboratory. The latter is CNBr-activated Sepharose 4B reacted with 1,6-diaminohexane (Svenson & Hynning, 1981).

2.1.2 Methods

2.1.2.1 Assay of enzyme activity

Amine oxidase activity was estimated spectrophotometrically. Assays were carried out at 25°C in 1 mL of 50 mM sodium phosphate buffer, pH 7.2, containing 3.33 mM benzylamine. The production of benzaldehyde was determined by monitoring the increase in absorbance at 250 nm. An extinction coefficient of 1.2×10^4 M^{-1} cm^{-1} was used to calculate the amount of benzaldehyde formed. One unit of activity was defined as the amount of enzyme catalyzing the production of 1 μmol of benzaldehyde per min under the standard assay conditions. Protein concentration was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as a protein standard. Spectral

measurements were made with a Beckman DU-8 spectrophotometer equipped with a Peltier temperature controller.

2.1.2.2 Enzyme purification

All steps were carried out at 4°C.

Step 1 Citration of blood and centrifugation.

Fresh pig blood (26 L) was mixed with 4.4 L of citrate solution (8 g of citric acid and 22 g of sodium citrate dihydrate/L) to prevent coagulation. The citrated blood was centrifuged at 6,400 x g for 25 min. The red and white blood cells were pelleted and discarded.

Step 2 $(NH_4)_2SO_4$ fractionation (35 - 55 %): salting out.

This process involves removal of proteins which precipitate before 35 % $(NH_4)_2SO_4$ saturation and proteins that are soluble beyond 55 % $(NH_4)_2SO_4$ saturation.

The supernatant (plasma, 16 L) was treated with solid ammonium sulfate to 35% saturation (209 g $(NH_4)_2SO_4$/L supernatant). The precipitate was separated by centrifugation at 6,400 x g for 25 min and discarded. The supernatant was brought to 55% saturation by the addition of 129 g $(NH_4)_2SO_4$/L and centrifuged at 11,300 x g for 25 min. The precipitate was dissolved in a minimal volume of 10 mM sodium phosphate buffer, pH 7.0, and was dialyzed against four changes of 16 L 10 mM sodium phosphate buffer, pH 7.0. After dialysis, the solution (2 L) was centrifuged at 17,700 x g for 25 min to remove solid particles.

Step 3 DE-52 anion exchange chromatography.

DEAE-cellulose is positively charged at pH 6-9, thus proteins with net negative surface charges would be adsorbed to the column while proteins with a positive charge or an overall charge near zero will pass through the column resulting in partitioning of the protein of interest. Since porcine plasma amine oxidase has an isoelectric point (pI) of 4.5 to 5.0, it will be negatively charged at pH 7.0 and adsorb to the resin.

One third of the clarified supernatant, i.e. 680 mL, was applied to a Whatman DE-52 cellulose column (5 x 50 cm) and the column was washed with 10 mM sodium phosphate buffer, pH 7.0, until the protein content of the eluate was close to baseline levels as monitored by the absorbance at 280 nm. Amine oxidase was eluted at a flow rate of 2.5 mL/min with 30 mM sodium phosphate buffer (1,200 mL), pH 7.0, followed by 400 mL of 100 mM sodium phosphate buffer, pH 7.0 (22 mL/fraction). Enzyme activity was found in both eluates (30 mM and 100 mM).

Step 4 Con A-Sepharose chromatography.

This step relies on the fact that Con A binds molecules which contain α-D-mannopyranosyl, α-D-glucopyranosyl and sterically related residues. Therefore, Con A-Sepharose is suitable for separation and purification of glycoproteins, glycolipids and polysaccharides from non-glycosylated proteins.

The fractions which eluted from the DE-52 column were concentrated by ultra-filtration (PM-10 membrane) and applied to a Con A-Sepharose column (5.5 x 23 cm) which had been equilibrated with a pH 7.0 buffer containing 10 mM sodium phosphate, 150 mM sodium chloride, 1 mM manganese chloride, and 1 mM calcium chloride. The column was washed with the starting buffer until the protein concentration dropped to baseline levels. Enzyme was eluted with 1,040 mL of starting buffer containing 100 mM methyl α-mannopyranoside (22 mL/fraction) at a flow rate of 0.76mL/min. The eluate was concentrated by ultrafiltration using a PM-30 membrane and dialyzed against 10 mM sodium phosphate buffer, pH 8.0.

Step 5 AH-Sepharose chromatography.

This affinity column is based on the fact that CNBr-activated AH-Sepharose contains an aminohexyl group which is an amine oxidase substrate analog and a charged group such as the isourea moiety which can introduce anion exchange character.

The dialyzed enzyme solution was applied to an AH-Sepharose column (2.5 x 23 cm) equilibrated with 10 mM sodium phosphate buffer, pH 8.0, and washed with the same buffer. Enzyme was eluted stepwise with 45 mM, 55 mM, 75 mM, and 100 mM potassium phosphate buffer, pH 8.0. Activity was found in all four eluates, with 64% of activity occurring in the 45 mM eluate. Steps 3-5 were carried out on the remaining two-thirds of the clarified supernatant from Step 2 as 650 mL and 670 mL volumes, respectively.

All eluates with activity were combined, concentrated by ultrafiltration, clarified by centrifugation, and dialyzed against 6 mM sodium phosphate buffer, pH 7.0. At this point the enzyme solution was divided into two portions of 100 mL each, and both were taken through Steps 6 and 7.

Step 6 Hydroxyapatite (crystalline calcium phosphate) column.

The mechanism of adsorption of proteins to hydroxyapatite is still not clear. It is believed to be that the adsorption of proteins to hydroxyapatite (HA) is due to polar dipole-dipole bonding between

H_3N^+ - protein
:
: and
:
PO_4^{3-} - HA

^-OOC - protein
:
: and
:
Ca^{2+} - HA

Generally, proteins that chromatograph well on anion exchangers also bind to HA, but low molecular weight proteins show very low affinity for hydroxyapatite.

A 100 mL aliquot of the dialyzed enzyme solution was loaded onto a hydroxyapatite column (2.5 x 52 cm) which had been equilibrated with the 6 mM sodium phosphate buffer, pH 7.0, and was eluted with 400 mL of 6 mM and 220 mL of 60 mM sodium phosphate buffer, pH 7.0. Activity was found in the 6 mM eluate. The eluate was concentrated in an Amicon ultrafiltration cell by using a PM-30 membrane and dialyzed against 10 mM sodium phosphate buffer, pH 7.0.

Step 7 Bio-Gel A 1.5 m (gel filtration) column.

This chromatography involves the separation of proteins in aqueous media according to size. The separation is due to the different length of time spent by the protein solutes within the liquid phase that is entrapped by the gel matrix. That is, the larger the molecule, the smaller is the fraction of the pores accessible to them. This results in a decrease in the time spent within the liquid phase inside the pores of the matrix. Therefore larger proteins elute from the colmn faster.

The concentrated enzyme solution (3 mL) was loaded onto a Bio-Gel A 1.5 m column (2.5 x 100 cm) and eluted with the starting buffe at a flow rate of 0.35 mL/min.

Step 8. Aliquots of sample after each purification step were assayed for activity and protein content. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was also carried out by the method of Laemmli (1970) using a Bio-Rad Mini Protean II vertical slab gel electrophoresis cell equipped with 1.5 mm spacers. The gels (11%) were run at 18 ma constant current for about 2 hr, and stained for protein with 0.35% Coomassie Brilliant Blue R-250 dissolved in 45:10:45 (v/v/v) methanol:acetic acid:water. Destaining was done by several changes of the same solvent.

2.2 Isolation of Sheep Serum Amine Oxidase

Sheep serum amine oxidase was isolated from sheep blood by the method of Rucker and Goettlich-Riemann (1972). These workers used a series of ammonium sulfate precipitations and calcium phosphate gel treatments to partially purify the enzyme. An additional series of chromatographic steps was implemented in this study to try to provide highly purified enzyme.

2.2.1 Materials

Fresh sheep blood was obtained from a slaughterhouse (Lamco, Innisfail, AB). Benzylamine was purchased from Sigma Chemical Company (St. Louis, MO). Blue

Sepharose CL-6B and Sephacryl S-300 SF were from Pharmacia Fine Chemicals (Uppsala, Sweden). Electrophoresis reagents were from Bio-Rad Laboratories (Mississauga, ON), and DE-52 cellulose was from Whatman (Maidstone, England). Calcium phosphate gel was prepared by the method of Kunitz (1952).

2.2.2 Methods

2.2.2.1 Assay of enzyme activity

Amine oxidase activity was assayed at 25°C in 1 mL of sodium phosphate buffer, pH 7.6, containing 3.33 mM benzylamine. One unit was defined as the amount of enzyme catalyzing the production of 1 μmol of benzaldehyde per min under assay conditions. The production of benzaldehyde was monitored spectrophotometrically at 250 nm and calculations for benzaldehyde production were based on a molar absorptivity of 1.2×10^4 M^{-1} cm^{-1}. Protein concentration was determined by the Bradford (1976) method using bovine serum albumin as a protein standard. Spectral measurements were made on a Hewlett-Packard 8451A diode array spectrophotometer thermostated with a circulating water bath.

2.2.2.2 Enzyme purification

All steps were carried out at 4°C. The procedure follows the flow chart (Figure 3.4) on page 28.

Step 1 Citration of blood and centrifugation.

Fresh sheep blood (12 L) was mixed with 2 L of citrate solution (8 g of citric acid and 22 g of sodium citrate dihydrate/L), the citrated blood was centrifuged at 10,000 rpm with a Beckman JA-10 rotor (17,700 x g) for 20 min, and the supernatant (serum, 7 L) was collected. High g value applied for sheep enzyme was due to the fact that

separation of pellet was not satisfactory at low centrifugal force probably because of a large amount of clotted blood.

Step 2 $(NH_4)_2SO_4$ fractionation (30-65%).

Solid ammonium sulfate was added to the serum (176 g $(NH_4)_2SO_4$/L serum) to 30% saturation, and the precipitate was removed after centrifugation at 17,700 x g for 20 min. The supernatant (7.2 L) was brought to 65% saturation by the addition of 235 g $(NH_4)_2SO_4$/L supernatant, and the precipitate was separated by centrifugation.

Step 3 $(NH_4)_2SO_4$ extraction (40%).

The precipitate was suspended in 40% saturated ammonium sulfate solution (900 mL) which was adjusted to pH 7.4. After standing for 30 min, the suspension (1 L) was centrifuged at 17,700 x g for 30 min and the particulate material was discarded. The supernatant was dialyzed against a 20-volume excess of 20 mM sodium phosphate buffer, pH 7.4, with three changes.

Step 4 Calcium phosphate gel adsorption.

The theory involved is the same as described for the hydroxyapatite column except that the gel is not suitable for chromatography because of low flow rate. It can be used in batch-wise adsorption treatments.

The dialyzed solution (1,240 mL) was treated with calcium phosphate gel (370 mL) at a ratio of 22.2 mL gel/g protein, and the precipitate was discarded. The supernatant, which contained all the enzyme activity, was treated with another portion of calcium phosphate gel (100 mL) and centrifuged. Most enzyme activity remained in the supernatant, instead of being adsorbed to calcium phosphate gel #2, therefore the calcium phosphate gel adsorption procedure was stopped at this stage.

Step 5 $(NH_4)_2SO_4$ extraction (55 %).

The supernatant (1.4 L) from Step 4 was treated with ammonium sulfate to 70% saturation (472 g $(NH_4)_2SO_4$/L supernatant). The precipitate which formed was separated by centrifugation and resuspended in 55% saturated ammonium sulfate solution (150 mL)

at pH 7.4. The 55% ammonium sulfate precipitate was collected by centrifuging at 17,700 x g for 30 min, dissolved in 50 mM phosphate buffer (105 mL), pH 7.4, and dialyzed against three changes of 3.5 L of 10 mM sodium phosphate buffer, pH 7.4.

Step 6 DE-52 anion exchange chromatography.

Half (90 mL) of the dialyzed enzyme solution was loaded onto a Whatman DE-52 cellulose column (5.5 x 23 cm) equilibrated with 10 mM phosphate buffer, pH 7.4, at a flow rate of 0.92 mL/min and washed with the starting buffer until the protein concentration was near baseline (approximately 200 mL of eluate collected) as monitored by absorbance at 280 nm. Amine oxidase was eluted with 30 mM sodium phosphate buffer, pH 7.4, again until the protein concentration reached baseline (1,360 mL of eluate), and then eluted with 100 mM sodium phosphate buffer (340 mL), pH 7.4. Enzyme activity was found in the 30 mM and 100 mM eluate as well as in the 10 mM wash. The remaining half (90 mL) of the dialyzed enzyme solution from Step 5 was loaded onto a Whatman DE-52 cellulose column and treated the same way as the first half of the dialyzed enzyme solution. High activity fractions from all eluates were pooled together into two groups according to specific activity (1.32×10^{-2} and 1.03×10^{-2} units/mg), concentrated by ultrafiltration (PM-30 membrane), and used in the stereochemical and kinetic studies. Low activity fractions from the 30 mM and 100 mM eluates were combined (3.12×10^{-3} units/mg), concentrated, and purified further. The final concentrated solution was blue in color, suggesting that ceruloplasmin was also present in the sample.

Step 7 Sephacryl S-300 SF (gel filtration) column #1.

Further purification of the low activity fraction was carried out as follows: The enzyme solution (8 mL) was loaded onto a Sephacryl S-300 SF column (2.5 x 100 cm) which had been equilibrated with 10 mM potassium phosphate buffer, pH 7.4, and was eluted with the same buffer at a flow rate of 0.55 mL/min. The eluates with activity (105 mL) were concentrated to 10 mL, dialyzed against 50 mM potassium phosphate buffer containing 100 mM KCl, pH 7.0.

Step 8 Blue Sepharose CL-6B (affinity) column.

The blue dye (Cibacron Blue F3GA) ligand has an affinity for enzymes that bind to purine nucleotides due to structural similarity between the two types of molecules. Albumin is also known to bind to the dye perhaps through electrostatic and hydrophobic interactions with the aromatic anionic ligand.

The eluates from Step 7 were applied to a Blue Sepharose CL-6B column (2.5 x 50 cm), and eluted with the starting buffer. The eluates with activity (50 mL) were pooled together, dialyzed against 10 mM potassium phosphate buffer, pH 8.0.

Step 9 AH-Sepharose column.

The dialyzed solution was loaded onto an AH-Sepharose column (2.5 x 23 cm). The column was washed with the starting buffer, and eluted with 45 mM, 55 mM, and gradient (50-300 mM) potassium phosphate buffer, pH 8.0. Activity was found in both the 45 mM (75 mL) and gradient eluates at approximately 75 mM (140 mL). The eluates with activity were combined and concentrated to 10 mL, 1 mL (approx. 1 unit) of the high activity fraction was added, samples were centrifuged to remove precipitate.

Step 10 Sephacryl S-300 SF (gel filtration) column #2.

The solution from Step 9 was loaded again onto a Sephacryl S-300 SF column (2.5 x 100 cm) equilibrated with 100 mM potassium phosphate buffer, pH 7.4. The enzyme was eluted with the starting buffer. Eluates (60 mL) from fractions #57 to #68 (5 mL/fraction) showed activity with elution volume of 280 mL.

Step 11

With the exception of Sephacryl column #1, SDS-PAGE was carried out on the eluates from DE-52 column to Sephacryl column #2, as described in Step 8 of Section 2.1.2.2.

2.3 Stereochemistry of Sheep Serum Amine Oxidase (SSAO) Reactions

2.3.1 Materials

Tyramine was purchased from Eastman Kodak Company (Rochester, NY). NADH (disodium salt, trihydrate), alcohol dehydrogenase from equine liver (1.6 units/mg) and catalase from bovine liver (11,000 units/mg) were obtained from Sigma Chemical Company (St. Louis, MO). (1*S*)- and (1*R*)-[^{2}H]tyramine were available from previous stereochemical studies (Coleman *et al.*, 1989). They were prepared by incubation of DL-[2-^{2}H]tyrosine with tyrosine decarboxylase (TDC) in protonated buffer or L-tyrosine with TDC in deuterated buffer. Deuterated buffer was prepared by making protonated buffer of the desired pH (7.0), evaporating solvent under vacuum, and dissolving the dried buffer salts in exactly the same volume of D_2O as the evaporated H_2O. The final pD of the buffer solution was 7.2, where pD refers to the uncorrected pH meter reading of the deuterated buffer. SSAO was exchanged into deuterated buffer by concentrating SSAO to 0.5 mL by ultrafiltration and resuspending in 0.5 mL of 100 mM potassium phosphate buffer, pD 7.2, and 5 mL of ^2H_2O. Concentration and resuspension were carried out three times to ensure the complete exchange of solvent.

2.3.2 Methods

All stereochemical studies were carried out in a coupled fashion by the method of Battersby *et al.* (1979) and Coleman *et al.* (1989) with slight modifications. The reaction was coupled to convert reactive aldehyde to stable alcohol and also to monitor the progress of the reaction by measuring decrease in absorbance at 340 nm.

2.3.2.1 Oxidation of monodeuterated tyramines

The following components were incubated in 1.0 mL of 100 mM potassium phosphate buffer, pH 7.2, at 25°C: 15-20 μmol of stereospecifically deuterated tyramine

(1*S*- or 1*R*-), sheep serum amine oxidase (0.3 units), 25-30 μmol NADH, 5-8 units of alcohol dehydrogenase, and 11,000 units of catalase. The conversion of amine to alcohol was monitored by the removal of aliquots (5 μL) at timed intervals, the aliquots were diluted with 995 μL of the buffer, and $\Delta Abs_{340\ nm}$ was measured.

At 50 or 100% completion of reaction, the incubation mixtures were diluted to 10 mL with water and loaded onto reverse-phase C_{18} Sep-pak cartridges which had been washed with 10 mL of methanol and 20 mL of water before use. The cartridges were rinsed with 5 mL of water, then the alcohol product was eluted with 10 mL of methanol. The methanol eluate was concentrated under reduced pressure, dissolved in 2 mL of ethyl acetate:*n*-hexane (2:1 v/v), and applied to a flash silica column (2g; 1 x 25 cm) which had been prewashed with 10 mL of ethyl acetate:*n*-hexane (2:1 v/v). The alcohol product (tyrosol) was eluted with 20 mL of the same solvent and collected in culture tubes (approx. 0.7 mL/fraction). This step effectively removed any remaining amine and NADH which remained bound to the column.

Aliquots of all the fractions were spotted on silica gel TLC plates, developed with the same solvent (R_f = 0.51 for *p*-hydroxyphenethyl alcohol), and the fractions containing the product were pooled. The tyrosol solution was concentrated to dryness under reduced pressure, dissolved in a solvent of $(^2H_3C)_2CO$: ^2H_2O (4:1 v/v), and ^1H NMR spectra were measured at 360 MHz on a Bruker WM-360 instrument operating at ambient temperature (22±1°C). The deuterium contents were estimated by integration of the ^1H NMR spectra. All stereochemical studies were done in triplicate.

2.3.2.2 Solvent exchange into product

To monitor solvent exchange into alcohol, incubations were carried out as described for the stereochemical studies with the chiral amines (Section 2.3.2.1), except that reactions were in 100 mM deuterated potassium phosphate buffer, pD 7.2, containing 15-20 μmol of unlabeled tyramine.

2.4 Kinetic Isotope Effects

2.4.1 Steady-state kinetics for benzylamine oxidation

2.4.1.1 Materials

Benzylamine was purchased from Sigma Chemical Company (St. Louis, MO). [α,α-^{2}H]benzylamine was synthesized by Dr. C. Swyngedouw in our laboratory by reduction of benzonitrile using $LiAl^2H_4$ in anhydrous tetrahydrofuran (Bardsley *et al.*, 1973). The sheep serum amine oxidase used for the kinetic studies was the enzyme prepared in Section 2.2.2.2.

2.4.1.2 Methods

Rucker and Goettlich-Riemann (1972) reported a K_m of 0.1 mM for benzylamine oxidation catalyzed by sheep serum amine oxidase. Therefore, six concentrations of substrate, ranging from 0.025 mM to 1.0 mM of either unlabeled or deuterated benzylamine, were used in the kinetic experiments. Initial rate measurements were carried out in a total volume of 0.99 mL containing 100 mM potassium phosphate buffer, pH 7.0, and substrate equilibrated to 25°C. Reaction was initiated by the addition of 10 μL of SSAO (0.012 units). The initial rate of reaction was estimated spectrophotometrically by monitoring the production of benzaldehyde, which results in an increase in absorbance at 250 nm. All kinetic experiments were done in duplicate.

The initial rate data obtained for the various substrate concentrations was fitted to the expression:

$$v = V_{max}[S] / (K_m + [S]) \qquad (2\text{-}1)$$

with a FORTRAN program based on the Cleland method (Cleland, 1979), where v is initial rate, V_{max} is maximal velocity, [S] is substrate concentration and K_m is Michaelis-Menten constant corresponding to the substrate concentration at half of the maximum velocity.

2.4.2 Steady-state kinetics for tyramine oxidation

2.4.2.1 Materials

Tyramine was purchased from Eastman Kodak Company (Rochester, NY). [α,α-^{2}H]Tyramine was synthesized by Dr. C. Swyngedouw in our laboratory by reduction of 4-(methoxyphenyl)acetonitrile using $LiAl^2H_4$ and subsequent deprotection of methoxyphenethylamine with HBr (Bardsley *et al.*, 1973, and Palcic *et al.*, 1983). Alcohol dehydrogenase (horse liver) and catalase (bovine liver) were obtained from Sigma Chemical Company (St. Louis, MO).

2.4.2.2 Methods

Based on the report of Palcic and Klinman (1983) that tyramine exhibited biphasic steady-state plots yielding K_m values of 1.3 mM and 52 mM for bovine plasma amine oxidase, six substrate concentrations ranging from 5 mM to 80 mM of unlabeled and deuterated tyramine were used for the sheep serum amine oxidase studies.

The rate of oxidation of tyramine to aldehyde catalyzed by SSAO was measured spectrophotometrically using a coupled enzyme system containing alcohol dehydrogenase (ADH) and NADH. In this coupled system, the 4-hydroxyphenylacetaldehyde produced by the amine oxidase is converted directly to 4-hydroxyphenethylalcohol (tyrosol) by alcohol dehydrogenase. NADH (molar absorptivity of 6.22×10^3 M^{-1} cm^{-1} at 340 nm) is converted to NAD^+ in the system, resulting in a decrease in absorbance at 340 nm.

Initial rate measurements were carried out in a total volume of 0.96 mL containing 100 mM potassium phosphate buffer, pH 7.0, and substrate equilibrated to 25°C. Reaction was initiated by the addition of 40 μL of SSAO (0.04 units). The initial rate data obtained for the various tyramine concentrations was fitted to Equation (2-1), as described in Section 2.4.1.2. All kinetic experiments were done in duplicate.

3. RESULTS AND DISCUSSION

3.1 Isolation of Porcine Plasma Amine Oxidase

A summary of the purification of porcine plasma amine oxidase is given in Table 3.1. The increase in enzyme purity at the various isolation steps is reflected in increasing specific activity (units/mg). The enzyme was purified 1,360 fold with an overall recovery of 25%.

All steps were carried out according to the literature (Falk *et al.*, 1983; Coleman *et al.*, 1989), except for the inclusion of AH-Sepharose chromatography, which had not been tried in the isolation of porcine plasma enzyme. The AH-Sepharose column was a new step introduced to modify the preparation, so serial elution with phosphate buffer of varying ionic strengths (45, 55, 75 and 100 mM) was attempted on AH-Sepharose. The resultant specific activity of 0.11 units/mg was an improvement compared to the 0.057 units/mg reported by Coleman *et al.* (1989) and equivalent to that of Falk *et al.* (1983), while the yield (25%) was much higher than that of the latter workers (5.3%).

The results of the gel electrophoresis at each purification step are shown in Figure 3.1. All gels were 11 % acrylamide. Approx. mol. wt. estimated from the gels of PPAO, which is a dimer, is 194,000 and this is in good agreement with values in literature (Falk *et al.*,1983). The final enzyme is highly purified as estimated by electrophoresis. This enzyme has been used by Ms. A. Coleman in our laboratory in stereochemical studies of the C-2 exchange process.

3.2 Isolation of Sheep Serum Amine Oxidase

A summary of the purification procedure is given in Table 3.2. The enzyme with the highest purity was purified about 24 fold. While the 40% saturated ammonium sulfate extraction was found to be the most effective purification step (6-fold increase in specific activity, compared with one after previous step), the calcium phosphate absorption step turned out to be disappointing. The concentrated enzyme solution after Step 6, the DE-52

Table 3.1 Purification of porcine plasma amine oxidase.

Purification Step	*Units*[a]	*Specific activity (units/mg)*	*Purification (fold)*	*Yield (%)*
Serum	60	8.1×10^{-5}	1	100
$(NH_4)_2SO_4$ fractionation (35-55%)	42	2.3×10^{-4}	3	70
DE-52 30 mM	17.2	3.2×10^{-4}	4	61
100 mM	19.4	6.7×10^{-4}	8	
Con A	34.2	5.9×10^{-3}	73	57
AH-Sepharose	24.9	1.6×10^{-2}	200	42
Hydroxyapatite	23.2	5.6×10^{-2}	690	38
Bio-Gel A 1.5 m	15.5	1.1×10^{-1}	1360	25

[a] 1 unit = 1 μmol product/min at 25°C

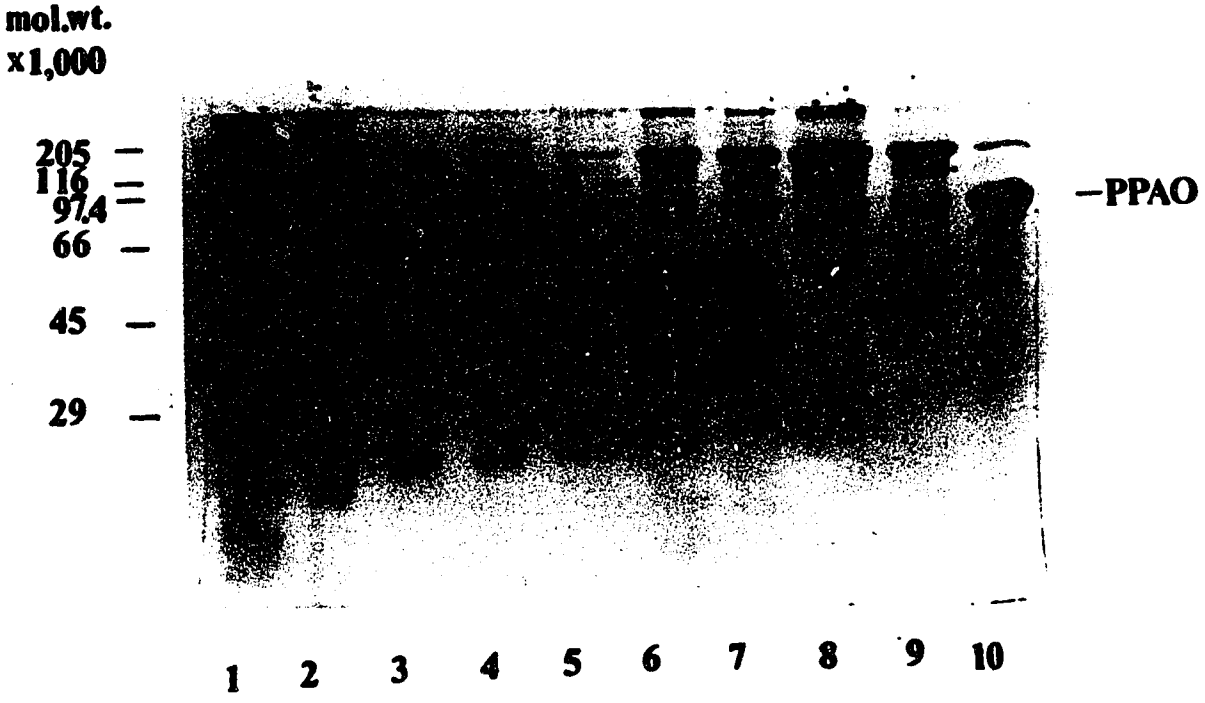


Figure 3.1. SDS-polyacrylamide gel electrophoresis of eluates after different purification steps in the porcine plasma amine oxidase isolation.

1. Standard (HMW)
2. Serum
3. & 4. Eluates of the DE-52 column
5. & 6. Eluates of the Con A column
7. Eluates of the AH-Sepharose column
8. & 9. Eluates of the Hydroxyapatite column
10. Eluates of the Bio-Gel A 1.5 m column

Table 3.2 Purification of sheep serum amine oxidase.

Purification Step		*Units*[a]	*Specific activity (units/mg)*	*Purification (fold)*	*Yield (%)*
Serum		139.0	5.5×10^{-4}	1	100
$(NH_4)_2SO_4$ fractionation (30-65%)		114.0	7.2×10^{-4}	1.3	82
$(NH_4)_2SO_4$ extraction (40%)		91.1	4.4×10^{-3}	8	66
Calcium phosphate gel adsorption		85.8	6.0×10^{-3}	11	62
$(NH_4)_2SO_4$ extraction (55%)		59.2	8.8×10^{-3}	16	43
DE-52 anion exchanger					
high activity fraction	1	19.8	1.3×10^{-2}	24	34
	2	23.0	1.0×10^{-2}	18	
low activity fraction		4.6	3.1×10^{-3}	6	

[a]1unit = 1μmol product/min at 25°C

anion exchange chromatography, was stored at 4°C for 2 months without any significant loss of activity. Steps up to #5 (Section 2.2.2.2) were according to the literature (Rucker *et al.*, 1972). DE-52 chromatography and the subsequent chromatographic steps were introduced by us. The SDS-PAGE of the low activity DE-52 eluate (Figure 3.2) shows that the major contaminating proteins are albumin with a mol.wt. of approx. 66,000 and a 45,000 band. Hemoglobin (16,000) is also present in the sample and three high mol. wt. bands at approx. 97,000, 116,000 and 130,000 are evident. The minor band at mol. wt. approx. 97,000 is the enzyme, SSAO, based on the fact that the relative band intensity of mol. wt. 97,000 increased with improved specific activity of the enzyme solution. The low activity fraction was subjected to an additional series of chromatographic steps. The results of these steps are summarized in Table 3.3.

The Sephacryl S-300 SF (gel filtration) column was chosen to try to remove hemoglobin, albumin and the 45,000 dalton band, all of which have a lower mol. wt. than SSAO. The resolution of proteins was poor on the initial Sephacryl S-300 SF column and there was only a marginal increase in specific activity after this step. Chromatography on Blue Sepharose CL-6B was carried out on the combined fractions since this resin has been reported to adsorb albumin (Travis *et al.*, 1976). The gel in Figure 3.3, which shows sequential fractions from loading and washing of the Blue Sepharose column, demonstrates partial removal of both albumin and the 45,000 dalton band. However, this would not be a practical method of removing albumin. Con A remains the method of choice for removing albumin and hemoglobin, which are not glycosylated, from glycoproteins such as SSAO.

All fractions with activity (#27-35) were combined and applied to an AH-Sepharose column. AH-Sepharose was used because it is an affinity support for other amine oxidases, such as bovine (Svenson and Hynning, 1981) and porcine (Section 2.1) AO . These steps for the further purification of SSAO had not been attempted before elsewhere, and some proved to be unsatisfactory. For instance, the poor resolution attained from the first

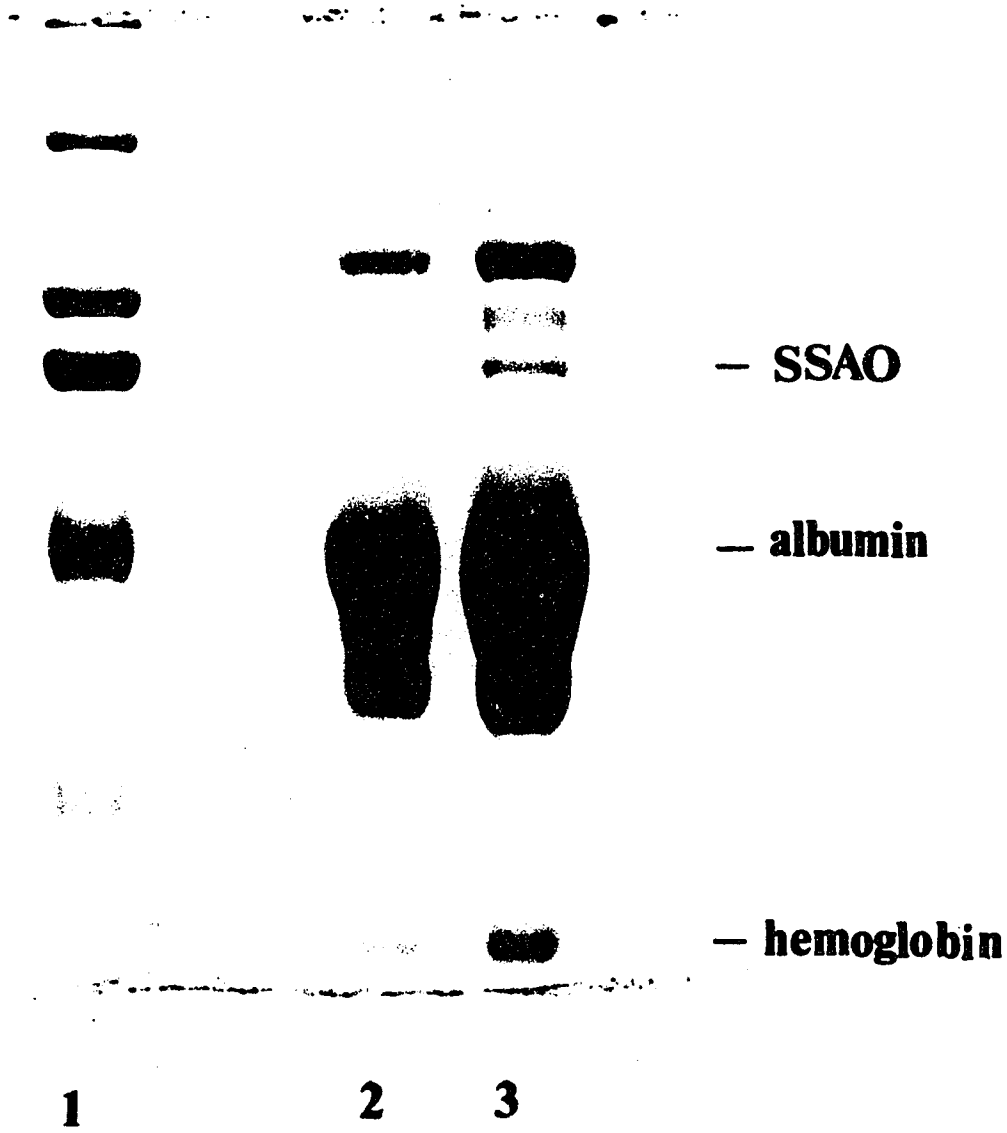


Figure 3.2. SDS-PAGE of low activity fraction from DE-52 of the sheep serum amine oxidase isolation.
1. Standard
2. & 3. Low activity fraction

Table 3.3 Further purification of SSAO.

Purification Step	*Units*	*Specific activity (units/mg)*	*Purification (fold)*	*Yield (%)*
DE-52 eluate (low activity fraction)	2.8	3.1×10^{-3}	1	100
Sephacryl S-300 SF #1	2.4	4.4×10^{-3}	1.4	86
Blue Sepharose CL-6B	1.8	1.1×10^{-2}	3.5	64
AH-Sepharose	1.2*	3.7×10^{-2}	12	43
Sephacryl S-300 SF #2	1.1	1.2×10^{-1}	39	29

* 1 mL (= 1 unit) of high activity fraction was added

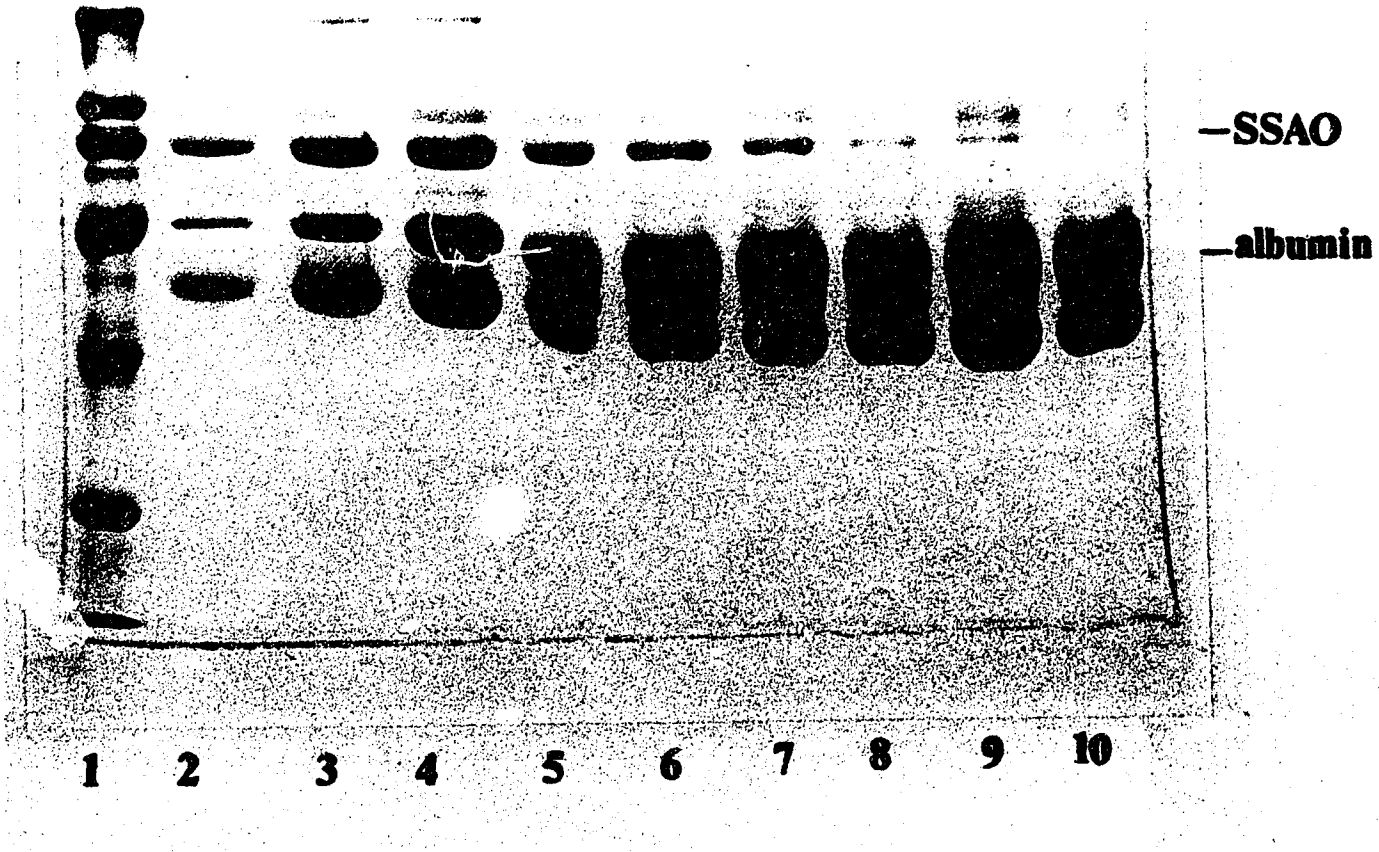


Figure 3.3. SDS-PAGE of Blue Sepharose eluates.

1. Standard
2. - 10. Fraction #27 → Fraction #35

Sephacryl S-300 column might be due to the low ionic strength (10 mM) of the buffer used. Eluates of the Blue Sepharose column at an early stage showed good resolution, while dimi[illegible] resolution towards the later stage of elution indicated that insufficient quantity of [illegible]harose CL-6B) was used (Figure 3.3). The cost of a larger column is pro[illegible]50 for 50 g of dry resin for the 2.5 x 50 cm column used in this preparation). [illegible] electrophoresis gels are included to illustrate the progress of the purification procedure. They are not of high quality due to an effort to make the SSAO band visible thereby resulting in considerable smearing of the major protein bands.

Though the AH-Sepharose column worked well, with a 3.4-fold increase in specific activity compared with the eluate from the Blue Sepharose column, the specific activity of the enzyme solution (0.037 units/mg) indicated that it might still contain a significant amount of contaminating protein. Therefore, the eluate of the AH-Sepharose column was rechromatographed onto the same Sephacryl S-300 SF column equilibrated with 100 mM buffer. The second gel filtration column gave an excellent resolution with a 3.2- fold purication. The high resolution of this column, compared with the first gel filtration column (10 mM buffer applied), might be due to the high ionic strength (100 mM) of the buffer. The Sephacryl gel contains carboxyl groups and thereby partial ionic interactions between the matrix and proteins can occur at low ionic strength. The specific activity of the final enzyme was 0.12 units/mg, which is the highest purification ever reported for SSAO.

Based on the results in this thesis a proposed scheme for the isolation of SSAO is: $(NH_4)_2SO_4$ fractionation (35-55%), then successive chromatography on DE-52 cellulose, Con A-Sepharose, hydroxyapatite and a Sephacryl S-300 SF column equilibrated with 100 mM potassium phosphate buffer, pH 7.4. It is suggested that the Con A-Sepharose column and hydroxyapatite column should be equilibrated and run as described for the porcine plasma amine oxidase, while the other steps are identical to those described for SSAO. An outline of the proposed isolation scheme for SSAO is given in Figure 3.5.

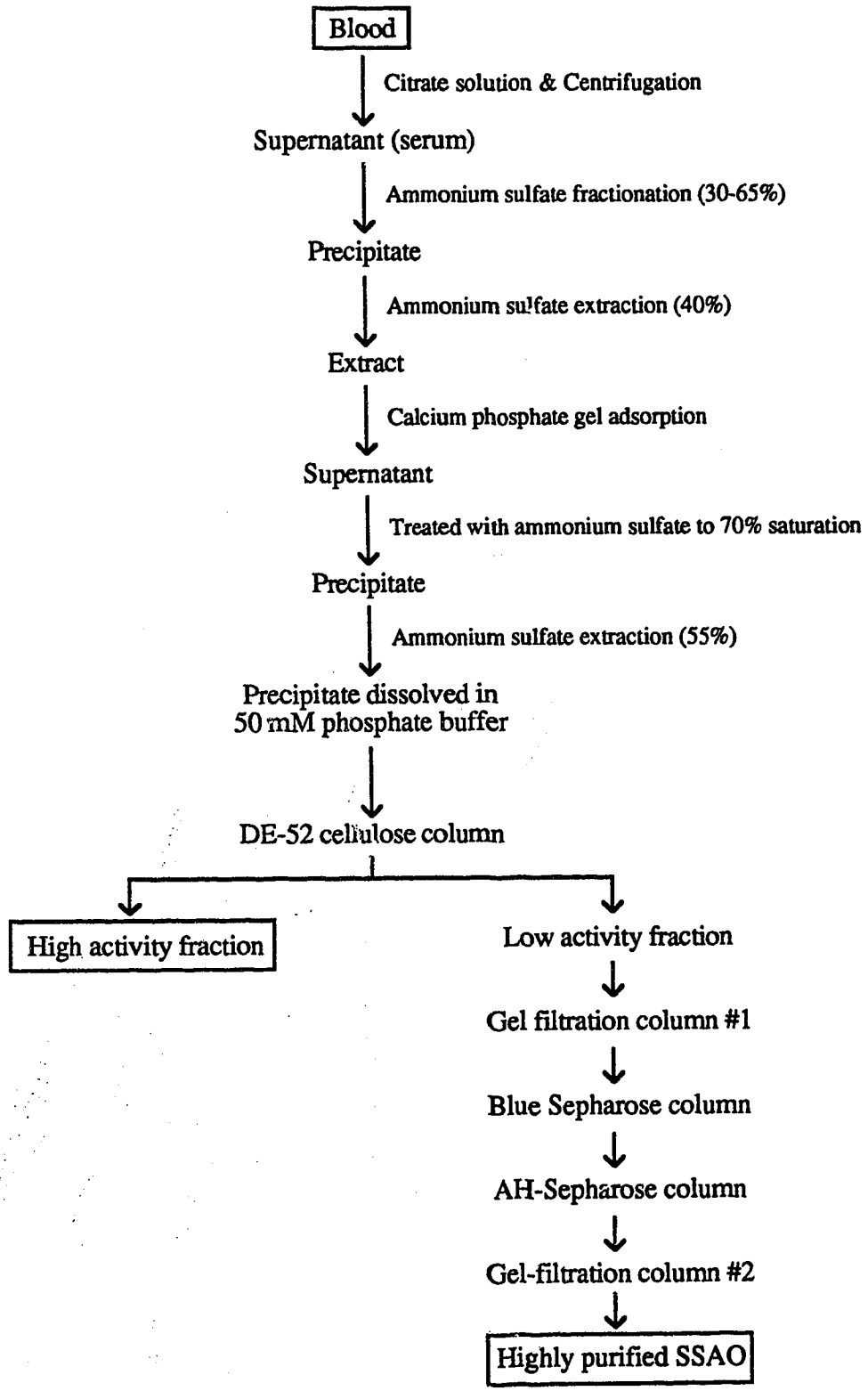


Figure 3.4. Outline of sheep serum amine oxidase isolation.

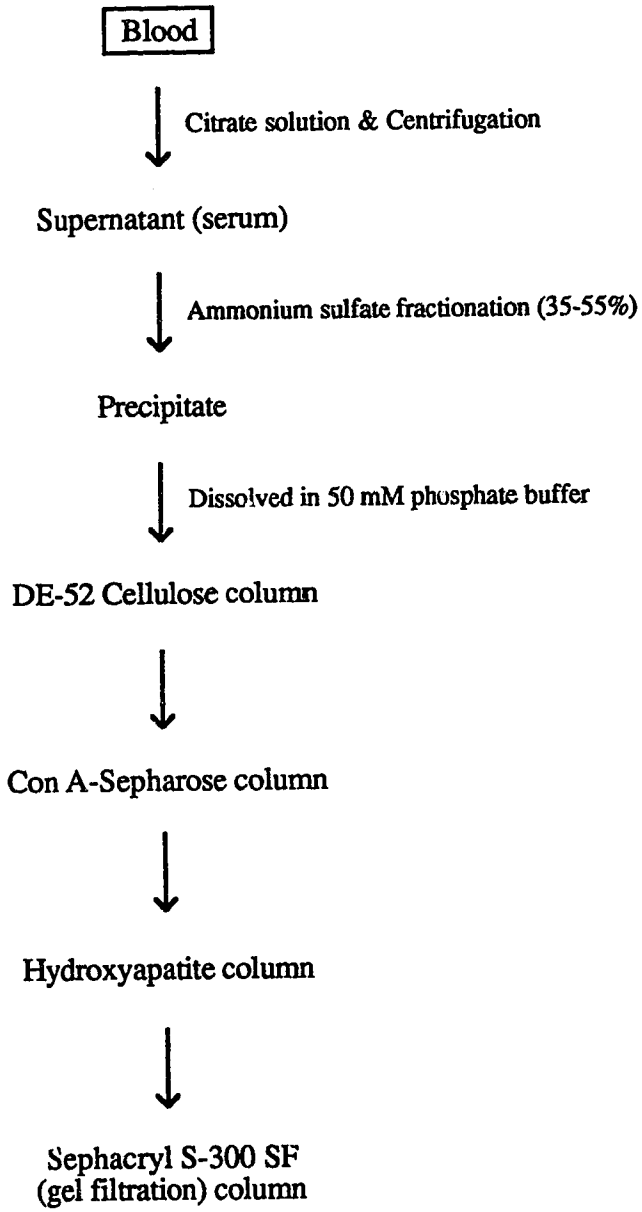


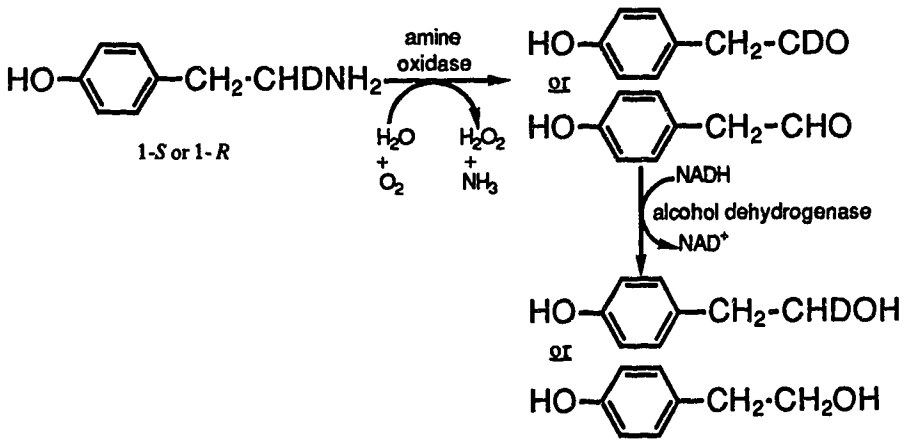
Figure 3.5. The proposed isolation scheme for SSAO.

3.3 Stereochemistry of Sheep Serum Amine Oxidase Reactions

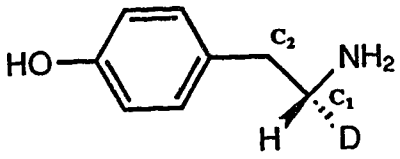
3.3.1 Oxidation of monodeuterated tyramines

Figures 3.6 and 3.7 show that the ^1H NMR spectra of stereospecifically deuterated tyramines isolated after incubation of L-tyrosine in ^2H_2O and DL-[2-^{2}H]tyrosine in H_2O, respectively, with tyrosine decarboxylase. The clean doublets at 2.89 ppm show that the deuteration was complete within the limits of sensitivity of the ^1H NMR spectra (≥98%) and the absolute configuration of the chiral tyramines are assigned based on the established stereospecificity of the tyrosine decarboxylase reaction with retention of configuration (Palcic and Floss, 1986).

Stereochemical studies with both chiral amines (1*S*- or 1*R*-tyramine) and sheep serum amine oxidase were carried out in a coupled manner such that the *p*-hydroxyphenyl acetaldehyde produced was reduced directly to *p*-hydroxyphenethyl alcohol by the action of alcohol dehydrogenase (ADH) and NADH.



Scheme 3-1



(1R)-[^{2}H]Tyramine

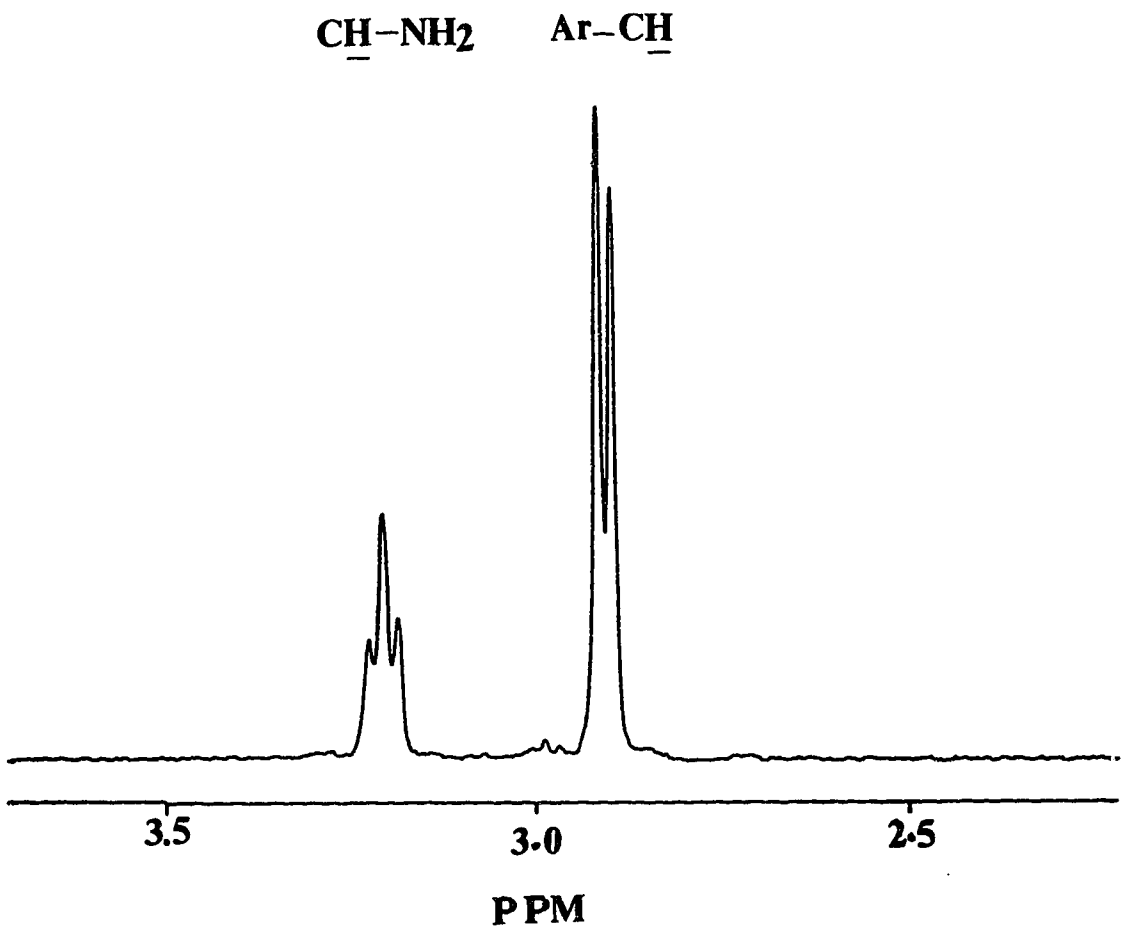
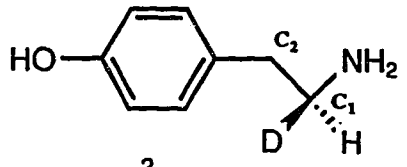


Figure 3.6. Partial 360 MHz ^{1}H NMR spectrum of (1*R*)-[^{2}H]tyramine derived from incubation of tyrosine decarboxylase with L-tyrosine in ^2H_2O.



(1S)-[^{2}H]Tyramine

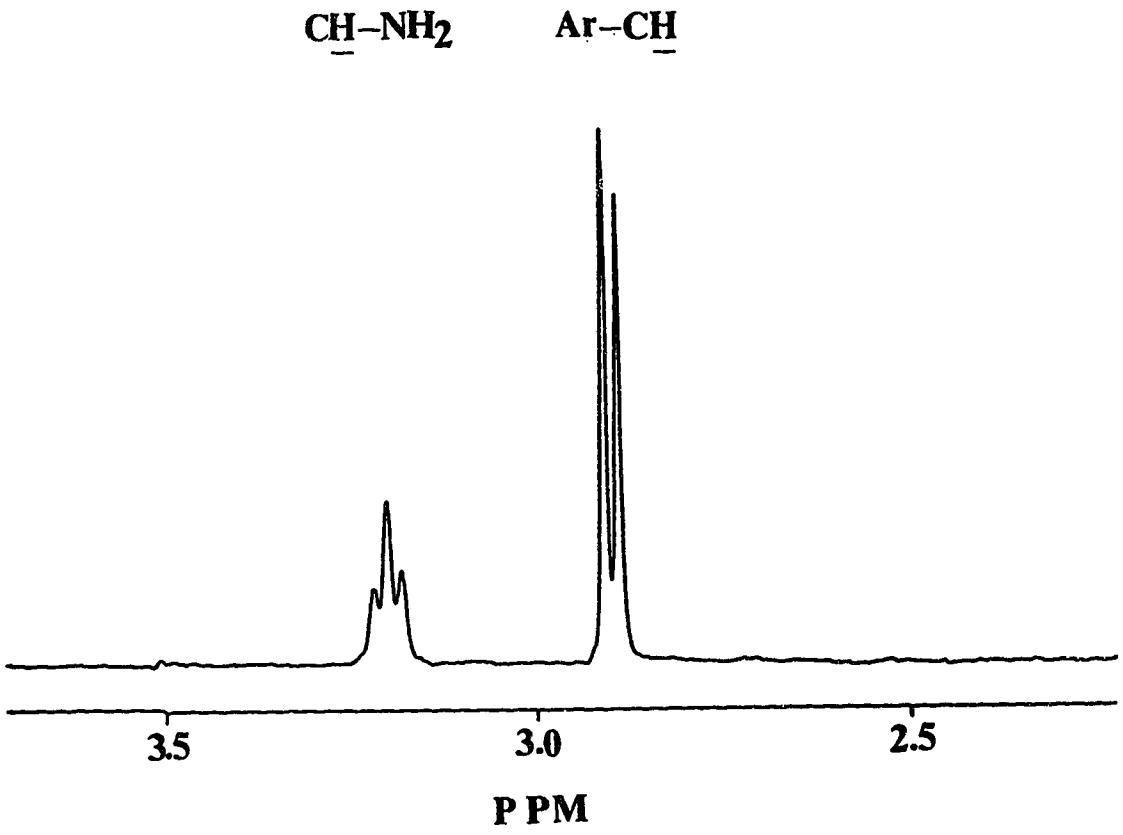


Figure 3.7. Partial 360 MHz ^{1}H NMR spectrum of (1*S*)-[^{2}H]tyramine derived from incubation of tyrosine decarboxylase with DL-[2-^{2}H]tyrosine in H_2O.

Figure 3.8 shows the ^1H NMR spectrum of *p*-hydroxyphenethyl alcohol (tyrosol) isolated from incubations with (1*S*)-[^2H]tyramine and sheep serum amine oxidase after the reaction was >90% complete. The spectral pattern shows a mixture of two alcohol products, one fully protonated and the other monodeuterated, with the latter being the major product. The pattern for this mixture is two overlapping triplets for the C-1 proton signals near 3.7 ppm and a doublet superimposed on a triplet for the C-2 protons near 2.8 ppm. The signals labeled N near 4.0 ppm are due to residual NADH, while that labeled X near 2.8 ppm is due to an unidentified impurity.

The minor triplet at 3.74 ppm arises from the product alcohol that is fully protonated at C-1. The major triplet at 3.72 ppm is from tyrosol that is monodeuterated at C-1. This signal is shifted about 0.02 ppm to higher field due to an α-deuterium isotope effect (Hansen, 1983). The signals for the C-2 protons are a triplet at 2.79 ppm exhibited by the minor product (fully protonated tyrosol). For the major product (monodeuterated tyrosol), a doublet for the C-2 protons is at 2.78 ppm, shifted upfield due to a ß-deuterium isotope effect. In this case, 89% of the deuterium of the original tyramine was retained in the product alcohols and 11% was lost, indicating that oxidation of the *S*-amine occurred in a partially nonstereospecific fashion.

The spectrum of the *p*-hydroxyphenethyl alcohol obtained from incubation of (1*R*)-[^2H]tyramine with sheep serum amine oxidase is shown in Figure 3.9. This sample also consists of a mixture of monodeuterated and fully protonated alcohols, with the monodeuterated product predominating. In this instance, 72% of the deuterium was retained in the product, indicating that reaction occurred in a partially nonstereospecific manner. These stereochemical studies demonstrate that sheep serum amine oxidase exhibits the rare mirror-image bindings and catalysis reported for bovine plasma amine oxidase (Summers *et al.*, 1979; Farnum *et al.*, 1986).

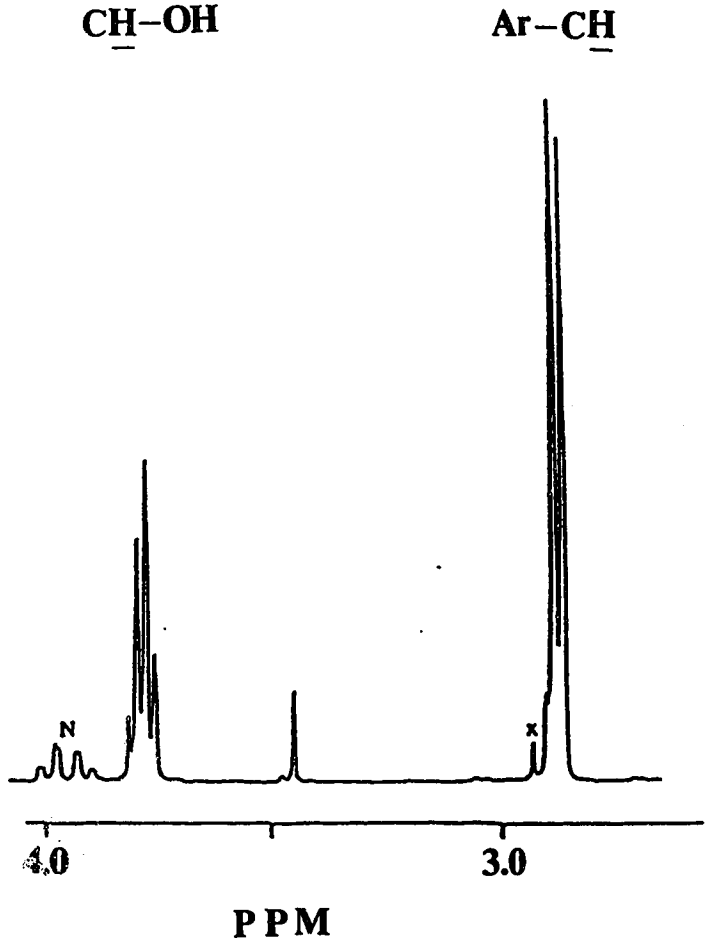


Figure 3.8. The ^{1}H NMR spectrum of alcohol derived from coupled incubation of (1*S*)-[^{2}H]tyramine and sheep serum amine oxidase with NADH and alcohol dehydrogenase.

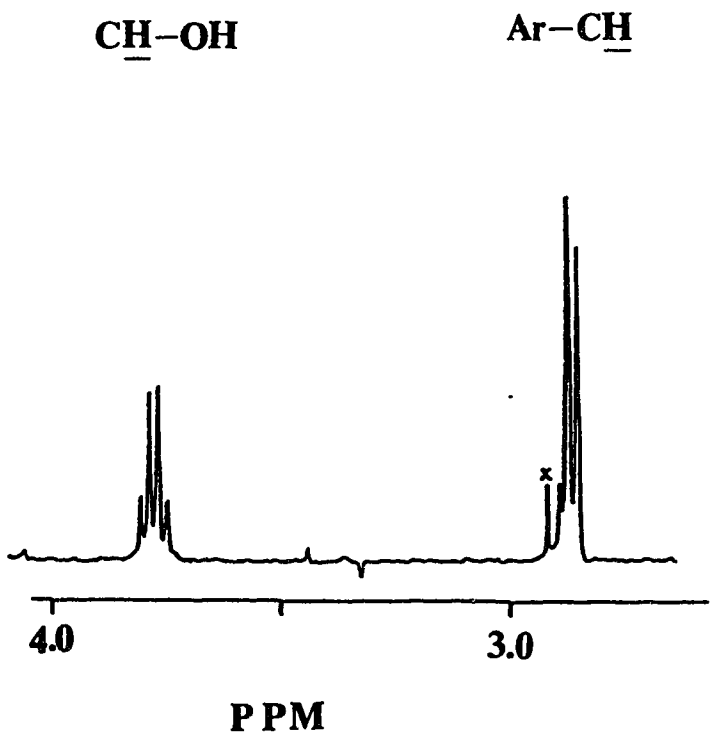
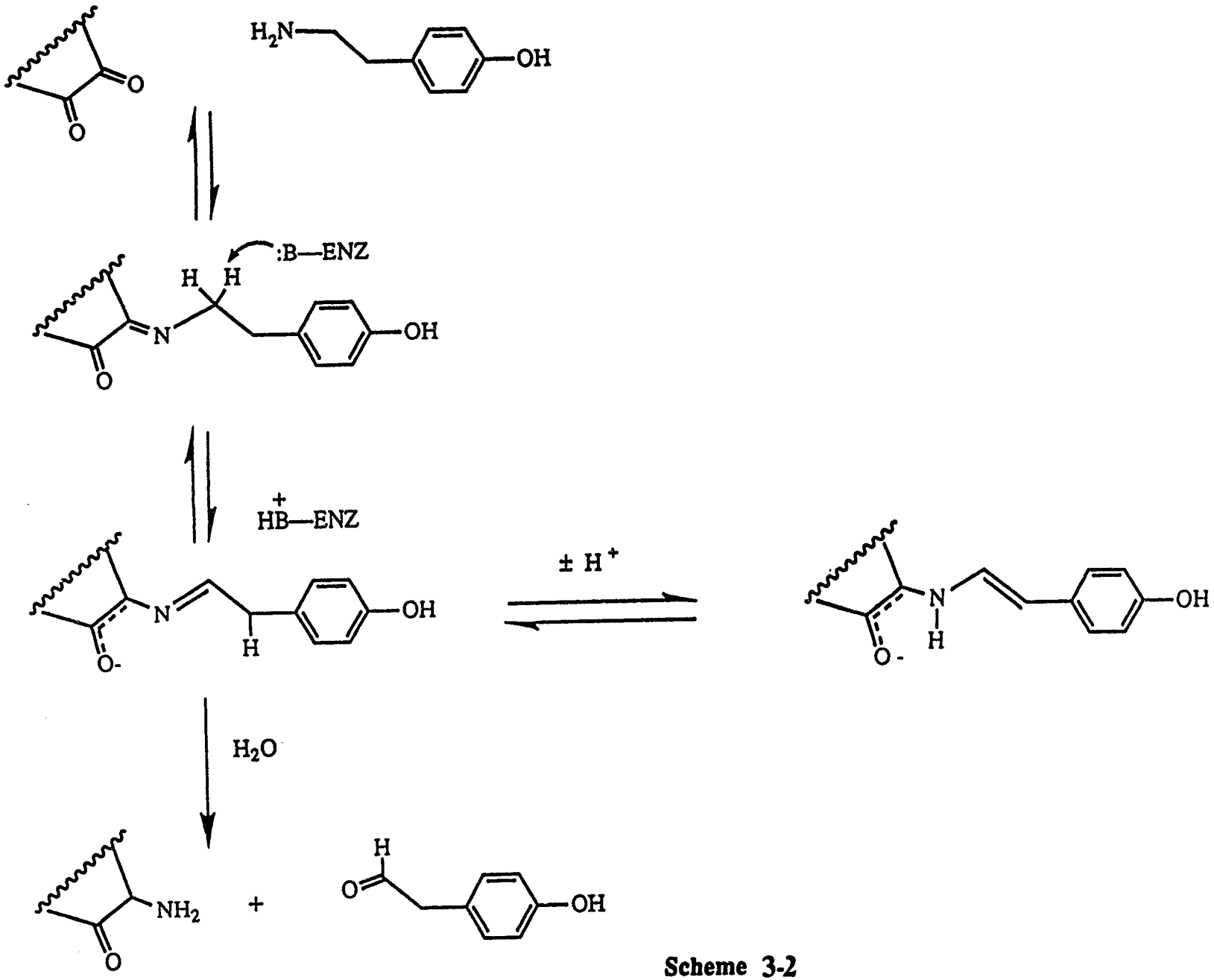


Figure 3.9. The ^{1}H NMR spectrum of alcohol derived from coupled incubation of (1*R*)-[^{2}H]tyramine and sheep serum amine oxidase with NADH and alcohol dehydrogenase.

A completely nonstereospecific reaction arising from equal flux through two catalytically competent modes, defined as pro-*S* or pro-*R* abstraction of hydrogen at C-1, would give a 1:1 mixture of monodeuterated and fully protonated alcohols. Any deviations from a ratio of 50/50 indicate preferred reaction through either the pro-*R* or pro-*S* abstraction mode. Preferred reaction through a mode can arise from a kinetic isotope effect (KIE) where the isotope effect results in a reduction in the rate of C-D bond cleavage relative to C-H bond cleavage. If a KIE of 6 is observed, the rate of reaction is 6-fold greater for C-H bond cleavage vs C-D bond cleavage. This would give products enriched in deuterium, i.e. 86% deuterated and 14% protonated. Differences in the deuterium content of the samples derived from (1*R*)- or (1*S*)-[^{2}H]tyramines arise from differences in the isotope effects in each mode.The KIE's estimated from the observed product ratios for the sheep serum amine oxidase are 8.1 for the *S* mode and 2.6 for the *R* mode. The reduced KIE for (1*R*)-tyramine indicates that a step other than C-H bond cleavage is partially rate-limiting in the *R* mode, which reduces the observed isotope effect.

An aminotransferase mechanism has been proposed for the copper amine oxidases, with initial Schiff base formation between the cofactor carbonyl and substrate amine (Scheme 3-2) (Farnum and Klinman, 1986). This is followed by base-catalyzed proton abstraction from C-1 of substrate such that the cofactor functions as an electron sink during catalysis, analogous to pyridoxal phosphate. Imine hydrolysis yields product aldehyde and an amino quinol as the reduced cofactor species (Hartman *et al.*, 1988).

For bovine plasma enzyme, the reduction in the observed isotope effect for the *R* mode has been attributed to a partially rate-limiting conformational change prior to imine hydrolysis. This reorientation does not occur in the *S* mode, hence the isotope effect is more fully expressed for these substrates.



Scheme 3-2

A summary of the stereochemical studies is shown in Table 3.4. A different KIE can be expressed for V_{max} (saturated substrate levels) or V_{max}/K_m conditions (sub-saturating substrate concentrations). If there is a difference in KIE for V_{max} and V_{max}/K_m, and the reaction goes from V_{max} to V_{max}/K_m control, there will be a difference in deuterium content in products isolated as a function of reaction progress. However, Table 3.4 shows that the amount of deuterium in products is constant for all tyrosols isolated after 50% reaction. This suggests that the KIE is the same for V_{max} and V_{max}/K_m or that the K_m for tyramine is high and reaction was under V_{max}/K_m control throughout.

3.3.2 Solvent exchange into product

Previous studies have reported that a solvent exchange pathway exists off the main catalytic pathway for several amine oxidases (Coleman *et al.*, 1989; Farnum *et al.*, 1986). This is attributed to reversible enamine formation after C-1 proton abstraction, thereby allowing for exchange of solvent into C-2 of product aldehydes (Scheme 3-2).

Figure 3.10 shows the spectrum of the alcohols obtained by incubation of sheep serum amine oxidase with fully protonated tyramine in ^2H_2O. Solvent is incorporated into products, evidenced by the overlapping triplets near 2.7 ppm and a doublet superimposed on a triplet near 3.7 ppm. The amount of deuterium incorporated into C-2 of products in duplicate experiments was 92 and 94% when reaction went to completion (>90%). The C-1 proton abstraction in Scheme 3-2 should be regarded as an irreversible step since the spectrum (Figure 3.10) indicates that no deuterium in ^2H_2O was incorporated into C-1 of products. Studies by Coleman *et al.* (1989) showed no solvent exchange in the case of the pro-*S* specific amine oxidases such as porcine kidney amine oxidase and chick pea amine oxidase. The absence of exchange can be attributed to kinetic or steric constraints for these enzymes. Kinetically, imine hydrolysis and solvent exchange via enamine formation are competing reactions (Scheme 3-2). Enzymes for which the rate of hydrolysis is rapid relative to enamine formation would function without an exchange

Table 3.4. Characteristics of ^1H NMR spectra of alcohols derived from coupled incubations of chiral amines with SSAO.

	Alcohols from S-tyramine		*Alcohols from R-tyramine*	
	diprotonated	*monodeuterated*	*diprotonated*	*monodeuterated*
Chemical shifts at				
C-1	3.74	3.72	3.73	3.71
C-2	2.79	2.78	2.79	2.78
Deuterium (%) at				
50% conversion		91		74
>90% conversion		87		70
KIE		8.1		2.6

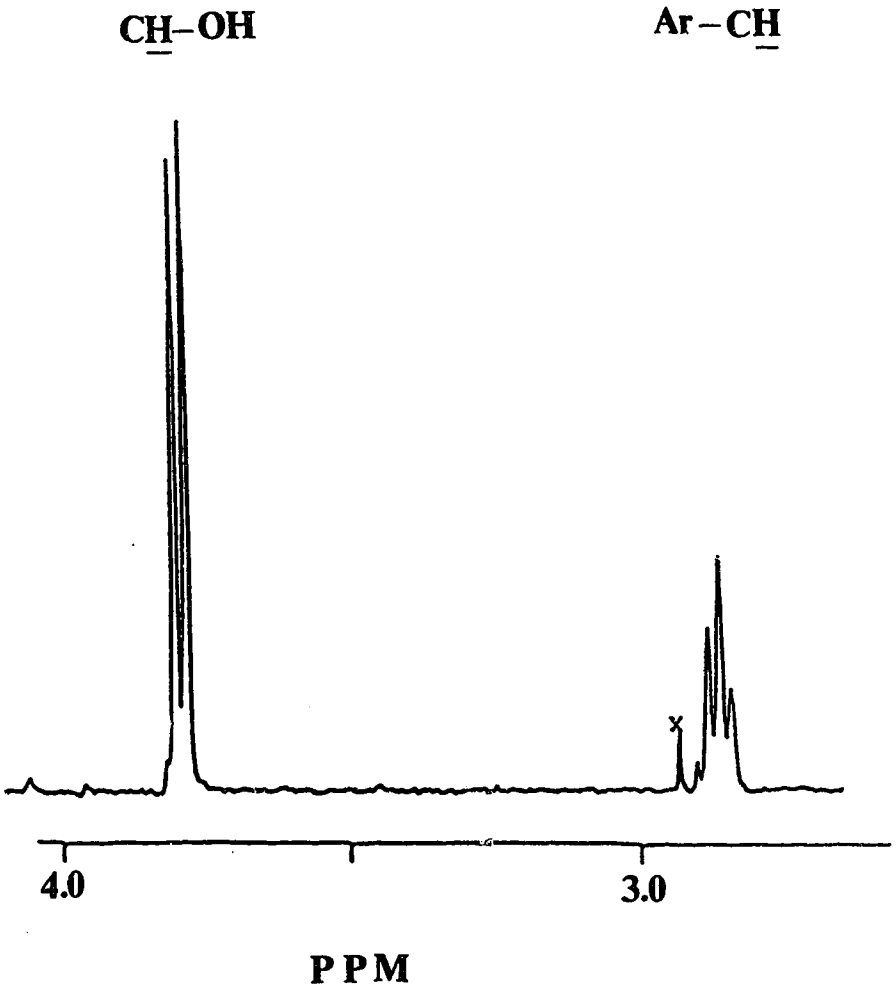


Figure 3.10. The ^{1}H NMR spectrum of alcohol derived from coupled incubation of sheep serum amine oxidase and tyramine in $^{2}H_2O$.

process. Alternatively, if enamine formation requires proton transfer to a residue of the enzyme active site, lack of a correctly positioned or unionized base to accept the proton would also preclude solvent exchange.

The nonstereospecificity of SSAO results from mirror-image binding and catalysis, which is a rare phenomenon in enzymology. There seems to be a correlation between the stereochemical course of proton abstraction at C-1 and a solvent exchange pathway into C-2 of products for all enzymes in the class. The pro-*R* specific and nonstereospecific enzymes have maintained the solvent wash-in pathway, while it has not been detected for any pro-*S* enzyme to date. More surprisingly, the modal preference for the nonstereospecific enzymes is uniform; the kinetic isotope effects for the pro-*R* abstraction mode are reduced relative to the pro-*S* mode for all nonstereospecific amine oxidases studied to date.

3.4 Kinetic Isotope Effects

The stereochemical studies with tyramine and sheep serum amine oxidase (SSAO) described in Section 3.3 demonstrated that two kinetically competent binding modes exist in the enzyme. One mode results in abstraction of the pro-*R* hydrogen of substrate, the other pro-*S* hydrogen abstraction. Furthermore, there is a difference in the kinetic isotope effect (KIE) for these modes, 8.1 for the *S* mode and 2.6 for the *R* mode. An isotope effect can be observed on V_{max} with substrate saturating and V_{max}/K_m with sub-saturating substrate concentrations. If a different KIE exists for V_{max} and V_{max}/K_m and the stereochemical studies are carried out starting with saturating substrate under V_{max} condition and shifted to V_{max}/K_m, the deuterium content in product can vary as a function of reaction extent.

To estimate the magnitude of KIE's, a series of steady-state kinetic measurements were carried out. The overall reaction catalyzed by SSAO occurs in two steps, as shown in Scheme 3-3 (Farnum *et al.*, 1986). This is a minimal kinetic scheme modeled on the bovine plasma amine oxidase reaction:

$$E_{ox}+RCH_2NH_3^+ \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_{ox}\bullet RCH_2NH_3^+ \underset{k_4}{\overset{k_3^*}{\rightleftharpoons}} E_{red}-N^+H=CHR \xrightarrow[H_2O]{k_5} E_{red}-NH_3^+(RCHO) \quad (3\text{-}1)$$

$$E_{red}-NH_3^+(RCHO) \xrightarrow[O_2]{k_6} E_{ox} + RCHO + NH_4^+ + H_2O_2 \quad (3\text{-}2)$$

Scheme 3-3

In the first step, after the formation of an E•S complex, proton abstraction and imine hydrolysis yield the aldehyde product and reduced enzyme (Eqn 3-1). This is followed by enzyme reoxidation by molecular oxygen, which gives H_2O_2 in the second step (Eqn 3-2).

Introduction of deuterium into substrate brings about unique perturbation of a single step in catalysis, k_3^*. For Scheme 3-3 above, the kinetic parameters and isotope effects on kinetic parameters are given as follows (Palcic *et al.*, 1983; Cleland, 1975):

$$V_{max} = \frac{k_3k_5k_6[E_t]}{k_3k_5 + k_3k_6 + k_6(k_4+k_5)} \quad (3\text{-}3)$$

$${}^DV_{max} = \frac{(V_{max})_H}{(V_{max})_D} = \frac{{}^Dk + k_{3H}/k_5 + k_{3H}/k_6 + {}^DK_{eq}k_{4H}/k_5}{1 + k_{3H}/k_5 + k_{3H}/k_6 + k_{4H}/k_5} \quad (3\text{-}4)$$

$$V_{max}/K_m = \frac{k_1k_3k_5[E_t]}{k_2(k_4+k_5) + k_3k_5} \quad (3\text{-}5)$$

$${}^DV_{max}/K_m = \frac{(V_{max}/K_m)_H}{(V_{max}/K_m)_D} = \frac{{}^Dk + k_{3H}/k_2 + {}^DK_{eq}k_{4H}/k_5}{1 + k_{3H}/k_2 + k_{4H}/k_5} \quad (3\text{-}6)$$

where ${}^DK_{eq} = \frac{(K_{eq})_H}{(K_{eq})_D} = \frac{k_{3H}k_{4D}}{k_{3D}k_{4H}}$ and ${}^Dk = \frac{k_{3H}}{k_{3D}}$

It can be seen that V_{max}/K_m includes all steps up to the first irreversible step, while V_{max} includes all steps after E_{ox}•S complex formation. ^Dk, the intrinsic isotope effect, is the full isotope effect originating from the single isotopically sensitive step of catalysis, exclusive of all effects from isotopically insensitive steps. An isotope effect is expressed as a decreased rate with deuterated substrates only if C-H bond cleavage is rate-limiting. Usually, in enzyme reactions bond cleavage is not fully rate-limiting, rather other slow steps such as product release, conformational changes, or reoxidation of the enzyme will reduce the magnitude of the observed isotope effect (Northrop, 1976).

The primary deuterium isotope effect for benzylamine oxidation catalyzed by sheep serum amine oxidase was determined by comparing reaction rates with unlabeled and dideuterated benzylamine (Figure 3.11). The kinetic paramaters obtained by computer analysis of this data using a program based on the Cleland method (Cleland, 1979) are listed in Table 3.5. It was shown that replacement of the hydrogen atoms at the α-carbon of benzylamine by deuterium decreased the steady-state maximum velocity by a factor of 1.44. The K_m was increased by a factor of 2.25, giving an isotope effect on V_{max}/K_m of 3.35.

The primary deuterium isotope effect for tyramine oxidation was obtained by comparing the reaction rates with unlabeled and dideuterated tyramine (Figure 3.12). The reaction was biphasic, therefore only the high substrate concentration points are shown. The data for low substrate concentrations showed considerable scatter. The kinetic parameters obtained by analysis of this data are also listed in Table 3.5. In this instance the kinetic isotope effect on V_{max} is 3.14 and there is no isotope effect on V_{max}/K_m. Because V_{max} and V_{max}/K_m are dependent on different components of the reaction mechanism (Eqn 3-4 and 3-6), the interpretation of isotope effects on V_{max} and V_{max}/K_m must also be different. Eqn 3-4 indicates that an isotope effect on V_{max} is governed by the forward ratios of catalysis, k_{3H}/k_5 and k_{3H}/k_6, while Eqn 3-6 shows that an isotope effect on V_{max}/K_m is influenced by the forward commitment to catalysis,

k_{3H}/k_2. Thus, $^{D}V_{max}$ of 1.44 and $^{D}V_{max}/K_m$ of 3.35 for benzylamine indicate that reoxidation of the enzyme is slow relative to the isotope-sensitive step (i.e. C-H bond cleavage), thereby abolishing the expression of the isotope effect by causing k_{3H}/k_6 to become large (Northrop, 1981). $^{D}V_{max}$ of 3.14 and $^{D}V_{max}/K_m$ of 0.9 (approx. 1.0) for tyramine suggest that imine hydrolysis and enzyme reoxidation are fast relative to C-H bond cleavage and that the slow step must be at dissociation of E•S complex suppressing the expression of the isotope effect by causing k_{3H}/k_2 to become large (Northrop, 1975).

The kinetic study of tyramine oxidation was initiated to try to account for the invariance in the deuterium content of the products isolated in the stereochemical studies. Two models were proposed. In the first, the K_m for tyramine is high and therefore the stereochemical studies were under V_{max}/K_m conditions throughout. Alternatively, the KIE on V_{max} and $V_{max}K_m$ is the same. The K_m's obtained in the kinetic study are 23 mM for unlabeled tyramine and 6.6 mM for dideuterated tyramine. Assuming the K_m for the monodeuterated tyramines will be in this range, the stereochemical study with initial substrate concentrations of 15 to 20 mM will be mainly under V_{max}/K_m control since substrate concentrations are well below saturating (10 x Km).

However, the KIE on V_{max}/K_m obtained in the kinetic study is approx. 1. For our nonstereospecific reaction an isotope effect of 1 would yield only products that retain 50 % deuterium. This contrasts with experimental values obtained of 89 % deuterium retention for the *S*- amine and 72 % for the *R*- amine. There is no straightforward explanation for this discrepancy. It can be noted that the kinetic experiments are initial rate measurements with negligible depletion of substrate while in the stereochemical study substrate concentration is continually being reduced. Estimates of the K_m for both (1*R*)- and (1*S*)-[^{2}H]tyramine should be obtained as well as estimates of $^{D}V_{max}$ and $^{D}V_{max}/K_m$ for these substrates. This study is

currently prohibitive with the limited supplies of chiral compound but will be carried out in the future.

Table 3.5 Kinetic parameters for the oxidation of benzylamine and tyramine.

	Benzylamine		*Tyramine*	
	Unlabeled	*Dideutero*	*Unlabeled*	*Dideutero*
K_m (mM)	0.12	0.27	23.0	6.6
V_{max} (nmole/min)	7.5	5.2	16.2	5.1
V_{max}/K_m (nmole/min•mM)	64.8	19.4	0.70	0.78
KIE on V_{max}	1.44		3.14	
KIE on V_{max}/K_m	3.35		0.90	

KIE on $V_{max} = (V_{max})_H/(V_{max})_D$

KIE on $V_{max}/K_m = (V_{max}/K_m)_H/(V_{max}/K_m)_D$

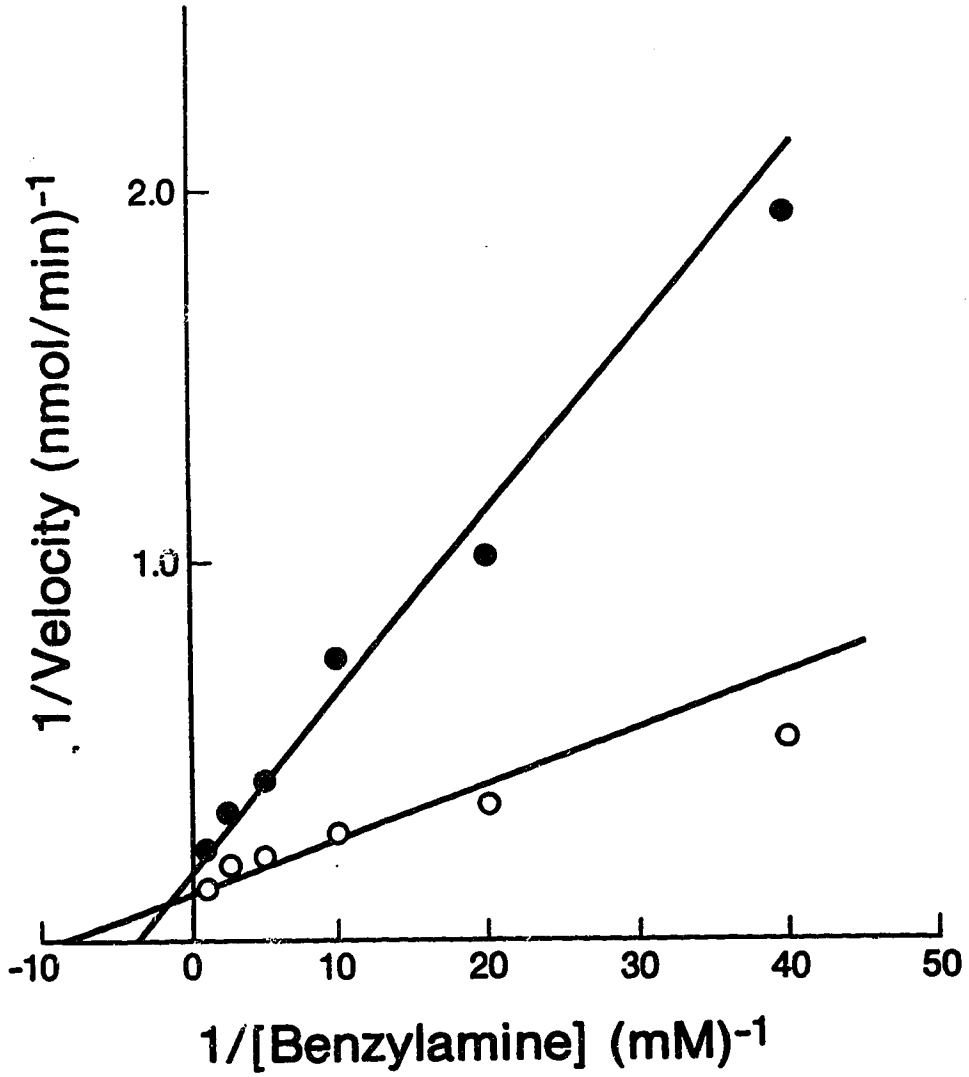


Figure 3.11. Double-reciprocal plot of initial rate data for the oxidation of benzylamine by sheep serum amine oxidase (0.012 units) in 100 mM potassium phosphate buffer, pH 7.0, at 25°C.The initial rates were measured by monitoring benzaldehyde formation at 250 nm. Open circles are for unlabeled benzylamine and closed circles are for dideuterated benzylamine.

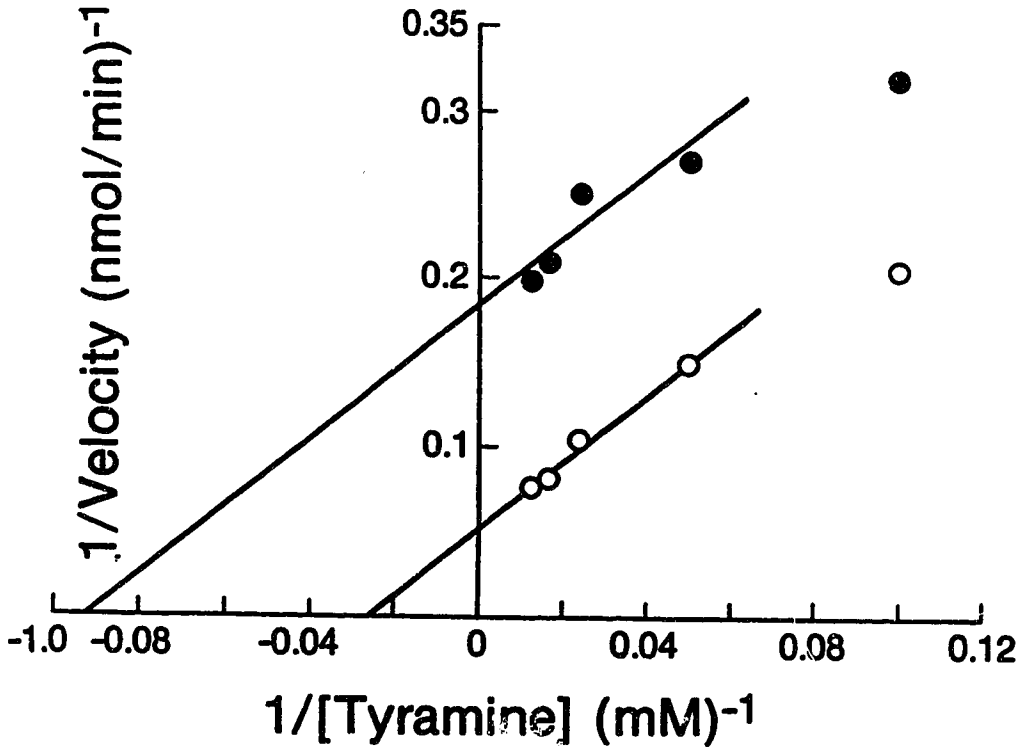


Figure 3.12. Double-reciprocal plot of initial rate data for the oxidation of tyramine by sheep serum amine oxidase (0.04 units) in a coupled system containing alcohol dehydrogenase and NADH in 100 mM potassium phosphate buffer, pH 7.0, at 25°C. The initial rates were measured by monitoring decrease in absorbance at 340 nm. Open circles are for unlabeled tyramine and closed circles are for dideuterated tyramine.

4. SUMMARY AND CONCLUSIONS

Porcine plasma amine oxidase was isolated from pig blood by applying ammonium sulfate fractionation (35-55%), chromatographic procedures using resins such as DE-52 cellulose, Con A-Sepharose, AH-Sepharose, hydroxyapatite, and finally a gel filtration colmn. The final enzyme, with a specific activity of 0.11 units/mg, was more than 90% pure as estimated by SDS-PAGE.

Sheep serum amine oxidase was isolated from sheep blood and purified by application of ammonium sulfate fractionation (30-65%), calcium phosphate gel adsorption, ammonium sulfate extraction (40 and 55%), a DE-52 anion exchanger, gel filtration (twice), Blue Sepharose CL-6B, and AH-Sepharose chromatography. The specific activity of the final enzyme was 0.12 units/mg.

The investigation carried out in this work showed that chiral tyramines were oxidized with a net nonstereospecific proton abstraction from C-1 by the sheep serum amine oxidase. The enzyme exhibited rare mirror-image binding (with differential flux) through two opposite and stereospecific reaction modes. Differential kinetic isotope effects were observed for each mode, 8.1 for the *S* mode and 2.6 for the *R* mode. The reduced KIE for (1*R*)-tyramine indicated that a step other than C-H bond cleavage is partially rate-limiting in the *R* mode, thus reducing the observed isotope effect.

The deuterium isotope effects on V_{max} and V_{max}/K_m with benzylamine and tyramine substrates were estimated in steady-state kinetic experiments. $^{D}V_{max}$ of 1.44 and $^{D}V_{max}/K_m$ of 3.35 for benzylamine indicate that reoxidation of the enzyme (SSAO) is slow relative to C-H bond cleavage, while $^{D}V_{max}$ of 3.14 and $^{D}V_{max}/K_m$ of 0.9 for tyramine show that the slow step must be at dissociation of E•S complex.

Although the above research revealed a great deal about this enzyme (SSAO), further studies are necessary on the stereochemistry of SSAO with other substrates, such as benzylamine, since bovine enzyme is stereospecific with benzylamine, but nonstereo-specific with dopamine and tyramine. Other areas deserving exploration include stereo-

chemistry of wash-in, amino acid sequence of active site, nature of the cofactor, and determination of intrinsic isotope effect.

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STEREOCHEMISTRY OF SHEEP SERUM AMINE OXIDASE REACTIONS

by

Young J. Kang (C)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF **Master of Science**

IN

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis

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submitted by **Young J. Kang**

in partial fulfillment of the requirements for the

degree of **Master of Science**

in **Food Chemistry**

M. Palcic, Supervisor

L. Ozimek

P. Sporns

M. Peppler

Date: Oct 11, 1990

DEDICATION

Dedicated to

My Mother, Father and Sisters

ABSTRACT

The stereochemical course of the oxidation of (1*R*)-[^{2}H]- and (1*S*)-[^{2}H]tyramine catalyzed by sheep serum amine oxidase has been investigated using ^{1}H NMR spectroscopy. Reactions were carried out in a coupled fashion such that the aldehydes produced by the amine oxidase were converted directly to alcohols with alcohol dehydrogenase. Both product alcohols obtained from incubations with the chiral amines and sheep serum amine oxidase contained deuterium, demonstrating that reaction occurred with net nonstereospecific proton abstraction from C-1. The enzyme also exhibited a solvent exchange pathway into C-2 of product. The magnitudes of kinetic isotope effects with different substrates were estimated in steady-state measurements by comparing the kinetic paramaters V_{max} and V_{max}/K_m obtained with fully protonated and dideuterated benzylamine and tyramine substrates. Benzylamine exhibited a kinetic isotope effect on V_{max} of 1.44 and V_{max}/K_m of 3.35, indicating that reoxidation of the enzyme is slow relative to the isotope sensitive step (i.e. C-H bond cleavage). Tyramine showed a kinetic isotope effect on V_{max} of 3.14 and V_{max}/K_m of 0.9, suggesting that reoxidation of the enzyme is rapid relative to the C-H bond cleavage step and the slow step must be at formation or dissociation of the intermediate E•S complex.

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Table of Contents

Chapter Page

1 INTRODUCTION 1

2 EXPERIMENTAL PROCEDURES 6

2.1 Isolation of Porcine Plasma Amine Oxidase 6

2.1.1 Materials 6

2.1.2 Methods 6

2.1.2.1 Assay of enzyme activity 6

2.1.2.2 Enzyme purification 7

2.2 Isolation of Sheep Serum Amine Oxidase 10

2.2.1 Materials 10

2.2.2 Methods 11

2.2.2.1 Assay of enzyme activity 11

2.2.2.2 Enzyme purification 11

2.3 Stereochemistry of Sheep Serum Amine Oxidase Reactions 15

2.3.1 Materials 15

2.3.2 Methods 15

2.3.2.1 Oxidation of monodeuterated tyramines 15

2.3.2.2 Solvent exchange into product 16

2.4 Kinetic Isotope Effects 17

2.4.1 Steady-state kinetics for benzylamine oxidation 17

2.4.1.1 Materials 17

2.4.1.2 Methods 17

2.4.2 Steady-state kinetics for tyramine oxidation 18

2.4.2.1 Materials 18

2.4.2.2 Methods 18

Chapter Page

3 RESULTS AND DISCUSSION....19

3.1 Isolation of Porcine Plasma Amine Oxidase....19

3.2 Isolation of Sheep Serum Amine Oxidase....19

3.3 Stereochemistry of Sheep Serum Amine Oxidase Reactions....30

3.3.1 Oxidation of monodeuterated tyramines....30

3.3.2 Solvent exchange into product....38

3.4 Kinetic Isotope Effects....41

4 SUMMARY AND CONCLUSIONS....49

5 REFERENCES....51

List of Tables

Table Page

3.1 Purification of porcine plasma amine oxidase.....20

3.2 Partial purification of sheep serum amine oxidase.....22

3.3 Further purification of sheep serum amine oxidase.....25

3.4 Characteristics of ^{1}H NMR spectra of alcohols derived from coupled incubations of chiral amines with sheep serum amine oxidase.....39

3.5 Kinetic parameters for the oxidation of benzylamine and tyramine.....46

List of Figures

Figure — Page

1.1 Structure of amine oxidase cofactors 5

3.1 SDS polyacrylamide gel electrophoresis of eluates after different purification steps in the porcine plasma amine oxidase isolation 21

3.2 SDS-PAGE of low activity fraction from DE-52 of the sheep serum amine oxidase isolation 24

3.3 SDS-PAGE of Blue Sepharose eluates 26

3.4 Outline of sheep serum amine oxidase isolation 28

3.5 The proposed isolation scheme for SSAO 29

3.6 Partial 360 MHz ^1H NMR spectrum of (1*R*)-[^2H]tyramine derived from incubation of tyrosine decarboxylase with L-tyrosine in ^2H_2O 31

3.7 Partial 360 MHz ^1H NMR spectrum of (1*S*)-[^2H]tyramine derived from incubation of tyrosine decarboxylase with DL-[2-^2H]tyrosine in H_2O 32

3.8 The ^1H NMR spectrum of alcohol derived from coupled incubation of (1*S*)-[^2H]tyramine and sheep serum amine oxidase with NADH and alcohol dehydrogenase 34

3.9 The ^1H NMR spectrum of alcohol derived from coupled incubation of (1*R*)-[^2H]tyramine and sheep serum amine oxidase with NADH and alcohol dehydrogenase 35

3.10 The ^1H NMR spectrum of alcohol derived from incubation of sheep serum amine oxidase and tyramine in ^2H_2O 40

3.11 Double-reciprocal plot of initial rate data for the oxidation of benzylamine by sheep serum amine oxidase (0.012 units) in 100 mM potassium phosphate buffer, pH 7.0, at 25°C 47

3.12 Double-reciprocal plot of initial rate data for the oxidation of tyramine by sheep serum amine oxidase (0.04 units) in a coupled system containing alcohol dehydrogenase and NADH in 100 mM potassium phosphate buffer, pH 7.0, at 25°C 48

List of Abbreviations

ADH	alcohol dehydrogenase
AO	amine oxidase
BPAO	bovine plasma amine oxidase
Con A	concanavalin A
DNA	deoxyribonucleic acid
E•S complex	enzyme•substrate complex
EC	Enzyme Commission
HMW	high molecular weight
^{D}k	intrinsic isotope effect
$^{D}k_{eq}$	isotope effect on equilibrium constant
$^{D}V_{max}$	isotope effect on V_{max}
$^{D}V_{max}/K_m$	isotope effect on V_{max}/K_m
KIE	kinetic isotope effect
V_{max}	maximal velocity
K_m	Michaelis constant
NADH	nicotinamide adenine dinucleotide (reduced form)
ppm	parts per million
PAGE	polyacrylamide gel electrophoresis
PPAO	porcine plasma amine oxidase
^{1}H NMR	proton nuclear magnetic resonance
R_f	relative mobility
RNA	ribonucleic acid
SSAO	sheep serum amine oxidase
SDS	sodium dodecyl sulfate
TLC	thin-layer chromatography
$[E_t]$	total enzyme concentration
TDC	tyrosine decarboxylase

1. INTRODUCTION

Amine oxidases catalyze the oxidative deamination of amines (mono-, di- and polyamines) with the stoichiometric formation of aldehyde as follows:

$$RCH_2NH_2 + O_2 + H_2O \longrightarrow RCHO + NH_3 + H_2O_2 \qquad (1\text{-}1)$$

These enzymes can be separated into three classes based on differences in cofactor, in substrate specificities and inhibitor sensitivities. The three classes are the flavin-containing monoamine oxidases (EC 1.4.3.4), the copper-containing amine oxidases (EC 1.4.3.6), and the semicarbazide-sensitive amine oxidases, which have not yet been given an Enzyme Commission designation (Mondovi, 1985).

Amine oxidases are ubiquitous, they are found in microorganisms as well as in higher forms of life. They are of great biological interest since many of their substrates are biogenic amines which are formed *in vivo* in various metabolic processes. Many of these biogenic amines appear to be involved in regulatory enzyme mechanisms, vital to higher mammals. For instance, polyamines such as spermine and spermidine seem to influence the synthesis of DNA and RNA (Kapeller-Adler, 1970 and Feuerstein & Marton, 1989).

The physiological role in neurotransmitter metabolism of the first class of enzymes, the flavin-containing monoamine oxidases, has made them the subject of intensive investigation, notably as targets for antidepressant drugs (Kapeller-Adler, 1970). The semicarbazide-sensitive amine oxidases found in vascular tissue and fat have not been well studied. At present, the nature of the prosthetic group is unknown, however, kinetically and stereochemically they appear to be distinct from the other two classes of amine oxidases (Callingham and Barrand, 1987).

The copper amine oxidases, which are the subject of this thesis, are widespread in nature — they are found in microorganisms, plant seedlings, serum, kidney, intestinal mucosa, liver and lung. In mammals they were first described as spermidine oxidase activity found in the serum of sheep (Hirsh, 1953) and oxen (Tabor *et al.*, 1953).

The first report of a copper amine oxidase in plants was made in 1948 by Werle and coworkers. They reported the occurrence of an enzyme that catalyzes the degradation of 1,4-diaminobutane, 1,5-diaminopentane, and histamine in extracts of some leguminous plants, sage and lavender.

An oxidative degradation of spermidine and spermine by bacterial preparations was first observed by Silverman and Evans in 1944, who used whole cells or lyophilized cell extracts of *Pseudomonas pyocyaneae (aeruginosa).*

Most copper amine oxidases lack strict substrate specificity. For instance, the classical serum amine oxidase catalyzes oxidative deamination of aromatic amines (such as tyramine and benzylamine) and peptidyl lysine, in addition to polyamines. Blaschko and coworkers (1959), who were some of the first investigators studying mammalian plasma amine oxidases, reported that ruminant enzyme acted on spermine and spermidine, while these were not significantly attacked by the non-ruminant plasma amine oxidase. The non-ruminant enzyme acted relatively rapidly on benzylamine and mescaline, but more slowly on tyramine and many monoamines. Blaschko also reported that the amine oxidases of mammalian plasma were inhibited by carbonyl reagents.

One of the best-characterized copper enzymes, bovine plasma amine oxidase, has a mol. wt. of about 170,000 and apparently consists of two identical subunits (Mondovi, 1985). Porcine plasma amine oxidase, which has a mol. wt. of approximately 195,000, is also a dimer (Falk *et al.*, 1983). A concentrated solution of either enzyme is pink in color.

When Hirsh (1953) first found spermine oxidase activity in sheep serum, it was a crude preparation, derived only from clotting and centrifugation of the blood. Sheep serum amine oxidase (SSAO) was isolated and purified by Rucker and Goettlich-Riemann (1972) through a series of steps involving ammonium sulfate fractionation, extraction and calcium adsorption. Though its purity (0.032 units/mg) was an improvement over previous preparations, it was considerably lower than those reported for bovine (0.36 units/mg) and

porcine (0.1 units/mg) plasma amine oxidases. Therefore, one objective of the research described in this thesis was to isolate and further purify SSAO.

Mammalian copper amine oxidases have long been recognized to play key roles in cellular processes. They catalyze the oxidative removal of biogenic amines from blood plasma, the crosslinking of collagen and elastin in connective tissue biogenesis, and the regulation of intracellular spermine and spermidine levels (Hartmann and Klinman, 1988).

The role of copper in these enzymes is not clear and mechanistic studies of these enzyme systems have been seriously hampered by the uncertainty as to the exact nature of the covalently bound cofactor. It was first believed to be pyridoxal phosphate (Mondovi *et al.*, 1967), and then thought to be pyrroloquinoline quinone (PQQ) (Moog *et al.*, 1986). More recently, bovine plasma amine oxidase has been shown to contain 3,4,6-trihydroxy-phenylalanine (Topa). Topa has been found in the pentapeptide at the active site:

Leu - Asn - X - Asp - Tyr

where X is Topa

Topa formation might represent a new posttranslational modification involving oxidation of an active site tyrosine (Janes *et al.*, 1990) (Figure 1.1).

Copper amine oxidases are characterized by unusual stereochemical patterns in their reactions. Dopamine and tyramine are oxidized with abstraction of the pro-*R* hydrogen at C-1 by the porcine plasma amine oxidase, the pro-*S* hydrogen by pea seedling amine oxidase, and a net nonstereospecific proton abstraction by the bovine plasma enzyme (Coleman *et al.*, 1989), while benzylamine oxidation catalyzed by the same enzyme (BPAO) is stereospecific with abstraction of the pro-*S* hydrogen at C-1. The pro-*R* (or pro-*S*) hydrogen refers to one of the chemically identical hydrogens at a prochiral centre i.e. the methylene carbon of benzylamine or C-1 of tyramine. If one of the methylene hydrogens at this centre is replaced by a deuterium, then a chiral centre is produced. The designation pro-*R* is used for the deuterium substitution that generates a chiral centre with an *R* configuration as defined by

Cahn- Ingold- Prelog system. The designation pro-*S* is used for the deuterium substitution that generates a chiral centre with an *S* configuration. For a review of stereochemical nomenclature used in this thesis see Walsh (1979). The copper amine oxidases provide the first example in which a reaction catalyzed by enzymes in the same formal class occurs by all three possible stereochemical routes. Solvent exchange profiles are consistent within each stereochemical class of enzyme: the pro-*R* and nonstereospecific enzymes exchange solvent into C-2 of product aldehydes, the pro-*S* enzymes do not.

In this study, the stereochemical course of oxidation of tyramine was established for another copper amine oxidase isolated from sheep serum (SSAO). The deuterium isotope effects with benzylamine and tyramine substrates were also estimated in steady-state kinetic experiments. In addition to these kinetic and stereochemical studies of SSAO, porcine plasma amine oxidase was isolated for use in other stereochemical studies and to gain experience in enzyme isolation methodologies.

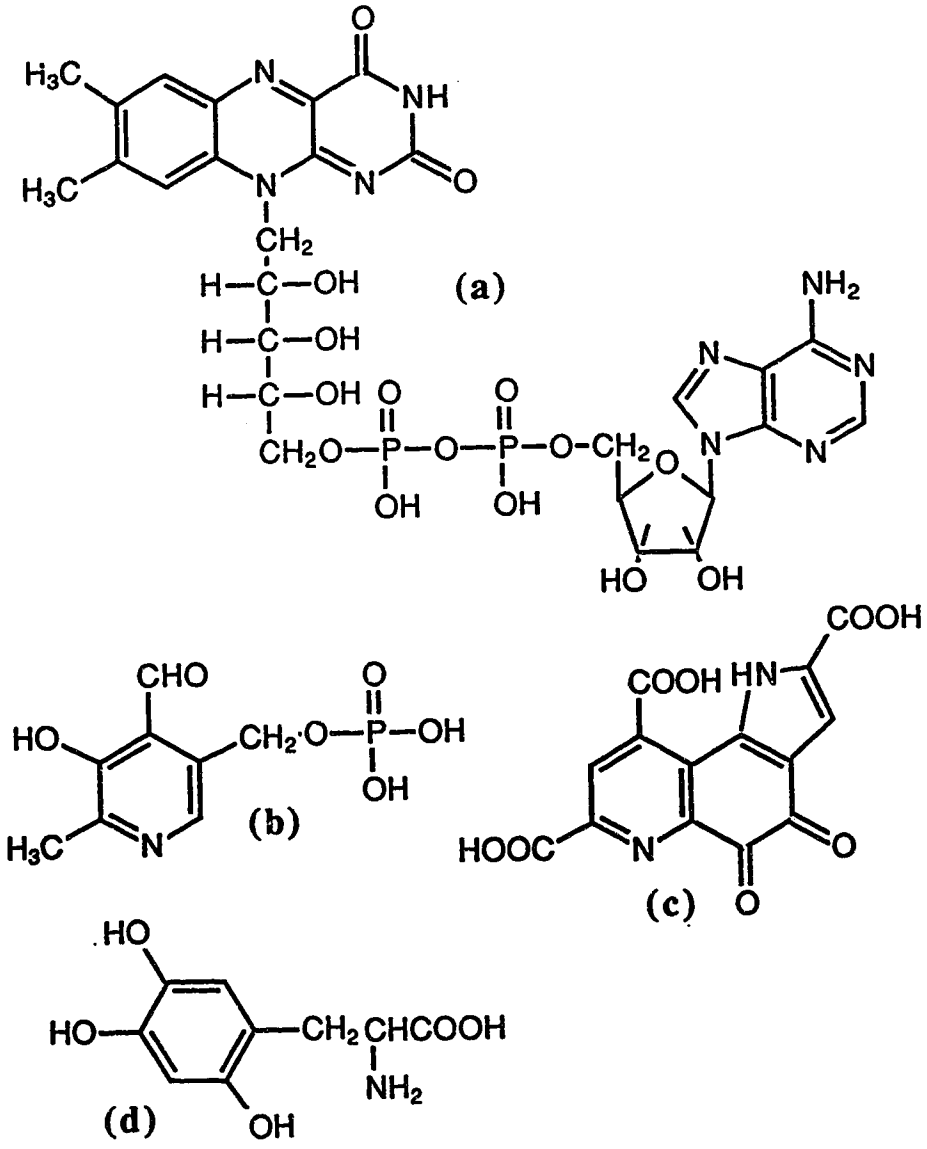


Figure 1.1. Structure of amine oxidase cofactors.
(a) Flavin adenine dinucleotide (FAD)
(b) Pyridoxal-5'-phosphate (PLP)
(c) Pyrroloquinoline quinone (PQQ)
(d) 3,4,6-Trihydroxyphenylalanine (Topa)

2. EXPERIMENTAL PROCEDURES

2.1 Isolation of Porcine Plasma Amine Oxidase (PPAO)

The enzyme was isolated from pig blood by the method of Falk *et al.* (1983) and Coleman *et al.* (1989) with some modifications. A new step (AH-Sepharose chromatography) was introduced to try to improve the preparation.

2.1.1 Materials

Fresh pig blood was obtained from a local slaughterhouse (Gainers, Edmonton, AB). Benzylamine was purchased from Sigma Chemical Company (St. Louis, MO). Hydroxyapatite (Bio-Gel HTP), Bio-Gel A 1.5 m and electrophoresis reagents were obtained from Bio-Rad Laboratories (Mississauga, ON). Con A-Sepharose was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), DE-52 cellulose was from Whatman (Maidstone, England), and AH-Sepharose was prepared by Ms. C. Scaman in our laboratory. The latter is CNBr-activated Sepharose 4B reacted with 1,6-diaminohexane (Svenson & Hynning, 1981).

2.1.2 Methods

2.1.2.1 Assay of enzyme activity

Amine oxidase activity was estimated spectrophotometrically. Assays were carried out at 25°C in 1 mL of 50 mM sodium phosphate buffer, pH 7.2, containing 3.33 mM benzylamine. The production of benzaldehyde was determined by monitoring the increase in absorbance at 250 nm. An extinction coefficient of 1.2×10^4 M^{-1} cm^{-1} was used to calculate the amount of benzaldehyde formed. One unit of activity was defined as the amount of enzyme catalyzing the production of 1 μmol of benzaldehyde per min under the standard assay conditions. Protein concentration was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as a protein standard. Spectral

measurements were made with a Beckman DU-8 spectrophotometer equipped with a Peltier temperature controller.

2.1.2.2 Enzyme purification

All steps were carried out at 4°C.

Step 1 Citration of blood and centrifugation.

Fresh pig blood (26 L) was mixed with 4.4 L of citrate solution (8 g of citric acid and 22 g of sodium citrate dihydrate/L) to prevent coagulation. The citrated blood was centrifuged at 6,400 x g for 25 min. The red and white blood cells were pelleted and discarded.

Step 2 $(NH_4)_2SO_4$ fractionation (35 - 55 %): salting out.

This process involves removal of proteins which precipitate before 35 % $(NH_4)_2SO_4$ saturation and proteins that are soluble beyond 55 % $(NH_4)_2SO_4$ saturation.

The supernatant (plasma, 16 L) was treated with solid ammonium sulfate to 35% saturation (209 g $(NH_4)_2SO_4$/L supernatant). The precipitate was separated by centrifugation at 6,400 x g for 25 min and discarded. The supernatant was brought to 55% saturation by the addition of 129 g $(NH_4)_2SO_4$/L and centrifuged at 11,300 x g for 25 min. The precipitate was dissolved in a minimal volume of 10 mM sodium phosphate buffer, pH 7.0, and was dialyzed against four changes of 16 L 10 mM sodium phosphate buffer, pH 7.0. After dialysis, the solution (2 L) was centrifuged at 17,700 x g for 25 min to remove solid particles.

Step 3 DE-52 anion exchange chromatography.

DEAE-cellulose is positively charged at pH 6-9, thus proteins with net negative surface charges would be adsorbed to the column while proteins with a positive charge or an overall charge near zero will pass through the column resulting in partitioning of the protein of interest. Since porcine plasma amine oxidase has an isoelectric point (pI) of 4.5 to 5.0, it will be negatively charged at pH 7.0 and adsorb to the resin.

One third of the clarified supernatant, i.e. 680 mL, was applied to a Whatman DE-52 cellulose column (5 x 50 cm) and the column was washed with 10 mM sodium phosphate buffer, pH 7.0, until the protein content of the eluate was close to baseline levels as monitored by the absorbance at 280 nm. Amine oxidase was eluted at a flow rate of 2.5 mL/min with 30 mM sodium phosphate buffer (1,200 mL), pH 7.0, followed by 400 mL of 100 mM sodium phosphate buffer, pH 7.0 (22 mL/fraction). Enzyme activity was found in both eluates (30 mM and 100 mM).

Step 4 Con A-Sepharose chromatography.

This step relies on the fact that Con A binds molecules which contain α-D-mannopyranosyl, α-D-glucopyranosyl and sterically related residues. Therefore, Con A-Sepharose is suitable for separation and purification of glycoproteins, glycolipids and polysaccharides from non-glycosylated proteins.

The fractions which eluted from the DE-52 column were concentrated by ultra-filtration (PM-10 membrane) and applied to a Con A-Sepharose column (5.5 x 23 cm) which had been equilibrated with a pH 7.0 buffer containing 10 mM sodium phosphate, 150 mM sodium chloride, 1 mM manganese chloride, and 1 mM calcium chloride. The column was washed with the starting buffer until the protein concentration dropped to baseline levels. Enzyme was eluted with 1,040 mL of starting buffer containing 100 mM methyl α-mannopyranoside (22 mL/fraction) at a flow rate of 0.76mL/min. The eluate was concentrated by ultrafiltration using a PM-30 membrane and dialyzed against 10 mM sodium phosphate buffer, pH 8.0.

Step 5 AH-Sepharose chromatography.

This affinity column is based on the fact that CNBr-activated AH-Sepharose contains an aminohexyl group which is an amine oxidase substrate analog and a charged group such as the isourea moiety which can introduce anion exchange character.

The dialyzed enzyme solution was applied to an AH-Sepharose column (2.5 x 23 cm) equilibrated with 10 mM sodium phosphate buffer, pH 8.0, and washed with the same buffer. Enzyme was eluted stepwise with 45 mM, 55 mM, 75 mM, and 100 mM potassium phosphate buffer, pH 8.0. Activity was found in all four eluates, with 64% of activity occurring in the 45 mM eluate. Steps 3-5 were carried out on the remaining two-thirds of the clarified supernatant from Step 2 as 650 mL and 670 mL volumes, respectively.

All eluates with activity were combined, concentrated by ultrafiltration, clarified by centrifugation, and dialyzed against 6 mM sodium phosphate buffer, pH 7.0. At this point the enzyme solution was divided into two portions of 100 mL each, and both were taken through Steps 6 and 7.

Step 6 Hydroxyapatite (crystalline calcium phosphate) column.

The mechanism of adsorption of proteins to hydroxyapatite is still not clear. It is believed to be that the adsorption of proteins to hydroxyapatite (HA) is due to polar dipole-dipole bonding between

H_3N^+ - protein
:
: and
:
PO_4^{3-} - HA

^-OOC - protein
:
: and
:
Ca^{2+} - HA

Generally, proteins that chromatograph well on anion exchangers also bind to HA, but low molecular weight proteins show very low affinity for hydroxyapatite.

A 100 mL aliquot of the dialyzed enzyme solution was loaded onto a hydroxyapatite column (2.5 x 52 cm) which had been equilibrated with the 6 mM sodium phosphate buffer, pH 7.0, and was eluted with 400 mL of 6 mM and 220 mL of 60 mM sodium phosphate buffer, pH 7.0. Activity was found in the 6 mM eluate. The eluate was concentrated in an Amicon ultrafiltration cell by using a PM-30 membrane and dialyzed against 10 mM sodium phosphate buffer, pH 7.0.

Step 7 Bio-Gel A 1.5 m (gel filtration) column.

This chromatography involves the separation of proteins in aqueous media according to size. The separation is due to the different length of time spent by the protein solutes within the liquid phase that is entrapped by the gel matrix. That is, the larger the molecule, the smaller is the fraction of the pores accessible to them. This results in a decrease in the time spent within the liquid phase inside the pores of the matrix. Therefore larger proteins elute from the colmn faster.

The concentrated enzyme solution (3 mL) was loaded onto a Bio-Gel A 1.5 m column (2.5 x 100 cm) and eluted with the starting buffe at a flow rate of 0.35 mL/min.

Step 8. Aliquots of sample after each purification step were assayed for activity and protein content. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was also carried out by the method of Laemmli (1970) using a Bio-Rad Mini Protean II vertical slab gel electrophoresis cell equipped with 1.5 mm spacers. The gels (11%) were run at 18 ma constant current for about 2 hr, and stained for protein with 0.35% Coomassie Brilliant Blue R-250 dissolved in 45:10:45 (v/v/v) methanol:acetic acid:water. Destaining was done by several changes of the same solvent.

2.2 Isolation of Sheep Serum Amine Oxidase

Sheep serum amine oxidase was isolated from sheep blood by the method of Rucker and Goettlich-Riemann (1972). These workers used a series of ammonium sulfate precipitations and calcium phosphate gel treatments to partially purify the enzyme. An additional series of chromatographic steps was implemented in this study to try to provide highly purified enzyme.

2.2.1 Materials

Fresh sheep blood was obtained from a slaughterhouse (Lamco, Innisfail, AB). Benzylamine was purchased from Sigma Chemical Company (St. Louis, MO). Blue

Sepharose CL-6B and Sephacryl S-300 SF were from Pharmacia Fine Chemicals (Uppsala, Sweden). Electrophoresis reagents were from Bio-Rad Laboratories (Mississauga, ON), and DE-52 cellulose was from Whatman (Maidstone, England). Calcium phosphate gel was prepared by the method of Kunitz (1952).

2.2.2 Methods

2.2.2.1 Assay of enzyme activity

Amine oxidase activity was assayed at 25°C in 1 mL of sodium phosphate buffer, pH 7.6, containing 3.33 mM benzylamine. One unit was defined as the amount of enzyme catalyzing the production of 1 μmol of benzaldehyde per min under assay conditions. The production of benzaldehyde was monitored spectrophotometrically at 250 nm and calculations for benzaldehyde production were based on a molar absorptivity of 1.2×10^4 M^{-1} cm^{-1}. Protein concentration was determined by the Bradford (1976) method using bovine serum albumin as a protein standard. Spectral measurements were made on a Hewlett-Packard 8451A diode array spectrophotometer thermostated with a circulating water bath.

2.2.2.2 Enzyme purification

All steps were carried out at 4°C. The procedure follows the flow chart (Figure 3.4) on page 28.

Step 1 Citration of blood and centrifugation.

Fresh sheep blood (12 L) was mixed with 2 L of citrate solution (8 g of citric acid and 22 g of sodium citrate dihydrate/L), the citrated blood was centrifuged at 10,000 rpm with a Beckman JA-10 rotor (17,700 x g) for 20 min, and the supernatant (serum, 7 L) was collected. High g value applied for sheep enzyme was due to the fact that

separation of pellet was not satisfactory at low centrifugal force probably because of a large amount of clotted blood.

Step 2 $(NH_4)_2SO_4$ fractionation (30-65%).

Solid ammonium sulfate was added to the serum (176 g $(NH_4)_2SO_4$/L serum) to 30% saturation, and the precipitate was removed after centrifugation at 17,700 x g for 20 min. The supernatant (7.2 L) was brought to 65% saturation by the addition of 235 g $(NH_4)_2SO_4$/L supernatant, and the precipitate was separated by centrifugation.

Step 3 $(NH_4)_2SO_4$ extraction (40%).

The precipitate was suspended in 40% saturated ammonium sulfate solution (900 mL) which was adjusted to pH 7.4. After standing for 30 min, the suspension (1 L) was centrifuged at 17,700 x g for 30 min and the particulate material was discarded. The supernatant was dialyzed against a 20-volume excess of 20 mM sodium phosphate buffer, pH 7.4, with three changes.

Step 4 Calcium phosphate gel adsorption.

The theory involved is the same as described for the hydroxyapatite column except that the gel is not suitable for chromatography because of low flow rate. It can be used in batch-wise adsorption treatments.

The dialyzed solution (1,240 mL) was treated with calcium phosphate gel (370 mL) at a ratio of 22.2 mL gel/g protein, and the precipitate was discarded. The supernatant, which contained all the enzyme activity, was treated with another portion of calcium phosphate gel (100 mL) and centrifuged. Most enzyme activity remained in the supernatant, instead of being adsorbed to calcium phosphate gel #2, therefore the calcium phosphate gel adsorption procedure was stopped at this stage.

Step 5 $(NH_4)_2SO_4$ extraction (55 %).

The supernatant (1.4 L) from Step 4 was treated with ammonium sulfate to 70% saturation (472 g $(NH_4)_2SO_4$/L supernatant). The precipitate which formed was separated by centrifugation and resuspended in 55% saturated ammonium sulfate solution (150 mL)

at pH 7.4. The 55% ammonium sulfate precipitate was collected by centrifuging at 17,700 x g for 30 min, dissolved in 50 mM phosphate buffer (105 mL), pH 7.4, and dialyzed against three changes of 3.5 L of 10 mM sodium phosphate buffer, pH 7.4.

Step 6 DE-52 anion exchange chromatography.

Half (90 mL) of the dialyzed enzyme solution was loaded onto a Whatman DE-52 cellulose column (5.5 x 23 cm) equilibrated with 10 mM phosphate buffer, pH 7.4, at a flow rate of 0.92 mL/min and washed with the starting buffer until the protein concentration was near baseline (approximately 200 mL of eluate collected) as monitored by absorbance at 280 nm. Amine oxidase was eluted with 30 mM sodium phosphate buffer, pH 7.4, again until the protein concentration reached baseline (1,360 mL of eluate), and then eluted with 100 mM sodium phosphate buffer (340 mL), pH 7.4. Enzyme activity was found in the 30 mM and 100 mM eluate as well as in the 10 mM wash. The remaining half (90 mL) of the dialyzed enzyme solution from Step 5 was loaded onto a Whatman DE-52 cellulose column and treated the same way as the first half of the dialyzed enzyme solution. High activity fractions from all eluates were pooled together into two groups according to specific activity (1.32×10^{-2} and 1.03×10^{-2} units/mg), concentrated by ultrafiltration (PM-30 membrane), and used in the stereochemical and kinetic studies. Low activity fractions from the 30 mM and 100 mM eluates were combined (3.12×10^{-3} units/mg), concentrated, and purified further. The final concentrated solution was blue in color, suggesting that ceruloplasmin was also present in the sample.

Step 7 Sephacryl S-300 SF (gel filtration) column #1.

Further purification of the low activity fraction was carried out as follows: The enzyme solution (8 mL) was loaded onto a Sephacryl S-300 SF column (2.5 x 100 cm) which had been equilibrated with 10 mM potassium phosphate buffer, pH 7.4, and was eluted with the same buffer at a flow rate of 0.55 mL/min. The eluates with activity (105 mL) were concentrated to 10 mL, dialyzed against 50 mM potassium phosphate buffer containing 100 mM KCl, pH 7.0.

Step 8 Blue Sepharose CL-6B (affinity) column.

The blue dye (Cibacron Blue F3GA) ligand has an affinity for enzymes that bind to purine nucleotides due to structural similarity between the two types of molecules. Albumin is also known to bind to the dye perhaps through electrostatic and hydrophobic interactions with the aromatic anionic ligand.

The eluates from Step 7 were applied to a Blue Sepharose CL-6B column (2.5 x 50 cm), and eluted with the starting buffer. The eluates with activity (50 mL) were pooled together, dialyzed against 10 mM potassium phosphate buffer, pH 8.0.

Step 9 AH-Sepharose column.

The dialyzed solution was loaded onto an AH-Sepharose column (2.5 x 23 cm). The column was washed with the starting buffer, and eluted with 45 mM, 55 mM, and gradient (50-300 mM) potassium phosphate buffer, pH 8.0. Activity was found in both the 45 mM (75 mL) and gradient eluates at approximately 75 mM (140 mL). The eluates with activity were combined and concentrated to 10 mL, 1 mL (approx. 1 unit) of the high activity fraction was added, samples were centrifuged to remove precipitate.

Step 10 Sephacryl S-300 SF (gel filtration) column #2.

The solution from Step 9 was loaded again onto a Sephacryl S-300 SF column (2.5 x 100 cm) equilibrated with 100 mM potassium phosphate buffer, pH 7.4. The enzyme was eluted with the starting buffer. Eluates (60 mL) from fractions #57 to #68 (5 mL/fraction) showed activity with elution volume of 280 mL.

Step 11

With the exception of Sephacryl column #1, SDS-PAGE was carried out on the eluates from DE-52 column to Sephacryl column #2, as described in Step 8 of Section 2.1.2.2.

2.3 Stereochemistry of Sheep Serum Amine Oxidase (SSAO) Reactions

2.3.1 Materials

Tyramine was purchased from Eastman Kodak Company (Rochester, NY). NADH (disodium salt, trihydrate), alcohol dehydrogenase from equine liver (1.6 units/mg) and catalase from bovine liver (11,000 units/mg) were obtained from Sigma Chemical Company (St. Louis, MO). (1*S*)- and (1*R*)-[^{2}H]tyramine were available from previous stereochemical studies (Coleman *et al.*, 1989). They were prepared by incubation of DL-[2-^{2}H]tyrosine with tyrosine decarboxylase (TDC) in protonated buffer or L-tyrosine with TDC in deuterated buffer. Deuterated buffer was prepared by making protonated buffer of the desired pH (7.0), evaporating solvent under vacuum, and dissolving the dried buffer salts in exactly the same volume of D_2O as the evaporated H_2O. The final pD of the buffer solution was 7.2, where pD refers to the uncorrected pH meter reading of the deuterated buffer. SSAO was exchanged into deuterated buffer by concentrating SSAO to 0.5 mL by ultrafiltration and resuspending in 0.5 mL of 100 mM potassium phosphate buffer, pD 7.2, and 5 mL of ^2H_2O. Concentration and resuspension were carried out three times to ensure the complete exchange of solvent.

2.3.2 Methods

All stereochemical studies were carried out in a coupled fashion by the method of Battersby *et al.* (1979) and Coleman *et al.* (1989) with slight modifications. The reaction was coupled to convert reactive aldehyde to stable alcohol and also to monitor the progress of the reaction by measuring decrease in absorbance at 340 nm.

2.3.2.1 Oxidation of monodeuterated tyramines

The following components were incubated in 1.0 mL of 100 mM potassium phosphate buffer, pH 7.2, at 25°C: 15-20 μmol of stereospecifically deuterated tyramine

(1*S*- or 1*R*-), sheep serum amine oxidase (0.3 units), 25-30 μmol NADH, 5-8 units of alcohol dehydrogenase, and 11,000 units of catalase. The conversion of amine to alcohol was monitored by the removal of aliquots (5 μL) at timed intervals, the aliquots were diluted with 995 μL of the buffer, and $\Delta Abs_{340\ nm}$ was measured.

At 50 or 100% completion of reaction, the incubation mixtures were diluted to 10 mL with water and loaded onto reverse-phase C_{18} Sep-pak cartridges which had been washed with 10 mL of methanol and 20 mL of water before use. The cartridges were rinsed with 5 mL of water, then the alcohol product was eluted with 10 mL of methanol. The methanol eluate was concentrated under reduced pressure, dissolved in 2 mL of ethyl acetate:*n*-hexane (2:1 v/v), and applied to a flash silica column (2g; 1 x 25 cm) which had been prewashed with 10 mL of ethyl acetate:*n*-hexane (2:1 v/v). The alcohol product (tyrosol) was eluted with 20 mL of the same solvent and collected in culture tubes (approx. 0.7 mL/fraction). This step effectively removed any remaining amine and NADH which remained bound to the column.

Aliquots of all the fractions were spotted on silica gel TLC plates, developed with the same solvent ($R_f = 0.51$ for *p*-hydroxyphenethyl alcohol), and the fractions containing the product were pooled. The tyrosol solution was concentrated to dryness under reduced pressure, dissolved in a solvent of $(^2H_3C)_2CO$: ^2H_2O (4:1 v/v), and ^1H NMR spectra were measured at 360 MHz on a Bruker WM-360 instrument operating at ambient temperature (22±1°C). The deuterium contents were estimated by integration of the ^1H NMR spectra. All stereochemical studies were done in triplicate.

2.3.2.2 Solvent exchange into product

To monitor solvent exchange into alcohol, incubations were carried out as described for the stereochemical studies with the chiral amines (Section 2.3.2.1), except that reactions were in 100 mM deuterated potassium phosphate buffer, pD 7.2, containing 15-20 μmol of unlabeled tyramine.

2.4 Kinetic Isotope Effects

2.4.1 Steady-state kinetics for benzylamine oxidation

2.4.1.1 Materials

Benzylamine was purchased from Sigma Chemical Company (St. Louis, MO). [α,α-^{2}H]benzylamine was synthesized by Dr. C. Swyngedouw in our laboratory by reduction of benzonitrile using $LiAl^2H_4$ in anhydrous tetrahydrofuran (Bardsley *et al.*, 1973). The sheep serum amine oxidase used for the kinetic studies was the enzyme prepared in Section 2.2.2.2.

2.4.1.2 Methods

Rucker and Goettlich-Riemann (1972) reported a K_m of 0.1 mM for benzylamine oxidation catalyzed by sheep serum amine oxidase. Therefore, six concentrations of substrate, ranging from 0.025 mM to 1.0 mM of either unlabeled or deuterated benzylamine, were used in the kinetic experiments. Initial rate measurements were carried out in a total volume of 0.99 mL containing 100 mM potassium phosphate buffer, pH 7.0, and substrate equilibrated to 25°C. Reaction was initiated by the addition of 10 µL of SSAO (0.012 units). The initial rate of reaction was estimated spectrophotometrically by monitoring the production of benzaldehyde, which results in an increase in absorbance at 250 nm. All kinetic experiments were done in duplicate.

The initial rate data obtained for the various substrate concentrations was fitted to the expression:

$$v = V_{max}[S] / (K_m + [S]) \qquad (2\text{-}1)$$

with a FORTRAN program based on the Cleland method (Cleland, 1979), where v is initial rate, V_{max} is maximal velocity, [S] is substrate concentration and K_m is Michaelis-Menten constant corresponding to the substrate concentration at half of the maximum velocity.

2.4.2 Steady-state kinetics for tyramine oxidation

2.4.2.1 Materials

Tyramine was purchased from Eastman Kodak Company (Rochester, NY). [α,α-^{2}H]Tyramine was synthesized by Dr. C. Swyngedouw in our laboratory by reduction of 4-(methoxyphenyl)acetonitrile using $LiAl^2H_4$ and subsequent deprotection of methoxy-phenethylamine with HBr (Bardsley *et al.*, 1973, and Palcic *et al.*, 1983). Alcohol dehydrogenase (horse liver) and catalase (bovine liver) were obtained from Sigma Chemical Company (St. Louis, MO).

2.4.2.2 Methods

Based on the report of Palcic and Klinman (1983) that tyramine exhibited biphasic steady-state plots yielding K_m values of 1.3 mM and 52 mM for bovine plasma amine oxidase, six substrate concentrations ranging from 5 mM to 80 mM of unlabeled and deuterated tyramine were used for the sheep serum amine oxidase studies.

The rate of oxidation of tyramine to aldehyde catalyzed by SSAO was measured spectrophotometrically using a coupled enzyme system containing alcohol dehydrogenase (ADH) and NADH. In this coupled system, the 4-hydroxyphenylacetaldehyde produced by the amine oxidase is converted directly to 4-hydroxyphenethylalcohol (tyrosol) by alcohol dehydrogenase. NADH (molar absorptivity of $6.22 \times 10^3\ M^{-1}\ cm^{-1}$ at 340 nm) is converted to NAD^+ in the system, resulting in a decrease in absorbance at 340 nm.

Initial rate measurements were carried out in a total volume of 0.96 mL containing 100 mM potassium phosphate buffer, pH 7.0, and substrate equilibrated to 25°C. Reaction was initiated by the addition of 40 μL of SSAO (0.04 units). The initial rate data obtained for the various tyramine concentrations was fitted to Equation (2-1), as described in Section 2.4.1.2. All kinetic experiments were done in duplicate.

3. RESULTS AND DISCUSSION

3.1 Isolation of Porcine Plasma Amine Oxidase

A summary of the purification of porcine plasma amine oxidase is given in Table 3.1. The increase in enzyme purity at the various isolation steps is reflected in increasing specific activity (units/mg). The enzyme was purified 1,360 fold with an overall recovery of 25%.

All steps were carried out according to the literature (Falk *et al.*, 1983; Coleman *et al.*, 1989), except for the inclusion of AH-Sepharose chromatography, which had not been tried in the isolation of porcine plasma enzyme. The AH-Sepharose column was a new step introduced to modify the preparation, so serial elution with phosphate buffer of varying ionic strengths (45, 55, 75 and 100 mM) was attempted on AH-Sepharose. The resultant specific activity of 0.11 units/mg was an improvement compared to the 0.057 units/mg reported by Coleman *et al.* (1989) and equivalent to that of Falk *et al.* (1983), while the yield (25%) was much higher than that of the latter workers (5.3%).

The results of the gel electrophoresis at each purification step are shown in Figure 3.1. All gels were 11 % acrylamide. Approx. mol. wt. estimated from the gels of PPAO, which is a dimer, is 194,000 and this is in good agreement with values in literature (Falk *et al.*,1983). The final enzyme is highly purified as estimated by electrophoresis. This enzyme has been used by Ms. A. Coleman in our laboratory in stereochemical studies of the C-2 exchange process.

3.2 Isolation of Sheep Serum Amine Oxidase

A summary of the purification procedure is given in Table 3.2. The enzyme with the highest purity was purified about 24 fold. While the 40% saturated ammonium sulfate extraction was found to be the most effective purification step (6-fold increase in specific activity, compared with one after previous step), the calcium phosphate absorption step turned out to be disappointing. The concentrated enzyme solution after Step 6, the DE-52

Table 3.1 Purification of porcine plasma amine oxidase.

Purification Step	*Units*[a]	*Specific activity (units/mg)*	*Purification (fold)*	*Yield (%)*
Serum	60	8.1×10^{-5}	1	100
$(NH_4)_2SO_4$ fractionation (35-55%)	42	2.3×10^{-4}	3	70
DE-52 30 mM	17.2	3.2×10^{-4}	4	61
100 mM	19.4	6.7×10^{-4}	8	
Con A	34.2	5.9×10^{-3}	73	57
AH-Sepharose	24.9	1.6×10^{-2}	200	42
Hydroxyapatite	23.2	5.6×10^{-2}	690	38
Bio-Gel A 1.5 m	15.5	1.1×10^{-1}	1360	25

[a] 1 unit = 1 μmol product/min at 25°C

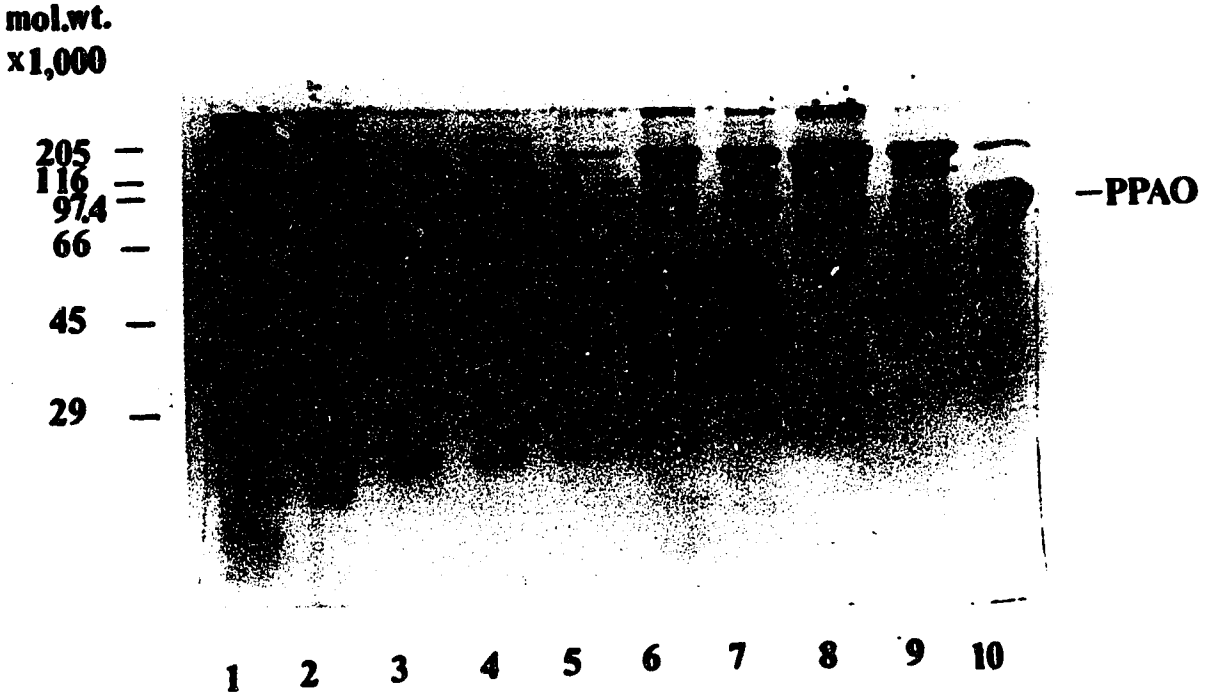


Figure 3.1. SDS-polyacrylamide gel electrophoresis of eluates after different purification steps in the porcine plasma amine oxidase isolation.

1. Standard (HMW)
2. Serum
3. & 4. Eluates of the DE-52 column
5. & 6. Eluates of the Con A column
7. Eluates of the AH-Sepharose column
8. & 9. Eluates of the Hydroxyapatite column
10. Eluates of the Bio-Gel A 1.5 m column

Table 3.2 Purification of sheep serum amine oxidase.

Purification Step		*Units*[a]	*Specific activity (units/mg)*	*Purification (fold)*	*Yield (%)*
Serum		139.0	5.5×10^{-4}	1	100
$(NH_4)_2SO_4$ fractionation (30-65%)		114.0	7.2×10^{-4}	1.3	82
$(NH_4)_2SO_4$ extraction (40%)		91.1	4.4×10^{-3}	8	66
Calcium phosphate gel adsorption		85.8	6.0×10^{-3}	11	62
$(NH_4)_2SO_4$ extraction (55%)		59.2	8.8×10^{-3}	16	43
DE-52 anion exchanger					
high activity fraction	1	19.8	1.3×10^{-2}	24	34
	2	23.0	1.0×10^{-2}	18	
low activity fraction		4.6	3.1×10^{-3}	6	

[a]1 unit = 1μmol product/min at 25°C

anion exchange chromatography, was stored at 4°C for 2 months without any significant loss of activity. Steps up to #5 (Section 2.2.2.2) were according to the literature (Rucker *et al.*, 1972). DE-52 chromatography and the subsequent chromatographic steps were introduced by us. The SDS-PAGE of the low activity DE-52 eluate (Figure 3.2) shows that the major contaminating proteins are albumin with a mol.wt. of approx. 66,000 and a 45,000 band. Hemoglobin (16,000) is also present in the sample and three high mol. wt. bands at approx. 97,000, 116,000 and 130,000 are evident. The minor band at mol. wt. approx. 97,000 is the enzyme, SSAO, based on the fact that the relative band intensity of mol. wt. 97,000 increased with improved specific activity of the enzyme solution. The low activity fraction was subjected to an additional series of chromatographic steps. The results of these steps are summarized in Table 3.3.

The Sephacryl S-300 SF (gel filtration) column was chosen to try to remove hemoglobin, albumin and the 45,000 dalton band, all of which have a lower mol. wt. than SSAO. The resolution of proteins was poor on the initial Sephacryl S-300 SF column and there was only a marginal increase in specific activity after this step. Chromatography on Blue Sepharose CL-6B was carried out on the combined fractions since this resin has been reported to adsorb albumin (Travis *et al.*, 1976). The gel in Figure 3.3, which shows sequential fractions from loading and washing of the Blue Sepharose column, demonstrates partial removal of both albumin and the 45,000 dalton band. However, this would not be a practical method of removing albumin. Con A remains the method of choice for removing albumin and hemoglobin, which are not glycosylated, from glycoproteins such as SSAO.

All fractions with activity (#27-35) were combined and applied to an AH-Sepharose column. AH-Sepharose was used because it is an affinity support for other amine oxidases, such as bovine (Svenson and Hynning, 1981) and porcine (Section 2.1) AO . These steps for the further purification of SSAO had not been attempted before elsewhere, and some proved to be unsatisfactory. For instance, the poor resolution attained from the first

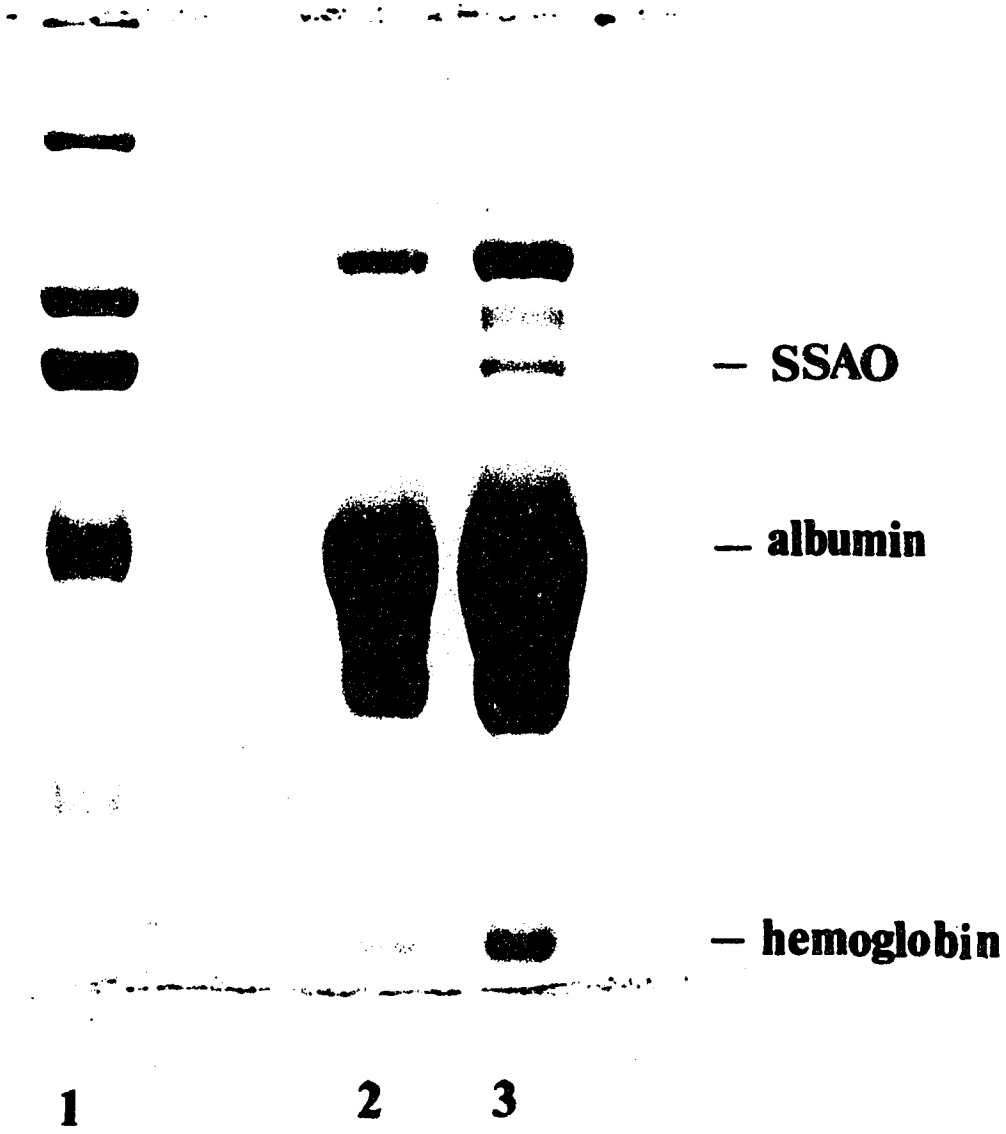


Figure 3.2. SDS-PAGE of low activity fraction from DE-52 of the sheep serum amine oxidase isolation.
1. Standard
2. & 3. Low activity fraction

Table 3.3 Further purification of SSAO.

Purification Step	*Units*	*Specific activity (units/mg)*	*Purification (fold)*	*Yield (%)*
DE-52 eluate (low activity fraction)	2.8	3.1×10^{-3}	1	100
Sephacryl S-300 SF #1	2.4	4.4×10^{-3}	1.4	86
Blue Sepharose CL-6B	1.8	1.1×10^{-2}	3.5	64
AH-Sepharose	1.2*	3.7×10^{-2}	12	43
Sephacryl S-300 SF #2	1.1	1.2×10^{-1}	39	29

* 1 mL (= 1 unit) of high activity fraction was added

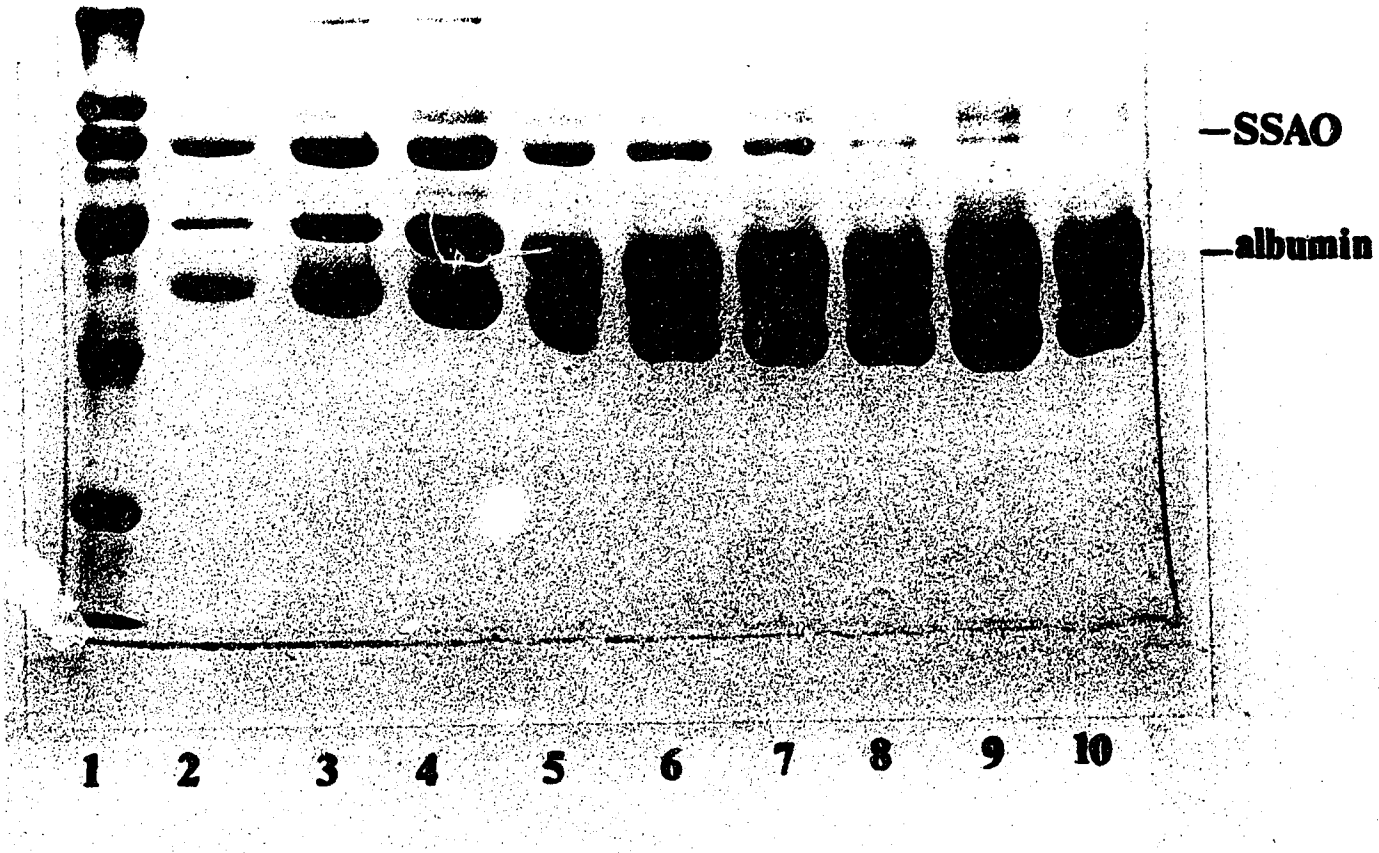


Figure 3.3. SDS-PAGE of Blue Sepharose eluates.
1. Standard
2. - 10. Fraction #27 → Fraction #35

Sephacryl S-300 column might be due to the low ionic strength (10 mM) of the buffer used. Eluates of the Blue Sepharose column at an early stage showed good resolution, while dimi[illegible] resolution towards the later stage of elution indicated that insufficient quantity of [illegible]harose CL-6B) was used (Figure 3.3). The cost of a larger column is pro[illegible]50 for 50 g of dry resin for the 2.5 x 50 cm column used in this preparation). [illegible] electrophoresis gels are included to illustrate the progress of the purification procedure. They are not of high quality due to an effort to make the SSAO band visible thereby resulting in considerable smearing of the major protein bands.

Though the AH-Sepharose column worked well, with a 3.4-fold increase in specific activity compared with the eluate from the Blue Sepharose column, the specific activity of the enzyme solution (0.037 units/mg) indicated that it might still contain a significant amount of contaminating protein. Therefore, the eluate of the AH-Sepharose column was rechromatographed onto the same Sephacryl S-300 SF column equilibrated with 100 mM buffer. The second gel filtration column gave an excellent resolution with a 3.2- fold purication. The high resolution of this column, compared with the first gel filtration column (10 mM buffer applied), might be due to the high ionic strength (100 mM) of the buffer. The Sephacryl gel contains carboxyl groups and thereby partial ionic interactions between the matrix and proteins can occur at low ionic strength. The specific activity of the final enzyme was 0.12 units/mg, which is the highest purification ever reported for SSAO.

Based on the results in this thesis a proposed scheme for the isolation of SSAO is: $(NH_4)_2SO_4$ fractionation (35-55%), then successive chromatography on DE-52 cellulose, Con A-Sepharose, hydroxyapatite and a Sephacryl S-300 SF column equilibrated with 100 mM potassium phosphate buffer, pH 7.4. It is suggested that the Con A-Sepharose column and hydroxyapatite column should be equilibrated and run as described for the porcine plasma amine oxidase, while the other steps are identical to those described for SSAO. An outline of the proposed isolation scheme for SSAO is given in Figure 3.5.

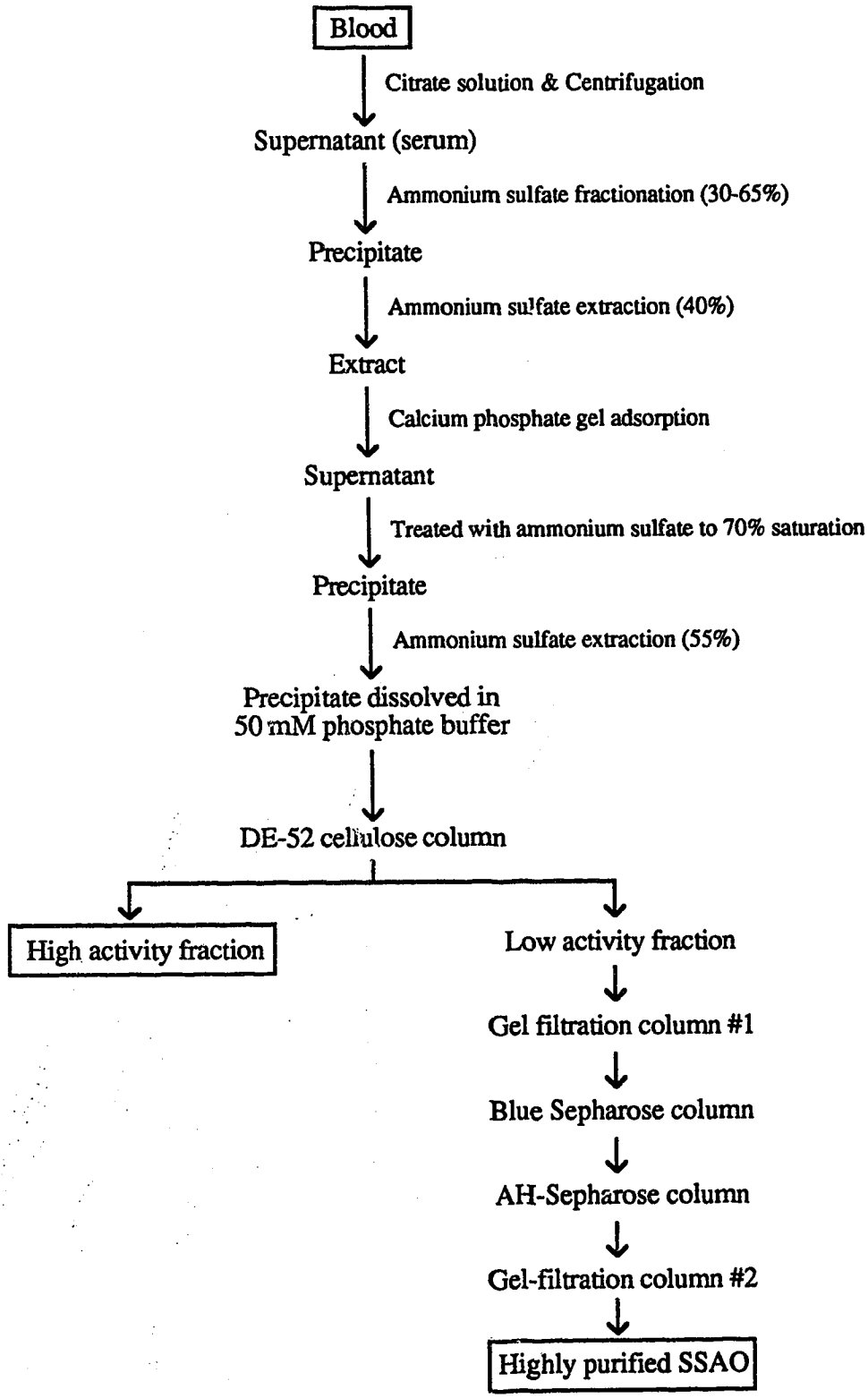


Figure 3.4. Outline of sheep serum amine oxidase isolation.

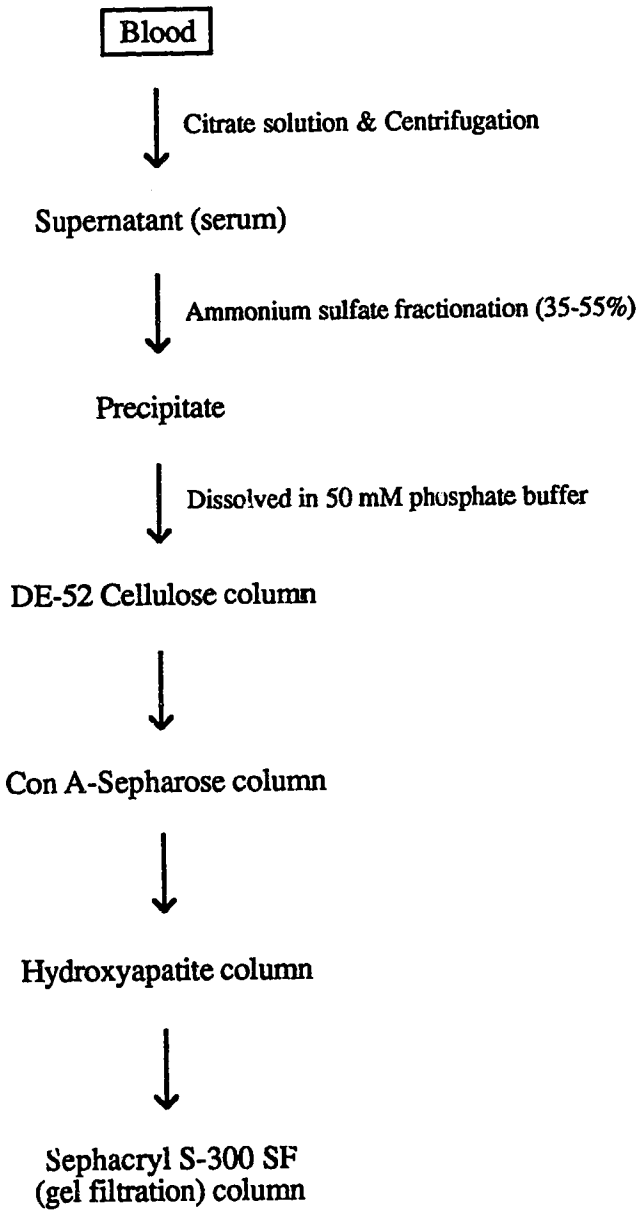


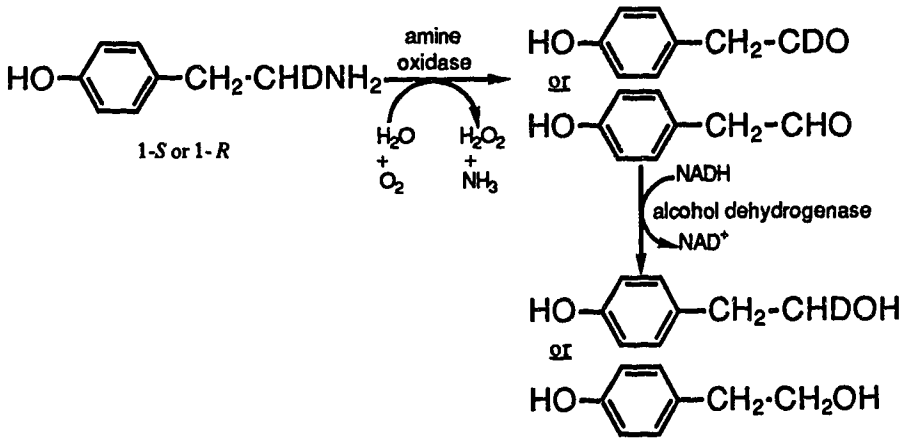
Figure 3.5. The proposed isolation scheme for SSAO.

3.3 Stereochemistry of Sheep Serum Amine Oxidase Reactions

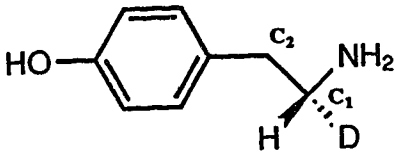
3.3.1 Oxidation of monodeuterated tyramines

Figures 3.6 and 3.7 show that the ^1H NMR spectra of stereospecifically deuterated tyramines isolated after incubation of L-tyrosine in ^2H_2O and DL-[2-^2H]tyrosine in H_2O, respectively, with tyrosine decarboxylase. The clean doublets at 2.89 ppm show that the deuteration was complete within the limits of sensitivity of the ^1H NMR spectra (≥98%) and the absolute configuration of the chiral tyramines are assigned based on the established stereospecificity of the tyrosine decarboxylase reaction with retention of configuration (Palcic and Floss, 1986).

Stereochemical studies with both chiral amines (1*S*- or 1*R*-tyramine) and sheep serum amine oxidase were carried out in a coupled manner such that the *p*-hydroxyphenyl acetaldehyde produced was reduced directly to *p*-hydroxyphenethyl alcohol by the action of alcohol dehydrogenase (ADH) and NADH.



Scheme 3-1



(1R)-[^{2}H]Tyramine

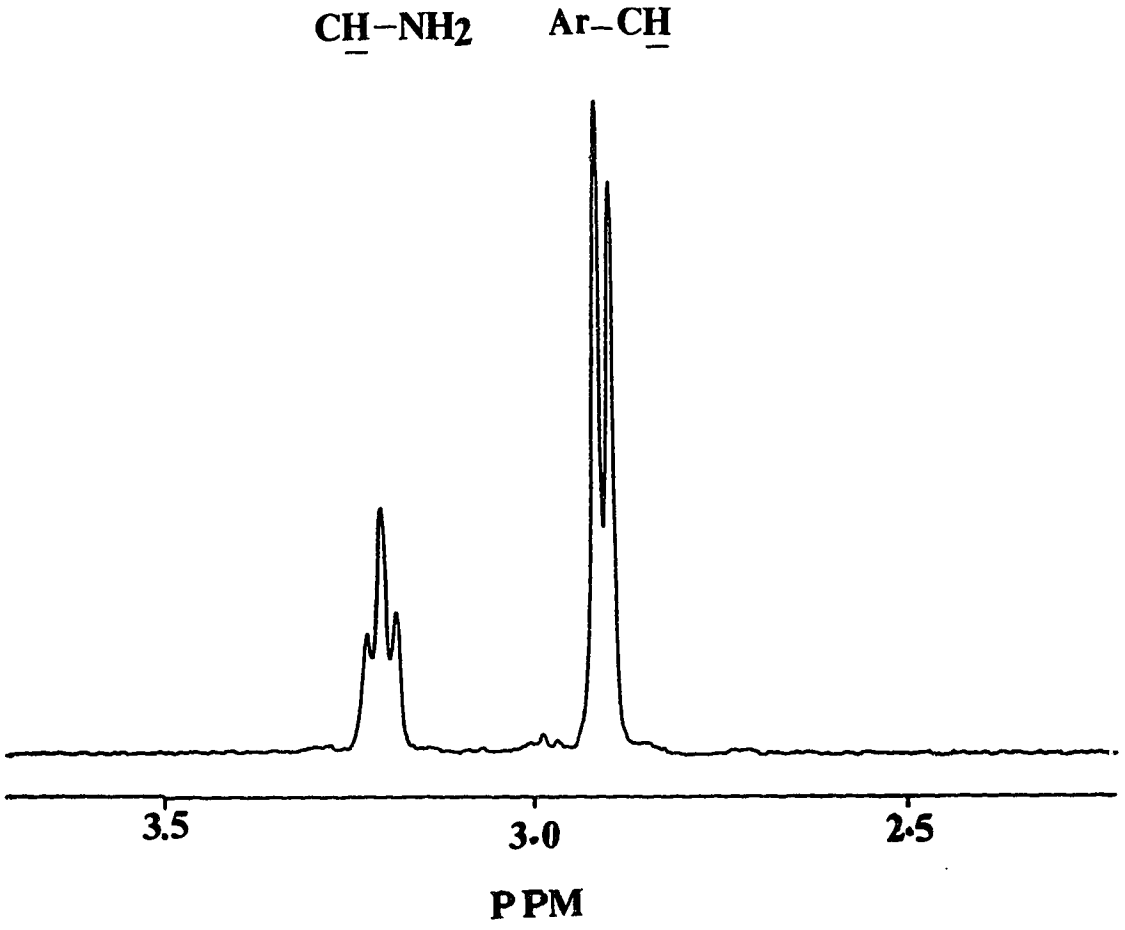
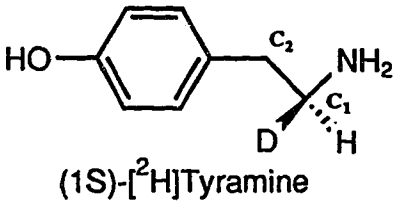


Figure 3.6. Partial 360 MHz ^{1}H NMR spectrum of (1*R*)-[^{2}H]tyramine derived from incubation of tyrosine decarboxylase with L-tyrosine in ^2H_2O.



(1S)-[^{2}H]Tyramine

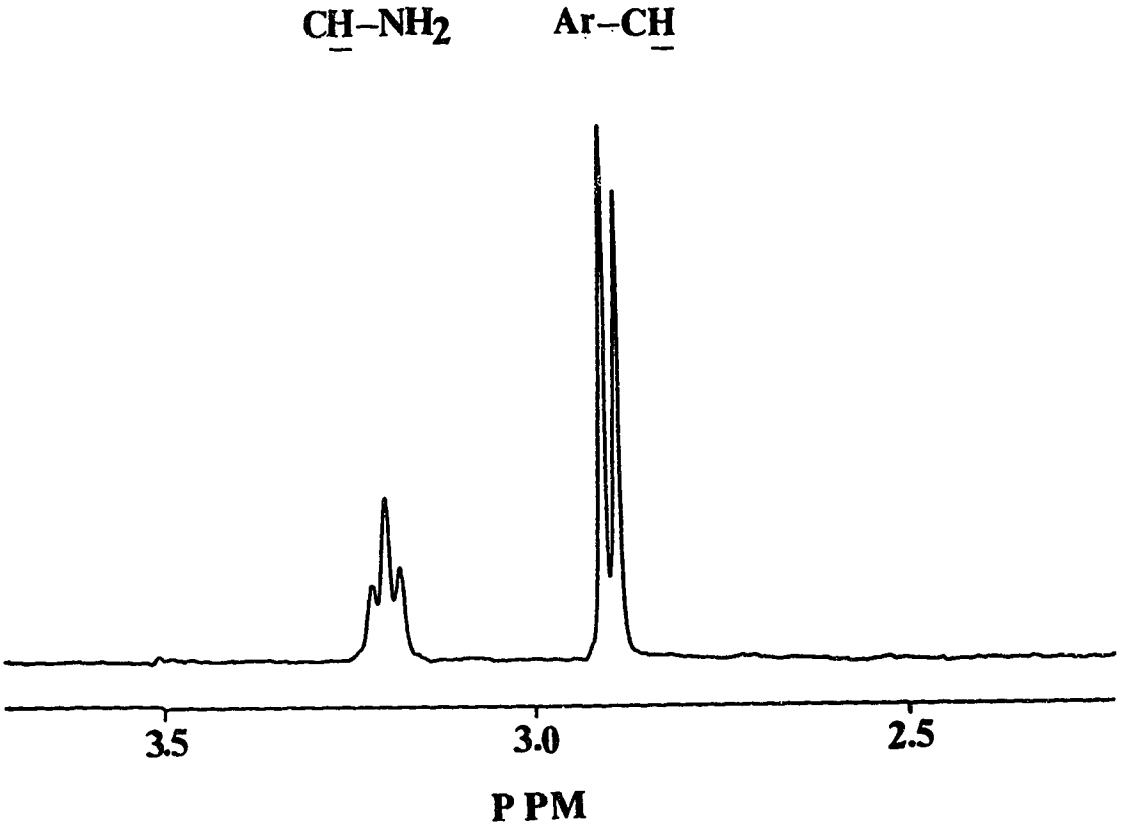


Figure 3.7. Partial 360 MHz ^{1}H NMR spectrum of (1*S*)-[^{2}H]tyramine derived from incubation of tyrosine decarboxylase with DL-[2-^{2}H]tyrosine in H_2O.

Figure 3.8 shows the ^1H NMR spectrum of *p*-hydroxyphenethyl alcohol (tyrosol) isolated from incubations with (1*S*)-[^2H]tyramine and sheep serum amine oxidase after the reaction was >90% complete. The spectral pattern shows a mixture of two alcohol products, one fully protonated and the other monodeuterated, with the latter being the major product. The pattern for this mixture is two overlapping triplets for the C-1 proton signals near 3.7 ppm and a doublet superimposed on a triplet for the C-2 protons near 2.8 ppm. The signals labeled N near 4.0 ppm are due to residual NADH, while that labeled X near 2.8 ppm is due to an unidentified impurity.

The minor triplet at 3.74 ppm arises from the product alcohol that is fully protonated at C-1. The major triplet at 3.72 ppm is from tyrosol that is monodeuterated at C-1. This signal is shifted about 0.02 ppm to higher field due to an α-deuterium isotope effect (Hansen, 1983). The signals for the C-2 protons are a triplet at 2.79 ppm exhibited by the minor product (fully protonated tyrosol). For the major product (monodeuterated tyrosol), a doublet for the C-2 protons is at 2.78 ppm, shifted upfield due to a ß-deuterium isotope effect. In this case, 89% of the deuterium of the original tyramine was retained in the product alcohols and 11% was lost, indicating that oxidation of the *S*-amine occurred in a partially nonstereospecific fashion.

The spectrum of the *p*-hydroxyphenethyl alcohol obtained from incubation of (1*R*)-[^2H]tyramine with sheep serum amine oxidase is shown in Figure 3.9. This sample also consists of a mixture of monodeuterated and fully protonated alcohols, with the monodeuterated product predominating. In this instance, 72% of the deuterium was retained in the product, indicating that reaction occurred in a partially nonstereospecific manner. These stereochemical studies demonstrate that sheep serum amine oxidase exhibits the rare mirror-image bindings and catalysis reported for bovine plasma amine oxidase (Summers *et al.*, 1979; Farnum *et al.*, 1986).

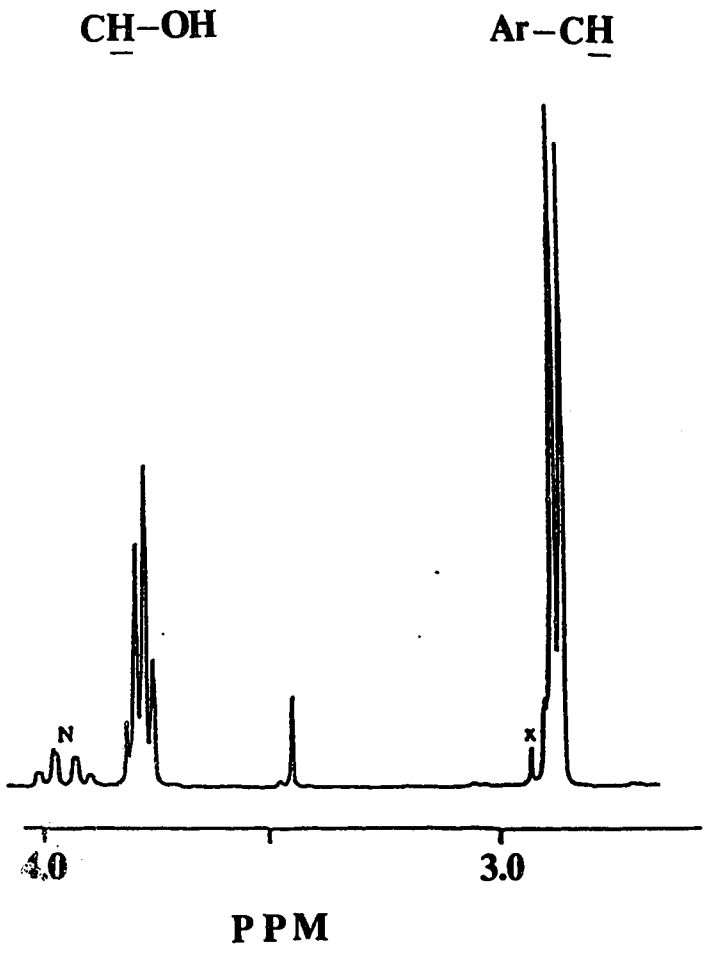


Figure 3.8. The ^{1}H NMR spectrum of alcohol derived from coupled incubation of (1*S*)-[^{2}H]tyramine and sheep serum amine oxidase with NADH and alcohol dehydrogenase.

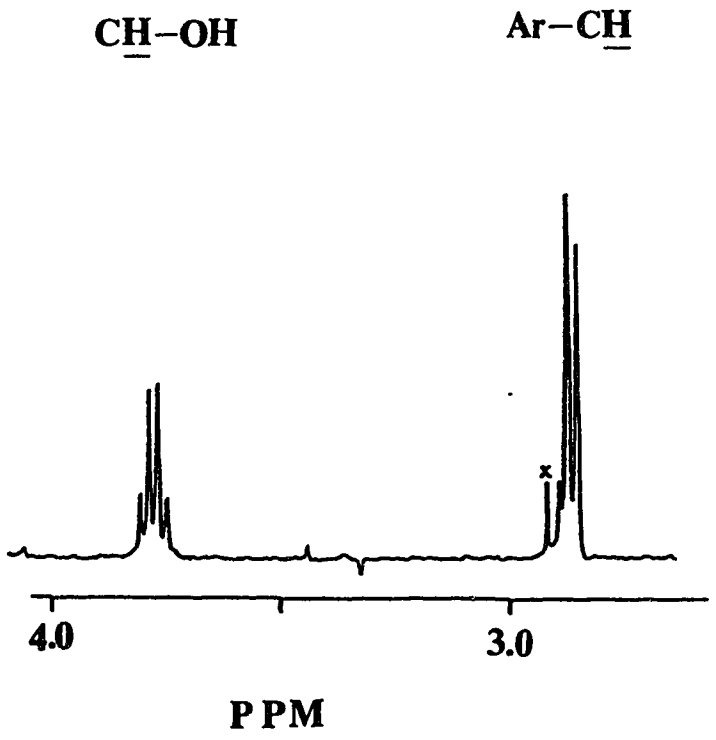
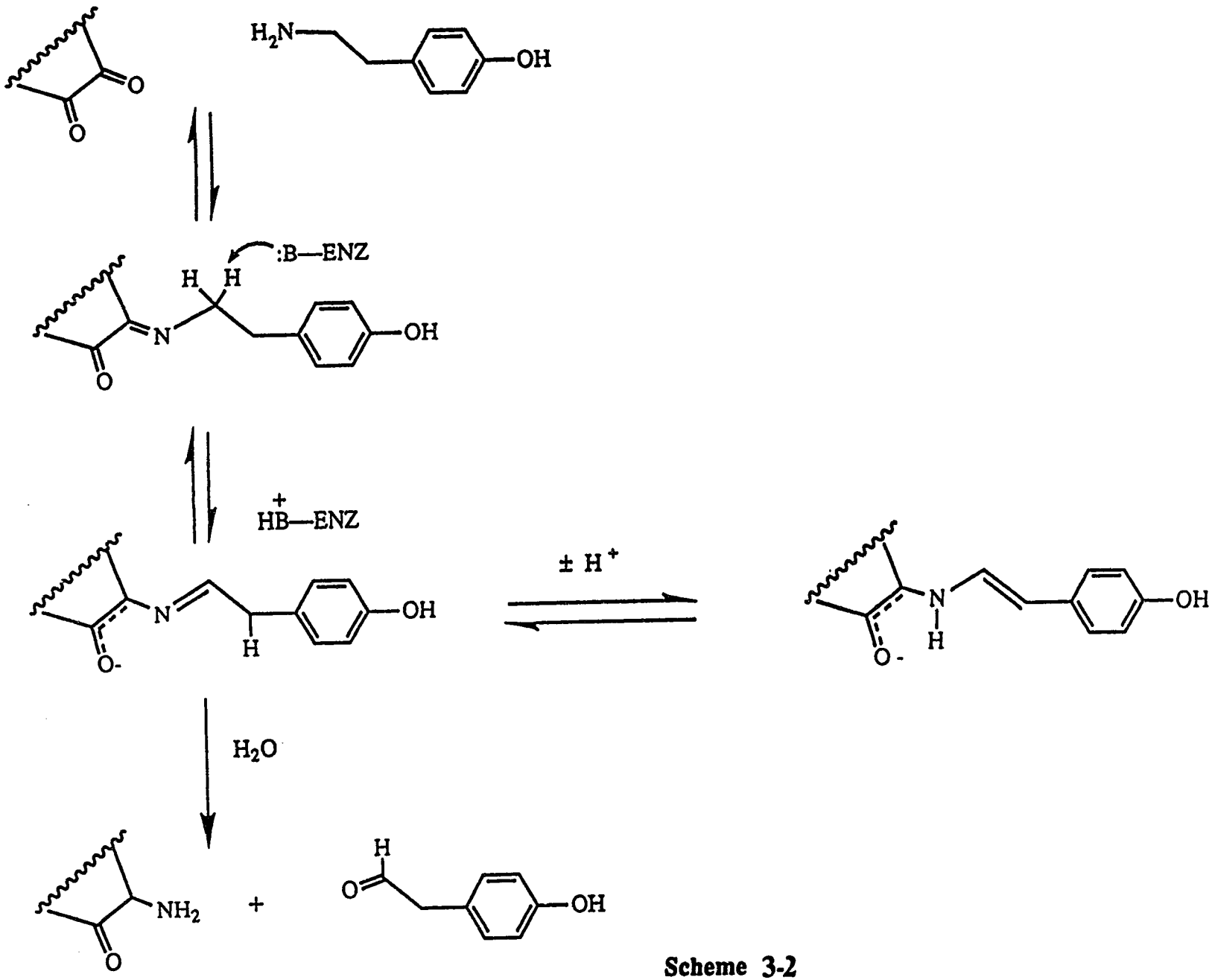


Figure 3.9. The ^{1}H NMR spectrum of alcohol derived from coupled incubation of (1*R*)-[^{2}H]tyramine and sheep serum amine oxidase with NADH and alcohol dehydrogenase.

A completely nonstereospecific reaction arising from equal flux through two catalytically competent modes, defined as pro-*S* or pro-*R* abstraction of hydrogen at C-1, would give a 1:1 mixture of monodeuterated and fully protonated alcohols. Any deviations from a ratio of 50/50 indicate preferred reaction through either the pro-*R* or pro-*S* abstraction mode. Preferred reaction through a mode can arise from a kinetic isotope effect (KIE) where the isotope effect results in a reduction in the rate of C-D bond cleavage relative to C-H bond cleavage. If a KIE of 6 is observed, the rate of reaction is 6-fold greater for C-H bond cleavage vs C-D bond cleavage. This would give products enriched in deuterium, i.e. 86% deuterated and 14% protonated. Differences in the deuterium content of the samples derived from (1*R*)- or (1*S*)-[^{2}H]tyramines arise from differences in the isotope effects in each mode.The KIE's estimated from the observed product ratios for the sheep serum amine oxidase are 8.1 for the *S* mode and 2.6 for the *R* mode. The reduced KIE for (1*R*)-tyramine indicates that a step other than C-H bond cleavage is partially rate-limiting in the *R* mode, which reduces the observed isotope effect.

An aminotransferase mechanism has been proposed for the copper amine oxidases, with initial Schiff base formation between the cofactor carbonyl and substrate amine (Scheme 3-2) (Farnum and Klinman, 1986). This is followed by base-catalyzed proton abstraction from C-1 of substrate such that the cofactor functions as an electron sink during catalysis, analogous to pyridoxal phosphate. Imine hydrolysis yields product aldehyde and an amino quinol as the reduced cofactor species (Hartman *et al.*, 1988).

For bovine plasma enzyme, the reduction in the observed isotope effect for the *R* mode has been attributed to a partially rate-limiting conformational change prior to imine hydrolysis. This reorientation does not occur in the *S* mode, hence the isotope effect is more fully expressed for these substrates.



Scheme 3-2

A summary of the stereochemical studies is shown in Table 3.4. A different KIE can be expressed for V_{max} (saturated substrate levels) or V_{max}/K_m conditions (sub-saturating substrate concentrations). If there is a difference in KIE for V_{max} and V_{max}/K_m, and the reaction goes from V_{max} to V_{max}/K_m control, there will be a difference in deuterium content in products isolated as a function of reaction progress. However, Table 3.4 shows that the amount of deuterium in products is constant for all tyrosols isolated after 50% reaction. This suggests that the KIE is the same for V_{max} and V_{max}/K_m or that the K_m for tyramine is high and reaction was under V_{max}/K_m control throughout.

3.3.2 Solvent exchange into product

Previous studies have reported that a solvent exchange pathway exists off the main catalytic pathway for several amine oxidases (Coleman *et al.*, 1989; Farnum *et al.*, 1986). This is attributed to reversible enamine formation after C-1 proton abstraction, thereby allowing for exchange of solvent into C-2 of product aldehydes (Scheme 3-2).

Figure 3.10 shows the spectrum of the alcohols obtained by incubation of sheep serum amine oxidase with fully protonated tyramine in ^2H_2O. Solvent is incorporated into products, evidenced by the overlapping triplets near 2.7 ppm and a doublet superimposed on a triplet near 3.7 ppm. The amount of deuterium incorporated into C-2 of products in duplicate experiments was 92 and 94% when reaction went to completion (>90%). The C-1 proton abstraction in Scheme 3-2 should be regarded as an irreversible step since the spectrum (Figure 3.10) indicates that no deuterium in ^2H_2O was incorporated into C-1 of products. Studies by Coleman *et al.* (1989) showed no solvent exchange in the case of the pro-*S* specific amine oxidases such as porcine kidney amine oxidase and chick pea amine oxidase. The absence of exchange can be attributed to kinetic or steric constraints for these enzymes. Kinetically, imine hydrolysis and solvent exchange via enamine formation are competing reactions (Scheme 3-2). Enzymes for which the rate of hydrolysis is rapid relative to enamine formation would function without an exchange

Table 3.4. Characteristics of ^1H NMR spectra of alcohols derived from coupled incubations of chiral amines with SSAO.

	Alcohols from S-tyramine		*Alcohols from R-tyramine*	
	diprotonated	*monodeuterated*	*diprotonated*	*monodeuterated*
Chemical shifts at				
C-1	3.74	3.72	3.73	3.71
C-2	2.79	2.78	2.79	2.78
Deuterium (%) at				
50% conversion		91		74
>90% conversion		87		70
KIE		8.1		2.6

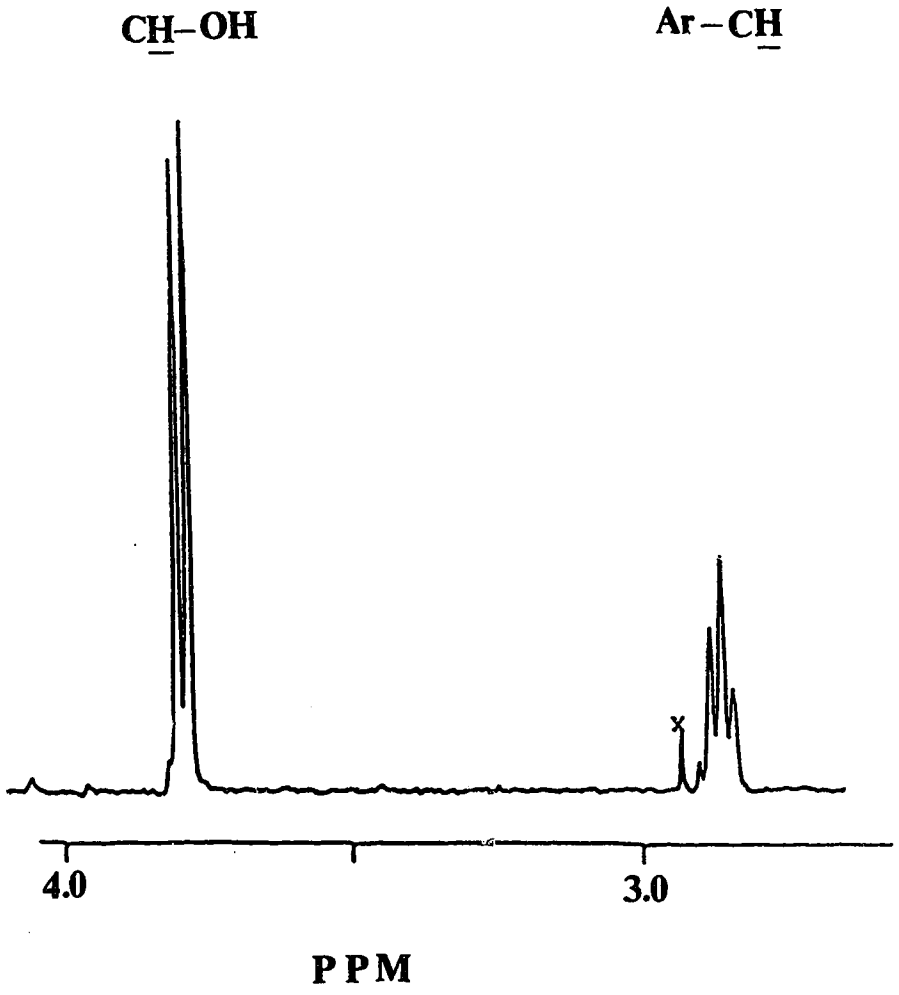


Figure 3.10. The ^{1}H NMR spectrum of alcohol derived from coupled incubation of sheep serum amine oxidase and tyramine in $^{2}H_2O$.

process. Alternatively, if enamine formation requires proton transfer to a residue of the enzyme active site, lack of a correctly positioned or unionized base to accept the proton would also preclude solvent exchange.

The nonstereospecificity of SSAO results from mirror-image binding and catalysis, which is a rare phenomenon in enzymology. There seems to be a correlation between the stereochemical course of proton abstraction at C-1 and a solvent exchange pathway into C-2 of products for all enzymes in the class. The pro-*R* specific and nonstereospecific enzymes have maintained the solvent wash-in pathway, while it has not been detected for any pro-*S* enzyme to date. More surprisingly, the modal preference for the nonstereospecific enzymes is uniform; the kinetic isotope effects for the pro-*R* abstraction mode are reduced relative to the pro-*S* mode for all nonstereospecific amine oxidases studied to date.

3.4 Kinetic Isotope Effects

The stereochemical studies with tyramine and sheep serum amine oxidase (SSAO) described in Section 3.3 demonstrated that two kinetically competent binding modes exist in the enzyme. One mode results in abstraction of the pro-*R* hydrogen of substrate, the other pro-*S* hydrogen abstraction. Furthermore, there is a difference in the kinetic isotope effect (KIE) for these modes, 8.1 for the *S* mode and 2.6 for the *R* mode. An isotope effect can be observed on V_{max} with substrate saturating and V_{max}/K_m with sub-saturating substrate concentrations. If a different KIE exists for V_{max} and V_{max}/K_m and the stereochemical studies are carried out starting with saturating substrate under V_{max} condition and shifted to V_{max}/K_m, the deuterium content in product can vary as a function of reaction extent.

To estimate the magnitude of KIE's, a series of steady-state kinetic measurements were carried out. The overall reaction catalyzed by SSAO occurs in two steps, as shown in Scheme 3-3 (Farnum *et al.*, 1986). This is a minimal kinetic scheme modeled on the bovine plasma amine oxidase reaction:

$$E_{ox}+RCH_2NH_3^+ \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_{ox}\cdot RCH_2NH_3^+ \underset{k_4}{\overset{k_3^*}{\rightleftharpoons}} E_{red}-N^+H=CHR \xrightarrow[H_2O]{k_5} E_{red}-NH_3^+(RCHO) \quad (3\text{-}1)$$

$$E_{red}-NH_3^+(RCHO) \xrightarrow[O_2]{k_6} E_{ox} + RCHO + NH_4^+ + H_2O_2 \quad (3\text{-}2)$$

Scheme 3-3

In the first step, after the formation of an E•S complex, proton abstraction and imine hydrolysis yield the aldehyde product and reduced enzyme (Eqn 3-1). This is followed by enzyme reoxidation by molecular oxygen, which gives H_2O_2 in the second step (Eqn 3-2).

Introduction of deuterium into substrate brings about unique perturbation of a single step in catalysis, k_3^*. For Scheme 3-3 above, the kinetic parameters and isotope effects on kinetic parameters are given as follows (Palcic *et al.*, 1983; Cleland, 1975):

$$V_{max} = \frac{k_3k_5k_6[E_t]}{k_3k_5 + k_3k_6 + k_6(k_4+k_5)} \quad (3\text{-}3)$$

$${}^DV_{max} = \frac{(V_{max})_H}{(V_{max})_D} = \frac{{}^Dk + k_{3H}/k_5 + k_{3H}/k_6 + {}^DK_{eq}k_{4H}/k_5}{1 + k_{3H}/k_5 + k_{3H}/k_6 + k_{4H}/k_5} \quad (3\text{-}4)$$

$$V_{max}/K_m = \frac{k_1k_3k_5[E_t]}{k_2(k_4+k_5) + k_3k_5} \quad (3\text{-}5)$$

$${}^DV_{max}/K_m = \frac{(V_{max}/K_m)_H}{(V_{max}/K_m)_D} = \frac{{}^Dk + k_{3H}/k_2 + {}^DK_{eq}k_{4H}/k_5}{1 + k_{3H}/k_2 + k_{4H}/k_5} \quad (3\text{-}6)$$

where ${}^DK_{eq} = \frac{(K_{eq})_H}{(K_{eq})_D} = \frac{k_{3H}k_{4D}}{k_{3D}k_{4H}}$ and ${}^Dk = \frac{k_{3H}}{k_{3D}}$

It can be seen that V_{max}/K_m includes all steps up to the first irreversible step, while V_{max} includes all steps after E_{ox}•S complex formation. ^Dk, the intrinsic isotope effect, is the full isotope effect originating from the single isotopically sensitive step of catalysis, exclusive of all effects from isotopically insensitive steps. An isotope effect is expressed as a decreased rate with deuterated substrates only if C-H bond cleavage is rate-limiting. Usually, in enzyme reactions bond cleavage is not fully rate-limiting, rather other slow steps such as product release, conformational changes, or reoxidation of the enzyme will reduce the magnitude of the observed isotope effect (Northrop, 1976).

The primary deuterium isotope effect for benzylamine oxidation catalyzed by sheep serum amine oxidase was determined by comparing reaction rates with unlabeled and dideuterated benzylamine (Figure 3.11). The kinetic paramaters obtained by computer analysis of this data using a program based on the Cleland method (Cleland, 1979) are listed in Table 3.5. It was shown that replacement of the hydrogen atoms at the α-carbon of benzylamine by deuterium decreased the steady-state maximum velocity by a factor of 1.44. The K_m was increased by a factor of 2.25, giving an isotope effect on V_{max}/K_m of 3.35.

The primary deuterium isotope effect for tyramine oxidation was obtained by comparing the reaction rates with unlabeled and dideuterated tyramine (Figure 3.12). The reaction was biphasic, therefore only the high substrate concentration points are shown. The data for low substrate concentrations showed considerable scatter. The kinetic parameters obtained by analysis of this data are also listed in Table 3.5. In this instance the kinetic isotope effect on V_{max} is 3.14 and there is no isotope effect on V_{max}/K_m. Because V_{max} and V_{max}/K_m are dependent on different components of the reaction mechanism (Eqn 3-4 and 3-6), the interpretation of isotope effects on V_{max} and V_{max}/K_m must also be different. Eqn 3-4 indicates that an isotope effect on V_{max} is governed by the forward ratios of catalysis, k_{3H}/k_5 and k_{3H}/k_6, while Eqn 3-6 shows that an isotope effect on V_{max}/K_m is influenced by the forward commitment to catalysis,

k_{3H}/k_2. Thus, $^{D}V_{max}$ of 1.44 and $^{D}V_{max}/K_m$ of 3.35 for benzylamine indicate that reoxidation of the enzyme is slow relative to the isotope-sensitive step (i.e. C-H bond cleavage), thereby abolishing the expression of the isotope effect by causing k_{3H}/k_6 to become large (Northrop, 1981). $^{D}V_{max}$ of 3.14 and $^{D}V_{max}/K_m$ of 0.9 (approx. 1.0) for tyramine suggest that imine hydrolysis and enzyme reoxidation are fast relative to C-H bond cleavage and that the slow step must be at dissociation of E•S complex suppressing the expression of the isotope effect by causing k_{3H}/k_2 to become large (Northrop, 1975).

The kinetic study of tyramine oxidation was initiated to try to account for the invariance in the deuterium content of the products isolated in the stereochemical studies. Two models were proposed. In the first, the K_m for tyramine is high and therefore the stereochemical studies were under V_{max}/K_m conditions throughout. Alternatively, the KIE on V_{max} and $V_{max}K_m$ is the same. The K_m's obtained in the kinetic study are 23 mM for unlabeled tyramine and 6.6 mM for dideuterated tyramine. Assuming the K_m for the monodeuterated tyramines will be in this range, the stereochemical study with initial substrate concentrations of 15 to 20 mM will be mainly under V_{max}/K_m control since substrate concentrations are well below saturating (10 x Km).

However, the KIE on V_{max}/K_m obtained in the kinetic study is approx. 1. For our nonstereospecific reaction an isotope effect of 1 would yield only products that retain 50 % deuterium. This contrasts with experimental values obtained of 89 % deuterium retention for the *S*- amine and 72 % for the *R*- amine. There is no straightforward explanation for this discrepancy. It can be noted that the kinetic experiments are initial rate measurements with negligible depletion of substrate while in the stereochemical study substrate concentration is continually being reduced. Estimates of the K_m for both (1*R*)- and (1*S*)-[^{2}H]tyramine should be obtained as well as estimates of $^{D}V_{max}$ and $^{D}V_{max}/K_m$ for these substrates. This study is

currently prohibitive with the limited supplies of chiral compound but will be carried out in the future.

Table 3.5 Kinetic parameters for the oxidation of benzylamine and tyramine.

	Benzylamine		*Tyramine*	
	Unlabeled	*Dideutero*	*Unlabeled*	*Dideutero*
K_m (mM)	0.12	0.27	23.0	6.6
V_{max} (nmole/min)	7.5	5.2	16.2	5.1
V_{max}/K_m (nmole/min•mM)	64.8	19.4	0.70	0.78
KIE on V_{max}	1.44		3.14	
KIE on V_{max}/K_m	3.35		0.90	

KIE on $V_{max} = (V_{max})_H/(V_{max})_D$

KIE on $V_{max}/K_m = (V_{max}/K_m)_H/(V_{max}/K_m)_D$

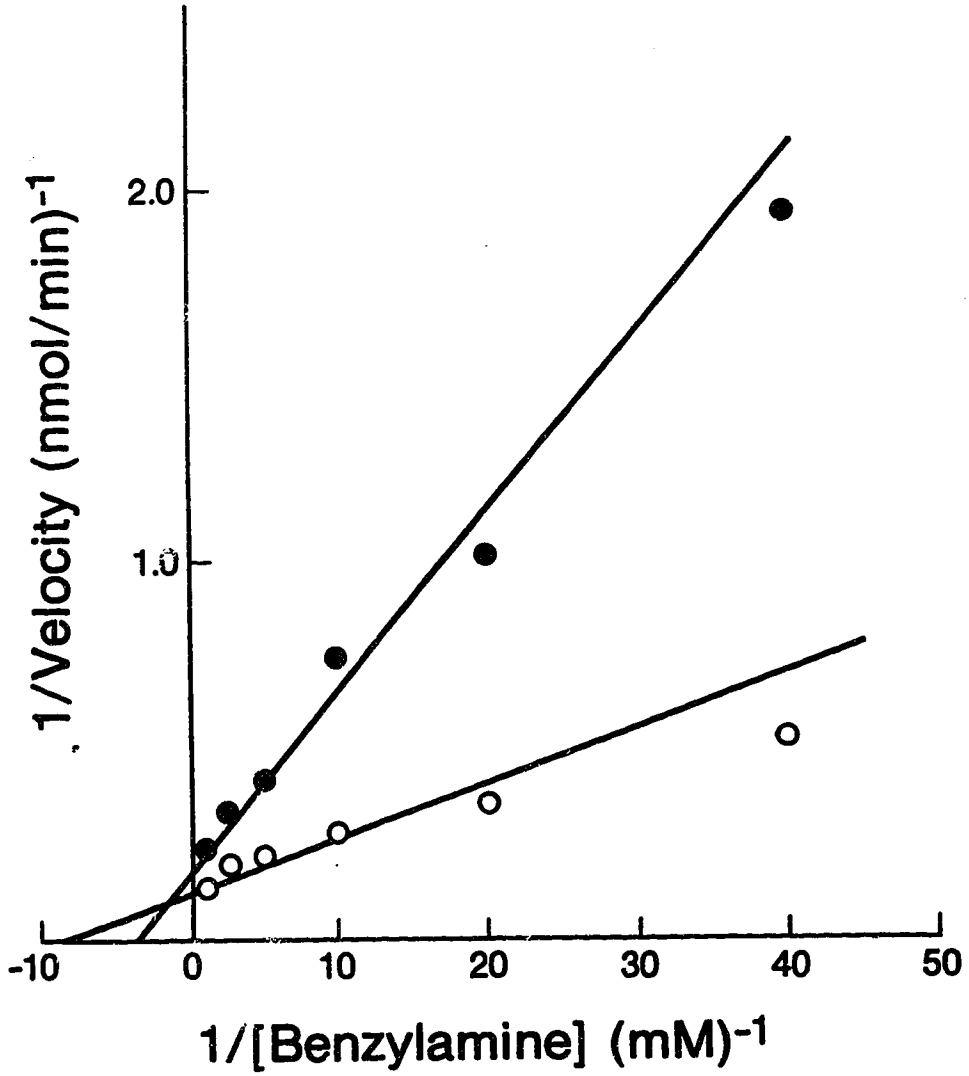


Figure 3.11. Double-reciprocal plot of initial rate data for the oxidation of benzylamine by sheep serum amine oxidase (0.012 units) in 100 mM potassium phosphate buffer, pH 7.0, at 25°C. The initial rates were measured by monitoring benzaldehyde formation at 250 nm. Open circles are for unlabeled benzylamine and closed circles are for dideuterated benzylamine.

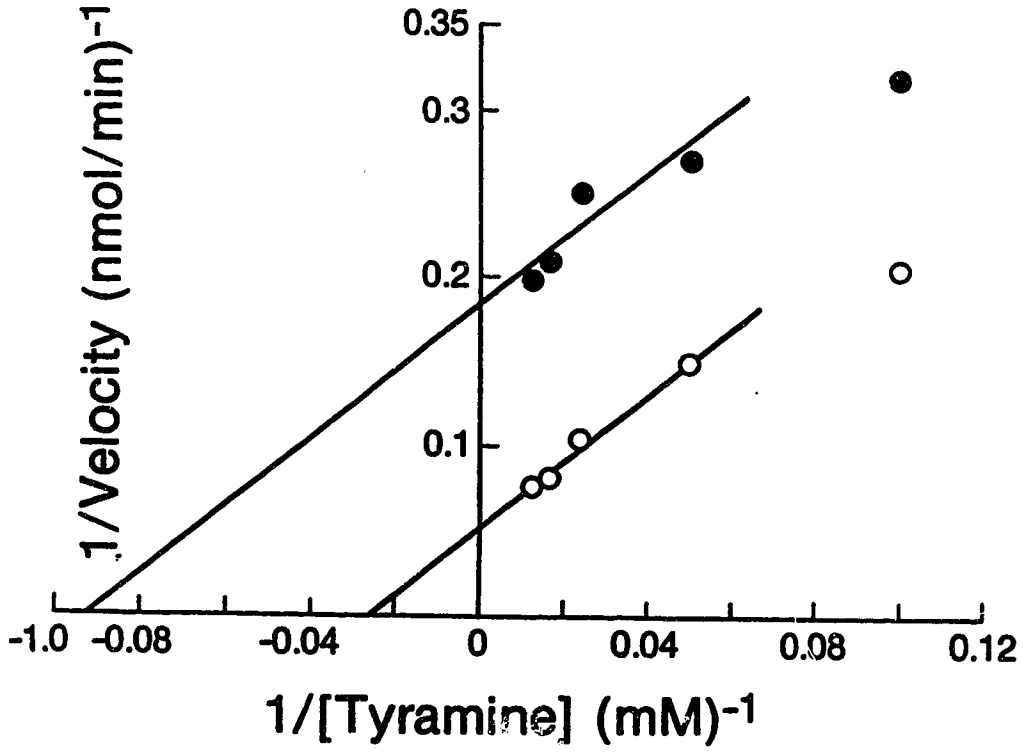


Figure 3.12. Double-reciprocal plot of initial rate data for the oxidation of tyramine by sheep serum amine oxidase (0.04 units) in a coupled system containing alcohol dehydrogenase and NADH in 100 mM potassium phosphate buffer, pH 7.0, at 25°C. The initial rates were measured by monitoring decrease in absorbance at 340 nm. Open circles are for unlabeled tyramine and closed circles are for dideuterated tyramine.

4. SUMMARY AND CONCLUSIONS

Porcine plasma amine oxidase was isolated from pig blood by applying ammonium sulfate fractionation (35-55%), chromatographic procedures using resins such as DE-52 cellulose, Con A-Sepharose, AH-Sepharose, hydroxyapatite, and finally a gel filtration colmn. The final enzyme, with a specific activity of 0.11 units/mg, was more than 90% pure as estimated by SDS-PAGE.

Sheep serum amine oxidase was isolated from sheep blood and purified by application of ammonium sulfate fractionation (30-65%), calcium phosphate gel adsorption, ammonium sulfate extraction (40 and 55%), a DE-52 anion exchanger, gel filtration (twice), Blue Sepharose CL-6B, and AH-Sepharose chromatography. The specific activity of the final enzyme was 0.12 units/mg.

The investigation carried out in this work showed that chiral tyramines were oxidized with a net nonstereospecific proton abstraction from C-1 by the sheep serum amine oxidase. The enzyme exhibited rare mirror-image binding (with differential flux) through two opposite and stereospecific reaction modes. Differential kinetic isotope effects were observed for each mode, 8.1 for the *S* mode and 2.6 for the *R* mode. The reduced KIE for (1*R*)-tyramine indicated that a step other than C-H bond cleavage is partially rate-limiting in the *R* mode, thus reducing the observed isotope effect.

The deuterium isotope effects on V_{max} and V_{max}/K_m with benzylamine and tyramine substrates were estimated in steady-state kinetic experiments. $^{D}V_{max}$ of 1.44 and $^{D}V_{max}/K_m$ of 3.35 for benzylamine indicate that reoxidation of the enzyme (SSAO) is slow relative to C-H bond cleavage, while $^{D}V_{max}$ of 3.14 and $^{D}V_{max}/K_m$ of 0.9 for tyramine show that the slow step must be at dissociation of E•S complex.

Although the above research revealed a great deal about this enzyme (SSAO), further studies are necessary on the stereochemistry of SSAO with other substrates, such as benzylamine, since bovine enzyme is stereospecific with benzylamine, but nonstereo-specific with dopamine and tyramine. Other areas deserving exploration include stereo-

chemistry of wash-in, amino acid sequence of active site, nature of the cofactor, and determination of intrinsic isotope effect.

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UNIVERSITY OF ALBERTA

STEREOCHEMISTRY OF SHEEP SERUM AMINE OXIDASE REACTIONS

by

Young J. Kang (C)

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SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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entitled **STEREOCHEMISTRY OF SHEEP SERUM AMINE OXIDASE REACTIONS**

submitted by **Young J. Kang**

in partial fulfillment of the requirements for the

degree of **Master of Science**

in **Food Chemistry**

M. Palcic, Supervisor

L. Ozimek

P. Sporns

M. Peppler

Date: Oct 11, 1990

DEDICATION

Dedicated to

My Mother, Father and Sisters

ABSTRACT

The stereochemical course of the oxidation of (1*R*)-[^{2}H]- and (1*S*)-[^{2}H]tyramine catalyzed by sheep serum amine oxidase has been investigated using ^{1}H NMR spectroscopy. Reactions were carried out in a coupled fashion such that the aldehydes produced by the amine oxidase were converted directly to alcohols with alcohol dehydrogenase. Both product alcohols obtained from incubations with the chiral amines and sheep serum amine oxidase contained deuterium, demonstrating that reaction occurred with net nonstereospecific proton abstraction from C-1. The enzyme also exhibited a solvent exchange pathway into C-2 of product. The magnitudes of kinetic isotope effects with different substrates were estimated in steady-state measurements by comparing the kinetic paramaters V_{max} and V_{max}/K_m obtained with fully protonated and dideuterated benzylamine and tyramine substrates. Benzylamine exhibited a kinetic isotope effect on V_{max} of 1.44 and V_{max}/K_m of 3.35, indicating that reoxidation of the enzyme is slow relative to the isotope sensitive step (i.e. C-H bond cleavage). Tyramine showed a kinetic isotope effect on V_{max} of 3.14 and V_{max}/K_m of 0.9, suggesting that reoxidation of the enzyme is rapid relative to the C-H bond cleavage step and the slow step must be at formation or dissociation of the intermediate E•S complex.

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Table of Contents

Chapter Page

1 INTRODUCTION 1

2 EXPERIMENTAL PROCEDURES 6

2.1 Isolation of Porcine Plasma Amine Oxidase 6

2.1.1 Materials 6

2.1.2 Methods 6

2.1.2.1 Assay of enzyme activity 6

2.1.2.2 Enzyme purification 7

2.2 Isolation of Sheep Serum Amine Oxidase 10

2.2.1 Materials 10

2.2.2 Methods 11

2.2.2.1 Assay of enzyme activity 11

2.2.2.2 Enzyme purification 11

2.3 Stereochemistry of Sheep Serum Amine Oxidase Reactions 15

2.3.1 Materials 15

2.3.2 Methods 15

2.3.2.1 Oxidation of monodeuterated tyramines 15

2.3.2.2 Solvent exchange into product 16

2.4 Kinetic Isotope Effects 17

2.4.1 Steady-state kinetics for benzylamine oxidation 17

2.4.1.1 Materials 17

2.4.1.2 Methods 17

2.4.2 Steady-state kinetics for tyramine oxidation 18

2.4.2.1 Materials 18

2.4.2.2 Methods 18

Chapter Page

3 RESULTS AND DISCUSSION....19

3.1 Isolation of Porcine Plasma Amine Oxidase....19

3.2 Isolation of Sheep Serum Amine Oxidase....19

3.3 Stereochemistry of Sheep Serum Amine Oxidase Reactions....30

3.3.1 Oxidation of monodeuterated tyramines....30

3.3.2 Solvent exchange into product....38

3.4 Kinetic Isotope Effects....41

4 SUMMARY AND CONCLUSIONS....49

5 REFERENCES....51

List of Tables

Table Page

3.1 Purification of porcine plasma amine oxidase.....20

3.2 Partial purification of sheep serum amine oxidase.....22

3.3 Further purification of sheep serum amine oxidase.....25

3.4 Characteristics of ^{1}H NMR spectra of alcohols derived from coupled incubations of chiral amines with sheep serum amine oxidase.....39

3.5 Kinetic parameters for the oxidation of benzylamine and tyramine.....46

List of Figures

Figure — Page

1.1 Structure of amine oxidase cofactors 5

3.1 SDS polyacrylamide gel electrophoresis of eluates after different purification steps in the porcine plasma amine oxidase isolation 21

3.2 SDS-PAGE of low activity fraction from DE-52 of the sheep serum amine oxidase isolation 24

3.3 SDS-PAGE of Blue Sepharose eluates 26

3.4 Outline of sheep serum amine oxidase isolation 28

3.5 The proposed isolation scheme for SSAO 29

3.6 Partial 360 MHz ^{1}H NMR spectrum of (1*R*)-[^{2}H]tyramine derived from incubation of tyrosine decarboxylase with L-tyrosine in ^2H_2O 31

3.7 Partial 360 MHz ^{1}H NMR spectrum of (1*S*)-[^{2}H]tyramine derived from incubation of tyrosine decarboxylase with DL-[2-^{2}H]tyrosine in H_2O 32

3.8 The ^{1}H NMR spectrum of alcohol derived from coupled incubation of (1*S*)-[^{2}H]tyramine and sheep serum amine oxidase with NADH and alcohol dehydrogenase 34

3.9 The ^{1}H NMR spectrum of alcohol derived from coupled incubation of (1*R*)-[^{2}H]tyramine and sheep serum amine oxidase with NADH and alcohol dehydrogenase 35

3.10 The ^{1}H NMR spectrum of alcohol derived from incubation of sheep serum amine oxidase and tyramine in ^2H_2O 40

3.11 Double-reciprocal plot of initial rate data for the oxidation of benzylamine by sheep serum amine oxidase (0.012 units) in 100 mM potassium phosphate buffer, pH 7.0, at 25°C 47

3.12 Double-reciprocal plot of initial rate data for the oxidation of tyramine by sheep serum amine oxidase (0.04 units) in a coupled system containing alcohol dehydrogenase and NADH in 100 mM potassium phosphate buffer, pH 7.0, at 25°C 48

List of Abbreviations

ADH	alcohol dehydrogenase
AO	amine oxidase
BPAO	bovine plasma amine oxidase
Con A	concanavalin A
DNA	deoxyribonucleic acid
E•S complex	enzyme•substrate complex
EC	Enzyme Commission
HMW	high molecular weight
^{D}k	intrinsic isotope effect
$^{D}k_{eq}$	isotope effect on equilibrium constant
$^{D}V_{max}$	isotope effect on V_{max}
$^{D}V_{max}/K_m$	isotope effect on V_{max}/K_m
KIE	kinetic isotope effect
V_{max}	maximal velocity
K_m	Michaelis constant
NADH	nicotinamide adenine dinucleotide (reduced form)
ppm	parts per million
PAGE	polyacrylamide gel electrophoresis
PPAO	porcine plasma amine oxidase
^{1}H NMR	proton nuclear magnetic resonance
R_f	relative mobility
RNA	ribonucleic acid
SSAO	sheep serum amine oxidase
SDS	sodium dodecyl sulfate
TLC	thin-layer chromatography
$[E_t]$	total enzyme concentration
TDC	tyrosine decarboxylase

1. INTRODUCTION

Amine oxidases catalyze the oxidative deamination of amines (mono-, di- and polyamines) with the stoichiometric formation of aldehyde as follows:

$$RCH_2NH_2 + O_2 + H_2O \longrightarrow RCHO + NH_3 + H_2O_2 \qquad (1\text{-}1)$$

These enzymes can be separated into three classes based on differences in cofactor, in substrate specificities and inhibitor sensitivities. The three classes are the flavin-containing monoamine oxidases (EC 1.4.3.4), the copper-containing amine oxidases (EC 1.4.3.6), and the semicarbazide-sensitive amine oxidases, which have not yet been given an Enzyme Commission designation (Mondovi, 1985).

Amine oxidases are ubiquitous, they are found in microorganisms as well as in higher forms of life. They are of great biological interest since many of their substrates are biogenic amines which are formed *in vivo* in various metabolic processes. Many of these biogenic amines appear to be involved in regulatory enzyme mechanisms, vital to higher mammals. For instance, polyamines such as spermine and spermidine seem to influence the synthesis of DNA and RNA (Kapeller-Adler, 1970 and Feuerstein & Marton, 1989).

The physiological role in neurotransmitter metabolism of the first class of enzymes, the flavin-containing monoamine oxidases, has made them the subject of intensive investigation, notably as targets for antidepressant drugs (Kapeller-Adler, 1970). The semicarbazide-sensitive amine oxidases found in vascular tissue and fat have not been well studied. At present, the nature of the prosthetic group is unknown, however, kinetically and stereochemically they appear to be distinct from the other two classes of amine oxidases (Callingham and Barrand, 1987).

The copper amine oxidases, which are the subject of this thesis, are widespread in nature — they are found in microorganisms, plant seedlings, serum, kidney, intestinal mucosa, liver and lung. In mammals they were first described as spermidine oxidase activity found in the serum of sheep (Hirsh, 1953) and oxen (Tabor *et al.*, 1953).

The first report of a copper amine oxidase in plants was made in 1948 by Werle and coworkers. They reported the occurrence of an enzyme that catalyzes the degradation of 1,4-diaminobutane, 1,5-diaminopentane, and histamine in extracts of some leguminous plants, sage and lavender.

An oxidative degradation of spermidine and spermine by bacterial preparations was first observed by Silverman and Evans in 1944, who used whole cells or lyophilized cell extracts of *Pseudomonas pyocyaneae (aeruginosa).*

Most copper amine oxidases lack strict substrate specificity. For instance, the classical serum amine oxidase catalyzes oxidative deamination of aromatic amines (such as tyramine and benzylamine) and peptidyl lysine, in addition to polyamines. Blaschko and coworkers (1959), who were some of the first investigators studying mammalian plasma amine oxidases, reported that ruminant enzyme acted on spermine and spermidine, while these were not significantly attacked by the non-ruminant plasma amine oxidase. The non-ruminant enzyme acted relatively rapidly on benzylamine and mescaline, but more slowly on tyramine and many monoamines. Blaschko also reported that the amine oxidases of mammalian plasma were inhibited by carbonyl reagents.

One of the best-characterized copper enzymes, bovine plasma amine oxidase, has a mol. wt. of about 170,000 and apparently consists of two identical subunits (Mondovi, 1985). Porcine plasma amine oxidase, which has a mol. wt. of approximately 195,000, is also a dimer (Falk *et al.*, 1983). A concentrated solution of either enzyme is pink in color.

When Hirsh (1953) first found spermine oxidase activity in sheep serum, it was a crude preparation, derived only from clotting and centrifugation of the blood. Sheep serum amine oxidase (SSAO) was isolated and purified by Rucker and Goettlich-Riemann (1972) through a series of steps involving ammonium sulfate fractionation, extraction and calcium adsorption. Though its purity (0.032 units/mg) was an improvement over previous preparations, it was considerably lower than those reported for bovine (0.36 units/mg) and

porcine (0.1 units/mg) plasma amine oxidases. Therefore, one objective of the research described in this thesis was to isolate and further purify SSAO.

Mammalian copper amine oxidases have long been recognized to play key roles in cellular processes. They catalyze the oxidative removal of biogenic amines from blood plasma, the crosslinking of collagen and elastin in connective tissue biogenesis, and the regulation of intracellular spermine and spermidine levels (Hartmann and Klinman, 1988).

The role of copper in these enzymes is not clear and mechanistic studies of these enzyme systems have been seriously hampered by the uncertainty as to the exact nature of the covalently bound cofactor. It was first believed to be pyridoxal phosphate (Mondovi *et al.*, 1967), and then thought to be pyrroloquinoline quinone (PQQ) (Moog *et al.*, 1986). More recently, bovine plasma amine oxidase has been shown to contain 3,4,6-trihydroxyphenylalanine (Topa). Topa has been found in the pentapeptide at the active site:

Leu - Asn - X - Asp - Tyr

where X is Topa

Topa formation might represent a new posttranslational modification involving oxidation of an active site tyrosine (Janes *et al.*, 1990) (Figure 1.1).

Copper amine oxidases are characterized by unusual stereochemical patterns in their reactions. Dopamine and tyramine are oxidized with abstraction of the pro-*R* hydrogen at C-1 by the porcine plasma amine oxidase, the pro-*S* hydrogen by pea seedling amine oxidase, and a net nonstereospecific proton abstraction by the bovine plasma enzyme (Coleman *et al.*, 1989), while benzylamine oxidation catalyzed by the same enzyme (BPAO) is stereospecific with abstraction of the pro-*S* hydrogen at C-1. The pro-*R* (or pro-*S*) hydrogen refers to one of the chemically identical hydrogens at a prochiral centre i.e. the methylene carbon of benzylamine or C-1 of tyramine. If one of the methylene hydrogens at this centre is replaced by a deuterium, then a chiral centre is produced. The designation pro-*R* is used for the deuterium substitution that generates a chiral centre with an *R* configuration as defined by

Cahn- Ingold- Prelog system. The designation pro-*S* is used for the deuterium substitution that generates a chiral centre with an *S* configuration. For a review of stereochemical nomenclature used in this thesis see Walsh (1979). The copper amine oxidases provide the first example in which a reaction catalyzed by enzymes in the same formal class occurs by all three possible stereochemical routes. Solvent exchange profiles are consistent within each stereochemical class of enzyme: the pro-*R* and nonstereospecific enzymes exchange solvent into C-2 of product aldehydes, the pro-*S* enzymes do not.

In this study, the stereochemical course of oxidation of tyramine was established for another copper amine oxidase isolated from sheep serum (SSAO). The deuterium isotope effects with benzylamine and tyramine substrates were also estimated in steady-state kinetic experiments. In addition to these kinetic and stereochemical studies of SSAO, porcine plasma amine oxidase was isolated for use in other stereochemical studies and to gain experience in enzyme isolation methodologies.

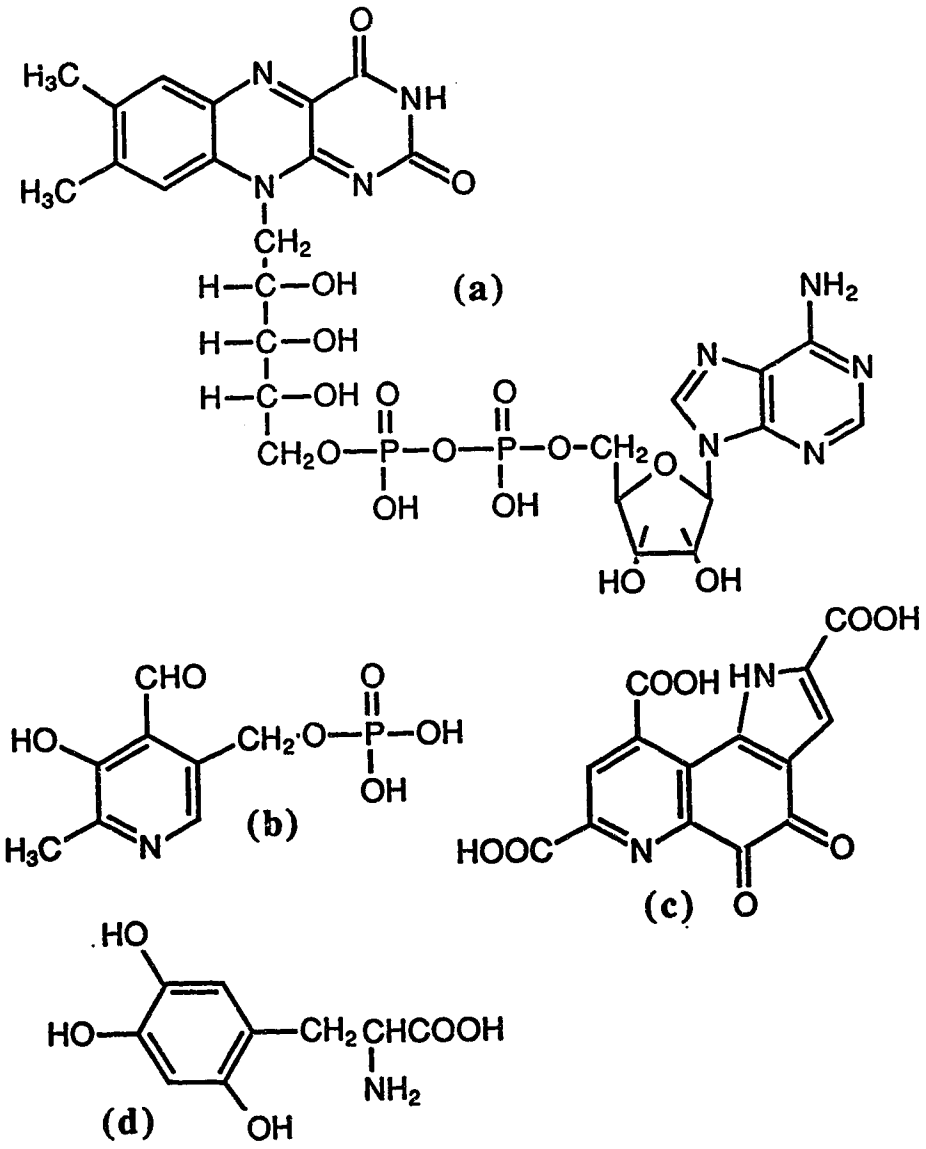


Figure 1.1. Structure of amine oxidase cofactors.
(a) Flavin adenine dinucleotide (FAD)
(b) Pyridoxal-5'-phosphate (PLP)
(c) Pyrroloquinoline quinone (PQQ)
(d) 3,4,6-Trihydroxyphenylalanine (Topa)

2. EXPERIMENTAL PROCEDURES

2.1 Isolation of Porcine Plasma Amine Oxidase (PPAO)

The enzyme was isolated from pig blood by the method of Falk *et al.* (1983) and Coleman *et al.* (1989) with some modifications. A new step (AH-Sepharose chromatography) was introduced to try to improve the preparation.

2.1.1 Materials

Fresh pig blood was obtained from a local slaughterhouse (Gainers, Edmonton, AB). Benzylamine was purchased from Sigma Chemical Company (St. Louis, MO). Hydroxyapatite (Bio-Gel HTP), Bio-Gel A 1.5 m and electrophoresis reagents were obtained from Bio-Rad Laboratories (Mississauga, ON). Con A-Sepharose was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), DE-52 cellulose was from Whatman (Maidstone, England), and AH-Sepharose was prepared by Ms. C. Scaman in our laboratory. The latter is CNBr-activated Sepharose 4B reacted with 1,6-diaminohexane (Svenson & Hynning, 1981).

2.1.2 Methods

2.1.2.1 Assay of enzyme activity

Amine oxidase activity was estimated spectrophotometrically. Assays were carried out at 25°C in 1 mL of 50 mM sodium phosphate buffer, pH 7.2, containing 3.33 mM benzylamine. The production of benzaldehyde was determined by monitoring the increase in absorbance at 250 nm. An extinction coefficient of 1.2×10^4 M^{-1} cm^{-1} was used to calculate the amount of benzaldehyde formed. One unit of activity was defined as the amount of enzyme catalyzing the production of 1 μmol of benzaldehyde per min under the standard assay conditions. Protein concentration was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as a protein standard. Spectral

measurements were made with a Beckman DU-8 spectrophotometer equipped with a Peltier temperature controller.

2.1.2.2 Enzyme purification

All steps were carried out at 4°C.

Step 1 Citration of blood and centrifugation.

Fresh pig blood (26 L) was mixed with 4.4 L of citrate solution (8 g of citric acid and 22 g of sodium citrate dihydrate/L) to prevent coagulation. The citrated blood was centrifuged at 6,400 x g for 25 min. The red and white blood cells were pelleted and discarded.

Step 2 $(NH_4)_2SO_4$ fractionation (35 - 55 %): salting out.

This process involves removal of proteins which precipitate before 35 % $(NH_4)_2SO_4$ saturation and proteins that are soluble beyond 55 % $(NH_4)_2SO_4$ saturation.

The supernatant (plasma, 16 L) was treated with solid ammonium sulfate to 35% saturation (209 g $(NH_4)_2SO_4$/L supernatant). The precipitate was separated by centrifugation at 6,400 x g for 25 min and discarded. The supernatant was brought to 55% saturation by the addition of 129 g $(NH_4)_2SO_4$/L and centrifuged at 11,300 x g for 25 min. The precipitate was dissolved in a minimal volume of 10 mM sodium phosphate buffer, pH 7.0, and was dialyzed against four changes of 16 L 10 mM sodium phosphate buffer, pH 7.0. After dialysis, the solution (2 L) was centrifuged at 17,700 x g for 25 min to remove solid particles.

Step 3 DE-52 anion exchange chromatography.

DEAE-cellulose is positively charged at pH 6-9, thus proteins with net negative surface charges would be adsorbed to the column while proteins with a positive charge or an overall charge near zero will pass through the column resulting in partitioning of the protein of interest. Since porcine plasma amine oxidase has an isoelectric point (pI) of 4.5 to 5.0, it will be negatively charged at pH 7.0 and adsorb to the resin.

One third of the clarified supernatant, i.e. 680 mL, was applied to a Whatman DE-52 cellulose column (5 x 50 cm) and the column was washed with 10 mM sodium phosphate buffer, pH 7.0, until the protein content of the eluate was close to baseline levels as monitored by the absorbance at 280 nm. Amine oxidase was eluted at a flow rate of 2.5 mL/min with 30 mM sodium phosphate buffer (1,200 mL), pH 7.0, followed by 400 mL of 100 mM sodium phosphate buffer, pH 7.0 (22 mL/fraction). Enzyme activity was found in both eluates (30 mM and 100 mM).

Step 4 Con A-Sepharose chromatography.

This step relies on the fact that Con A binds molecules which contain α-D-mannopyranosyl, α-D-glucopyranosyl and sterically related residues. Therefore, Con A-Sepharose is suitable for separation and purification of glycoproteins, glycolipids and polysaccharides from non-glycosylated proteins.

The fractions which eluted from the DE-52 column were concentrated by ultrafiltration (PM-10 membrane) and applied to a Con A-Sepharose column (5.5 x 23 cm) which had been equilibrated with a pH 7.0 buffer containing 10 mM sodium phosphate, 150 mM sodium chloride, 1 mM manganese chloride, and 1 mM calcium chloride. The column was washed with the starting buffer until the protein concentration dropped to baseline levels. Enzyme was eluted with 1,040 mL of starting buffer containing 100 mM methyl α-mannopyranoside (22 mL/fraction) at a flow rate of 0.76mL/min. The eluate was concentrated by ultrafiltration using a PM-30 membrane and dialyzed against 10 mM sodium phosphate buffer, pH 8.0.

Step 5 AH-Sepharose chromatography.

This affinity column is based on the fact that CNBr-activated AH-Sepharose contains an aminohexyl group which is an amine oxidase substrate analog and a charged group such as the isourea moiety which can introduce anion exchange character.

The dialyzed enzyme solution was applied to an AH-Sepharose column (2.5 x 23 cm) equilibrated with 10 mM sodium phosphate buffer, pH 8.0, and washed with the same buffer. Enzyme was eluted stepwise with 45 mM, 55 mM, 75 mM, and 100 mM potassium phosphate buffer, pH 8.0. Activity was found in all four eluates, with 64% of activity occurring in the 45 mM eluate. Steps 3-5 were carried out on the remaining two-thirds of the clarified supernatant from Step 2 as 650 mL and 670 mL volumes, respectively.

All eluates with activity were combined, concentrated by ultrafiltration, clarified by centrifugation, and dialyzed against 6 mM sodium phosphate buffer, pH 7.0. At this point the enzyme solution was divided into two portions of 100 mL each, and both were taken through Steps 6 and 7.

Step 6 Hydroxyapatite (crystalline calcium phosphate) column.

The mechanism of adsorption of proteins to hydroxyapatite is still not clear. It is believed to be that the adsorption of proteins to hydroxyapatite (HA) is due to polar dipole-dipole bonding between

H_3N^+ - protein
:
: and
:
PO_4^{3-} - HA

^-OOC - protein
:
: and
:
Ca^{2+} - HA

Generally, proteins that chromatograph well on anion exchangers also bind to HA, but low molecular weight proteins show very low affinity for hydroxyapatite.

A 100 mL aliquot of the dialyzed enzyme solution was loaded onto a hydroxyapatite column (2.5 x 52 cm) which had been equilibrated with the 6 mM sodium phosphate buffer, pH 7.0, and was eluted with 400 mL of 6 mM and 220 mL of 60 mM sodium phosphate buffer, pH 7.0. Activity was found in the 6 mM eluate. The eluate was concentrated in an Amicon ultrafiltration cell by using a PM-30 membrane and dialyzed against 10 mM sodium phosphate buffer, pH 7.0.

Step 7 Bio-Gel A 1.5 m (gel filtration) column.

This chromatography involves the separation of proteins in aqueous media according to size. The separation is due to the different length of time spent by the protein solutes within the liquid phase that is entrapped by the gel matrix. That is, the larger the molecule, the smaller is the fraction of the pores accessible to them. This results in a decrease in the time spent within the liquid phase inside the pores of the matrix. Therefore larger proteins elute from the colmn faster.

The concentrated enzyme solution (3 mL) was loaded onto a Bio-Gel A 1.5 m column (2.5 x 100 cm) and eluted with the starting buffe at a flow rate of 0.35 mL/min.

Step 8. Aliquots of sample after each purification step were assayed for activity and protein content. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was also carried out by the method of Laemmli (1970) using a Bio-Rad Mini Protean II vertical slab gel electrophoresis cell equipped with 1.5 mm spacers. The gels (11%) were run at 18 ma constant current for about 2 hr, and stained for protein with 0.35% Coomassie Brilliant Blue R-250 dissolved in 45:10:45 (v/v/v) methanol:acetic acid:water. Destaining was done by several changes of the same solvent.

2.2 Isolation of Sheep Serum Amine Oxidase

Sheep serum amine oxidase was isolated from sheep blood by the method of Rucker and Goettlich-Riemann (1972). These workers used a series of ammonium sulfate precipitations and calcium phosphate gel treatments to partially purify the enzyme. An additional series of chromatographic steps was implemented in this study to try to provide highly purified enzyme.

2.2.1 Materials

Fresh sheep blood was obtained from a slaughterhouse (Lamco, Innisfail, AB). Benzylamine was purchased from Sigma Chemical Company (St. Louis, MO). Blue

Sepharose CL-6B and Sephacryl S-300 SF were from Pharmacia Fine Chemicals (Uppsala, Sweden). Electrophoresis reagents were from Bio-Rad Laboratories (Mississauga, ON), and DE-52 cellulose was from Whatman (Maidstone, England). Calcium phosphate gel was prepared by the method of Kunitz (1952).

2.2.2 Methods

2.2.2.1 Assay of enzyme activity

Amine oxidase activity was assayed at 25°C in 1 mL of sodium phosphate buffer, pH 7.6, containing 3.33 mM benzylamine. One unit was defined as the amount of enzyme catalyzing the production of 1 μmol of benzaldehyde per min under assay conditions. The production of benzaldehyde was monitored spectrophotometrically at 250 nm and calculations for benzaldehyde production were based on a molar absorptivity of 1.2×10^4 M^{-1} cm^{-1}. Protein concentration was determined by the Bradford (1976) method using bovine serum albumin as a protein standard. Spectral measurements were made on a Hewlett-Packard 8451A diode array spectrophotometer thermostated with a circulating water bath.

2.2.2.2 Enzyme purification

All steps were carried out at 4°C. The procedure follows the flow chart (Figure 3.4) on page 28.

Step 1 Citration of blood and centrifugation.

Fresh sheep blood (12 L) was mixed with 2 L of citrate solution (8 g of citric acid and 22 g of sodium citrate dihydrate/L), the citrated blood was centrifuged at 10,000 rpm with a Beckman JA-10 rotor (17,700 x g) for 20 min, and the supernatant (serum, 7 L) was collected. High g value applied for sheep enzyme was due to the fact that

separation of pellet was not satisfactory at low centrifugal force probably because of a large amount of clotted blood.

Step 2 $(NH_4)_2SO_4$ fractionation (30-65%).

Solid ammonium sulfate was added to the serum (176 g $(NH_4)_2SO_4$/L serum) to 30% saturation, and the precipitate was removed after centrifugation at 17,700 x g for 20 min. The supernatant (7.2 L) was brought to 65% saturation by the addition of 235 g $(NH_4)_2SO_4$/L supernatant, and the precipitate was separated by centrifugation.

Step 3 $(NH_4)_2SO_4$ extraction (40%).

The precipitate was suspended in 40% saturated ammonium sulfate solution (900 mL) which was adjusted to pH 7.4. After standing for 30 min, the suspension (1 L) was centrifuged at 17,700 x g for 30 min and the particulate material was discarded. The supernatant was dialyzed against a 20-volume excess of 20 mM sodium phosphate buffer, pH 7.4, with three changes.

Step 4 Calcium phosphate gel adsorption.

The theory involved is the same as described for the hydroxyapatite column except that the gel is not suitable for chromatography because of low flow rate. It can be used in batch-wise adsorption treatments.

The dialyzed solution (1,240 mL) was treated with calcium phosphate gel (370 mL) at a ratio of 22.2 mL gel/g protein, and the precipitate was discarded. The supernatant, which contained all the enzyme activity, was treated with another portion of calcium phosphate gel (100 mL) and centrifuged. Most enzyme activity remained in the supernatant, instead of being adsorbed to calcium phosphate gel #2, therefore the calcium phosphate gel adsorption procedure was stopped at this stage.

Step 5 $(NH_4)_2SO_4$ extraction (55 %).

The supernatant (1.4 L) from Step 4 was treated with ammonium sulfate to 70% saturation (472 g $(NH_4)_2SO_4$/L supernatant). The precipitate which formed was separated by centrifugation and resuspended in 55% saturated ammonium sulfate solution (150 mL)

at pH 7.4. The 55% ammonium sulfate precipitate was collected by centrifuging at 17,700 x g for 30 min, dissolved in 50 mM phosphate buffer (105 mL), pH 7.4, and dialyzed against three changes of 3.5 L of 10 mM sodium phosphate buffer, pH 7.4.

Step 6 DE-52 anion exchange chromatography.

Half (90 mL) of the dialyzed enzyme solution was loaded onto a Whatman DE-52 cellulose column (5.5 x 23 cm) equilibrated with 10 mM phosphate buffer, pH 7.4, at a flow rate of 0.92 mL/min and washed with the starting buffer until the protein concentration was near baseline (approximately 200 mL of eluate collected) as monitored by absorbance at 280 nm. Amine oxidase was eluted with 30 mM sodium phosphate buffer, pH 7.4, again until the protein concentration reached baseline (1,360 mL of eluate), and then eluted with 100 mM sodium phosphate buffer (340 mL), pH 7.4. Enzyme activity was found in the 30 mM and 100 mM eluate as well as in the 10 mM wash. The remaining half (90 mL) of the dialyzed enzyme solution from Step 5 was loaded onto a Whatman DE-52 cellulose column and treated the same way as the first half of the dialyzed enzyme solution. High activity fractions from all eluates were pooled together into two groups according to specific activity (1.32×10^{-2} and 1.03×10^{-2} units/mg), concentrated by ultrafiltration (PM-30 membrane), and used in the stereochemical and kinetic studies. Low activity fractions from the 30 mM and 100 mM eluates were combined (3.12×10^{-3} units/mg), concentrated, and purified further. The final concentrated solution was blue in color, suggesting that ceruloplasmin was also present in the sample.

Step 7 Sephacryl S-300 SF (gel filtration) column #1.

Further purification of the low activity fraction was carried out as follows: The enzyme solution (8 mL) was loaded onto a Sephacryl S-300 SF column (2.5 x 100 cm) which had been equilibrated with 10 mM potassium phosphate buffer, pH 7.4, and was eluted with the same buffer at a flow rate of 0.55 mL/min. The eluates with activity (105 mL) were concentrated to 10 mL, dialyzed against 50 mM potassium phosphate buffer containing 100 mM KCl, pH 7.0.

Step 8 Blue Sepharose CL-6B (affinity) column.

The blue dye (Cibacron Blue F3GA) ligand has an affinity for enzymes that bind to purine nucleotides due to structural similarity between the two types of molecules. Albumin is also known to bind to the dye perhaps through electrostatic and hydrophobic interactions with the aromatic anionic ligand.

The eluates from Step 7 were applied to a Blue Sepharose CL-6B column (2.5 x 50 cm), and eluted with the starting buffer. The eluates with activity (50 mL) were pooled together, dialyzed against 10 mM potassium phosphate buffer, pH 8.0.

Step 9 AH-Sepharose column.

The dialyzed solution was loaded onto an AH-Sepharose column (2.5 x 23 cm). The column was washed with the starting buffer, and eluted with 45 mM, 55 mM, and gradient (50-300 mM) potassium phosphate buffer, pH 8.0. Activity was found in both the 45 mM (75 mL) and gradient eluates at approximately 75 mM (140 mL). The eluates with activity were combined and concentrated to 10 mL, 1 mL (approx. 1 unit) of the high activity fraction was added, samples were centrifuged to remove precipitate.

Step 10 Sephacryl S-300 SF (gel filtration) column #2.

The solution from Step 9 was loaded again onto a Sephacryl S-300 SF column (2.5 x 100 cm) equilibrated with 100 mM potassium phosphate buffer, pH 7.4. The enzyme was eluted with the starting buffer. Eluates (60 mL) from fractions #57 to #68 (5 mL/fraction) showed activity with elution volume of 280 mL.

Step 11

With the exception of Sephacryl column #1, SDS-PAGE was carried out on the eluates from DE-52 column to Sephacryl column #2, as described in Step 8 of Section 2.1.2.2.

2.3 Stereochemistry of Sheep Serum Amine Oxidase (SSAO) Reactions

2.3.1 Materials

Tyramine was purchased from Eastman Kodak Company (Rochester, NY). NADH (disodium salt, trihydrate), alcohol dehydrogenase from equine liver (1.6 units/mg) and catalase from bovine liver (11,000 units/mg) were obtained from Sigma Chemical Company (St. Louis, MO). (1*S*)- and (1*R*)-[^{2}H]tyramine were available from previous stereochemical studies (Coleman *et al.*, 1989). They were prepared by incubation of DL-[2-^{2}H]tyrosine with tyrosine decarboxylase (TDC) in protonated buffer or L-tyrosine with TDC in deuterated buffer. Deuterated buffer was prepared by making protonated buffer of the desired pH (7.0), evaporating solvent under vacuum, and dissolving the dried buffer salts in exactly the same volume of D_2O as the evaporated H_2O. The final pD of the buffer solution was 7.2, where pD refers to the uncorrected pH meter reading of the deuterated buffer. SSAO was exchanged into deuterated buffer by concentrating SSAO to 0.5 mL by ultrafiltration and resuspending in 0.5 mL of 100 mM potassium phosphate buffer, pD 7.2, and 5 mL of ^2H_2O. Concentration and resuspension were carried out three times to ensure the complete exchange of solvent.

2.3.2 Methods

All stereochemical studies were carried out in a coupled fashion by the method of Battersby *et al.* (1979) and Coleman *et al.* (1989) with slight modifications. The reaction was coupled to convert reactive aldehyde to stable alcohol and also to monitor the progress of the reaction by measuring decrease in absorbance at 340 nm.

2.3.2.1 Oxidation of monodeuterated tyramines

The following components were incubated in 1.0 mL of 100 mM potassium phosphate buffer, pH 7.2, at 25°C: 15-20 μmol of stereospecifically deuterated tyramine

(1*S*- or 1*R*-), sheep serum amine oxidase (0.3 units), 25-30 μmol NADH, 5-8 units of alcohol dehydrogenase, and 11,000 units of catalase. The conversion of amine to alcohol was monitored by the removal of aliquots (5 μL) at timed intervals, the aliquots were diluted with 995 μL of the buffer, and $\Delta Abs_{340\ nm}$ was measured.

At 50 or 100% completion of reaction, the incubation mixtures were diluted to 10 mL with water and loaded onto reverse-phase C_{18} Sep-pak cartridges which had been washed with 10 mL of methanol and 20 mL of water before use. The cartridges were rinsed with 5 mL of water, then the alcohol product was eluted with 10 mL of methanol. The methanol eluate was concentrated under reduced pressure, dissolved in 2 mL of ethyl acetate:*n*-hexane (2:1 v/v), and applied to a flash silica column (2g; 1 x 25 cm) which had been prewashed with 10 mL of ethyl acetate:*n*-hexane (2:1 v/v). The alcohol product (tyrosol) was eluted with 20 mL of the same solvent and collected in culture tubes (approx. 0.7 mL/fraction). This step effectively removed any remaining amine and NADH which remained bound to the column.

Aliquots of all the fractions were spotted on silica gel TLC plates, developed with the same solvent ($R_f = 0.51$ for *p*-hydroxyphenethyl alcohol), and the fractions containing the product were pooled. The tyrosol solution was concentrated to dryness under reduced pressure, dissolved in a solvent of $(^2H_3C)_2CO$: ^2H_2O (4:1 v/v), and ^1H NMR spectra were measured at 360 MHz on a Bruker WM-360 instrument operating at ambient temperature (22±1°C). The deuterium contents were estimated by integration of the ^1H NMR spectra. All stereochemical studies were done in triplicate.

2.3.2.2 Solvent exchange into product

To monitor solvent exchange into alcohol, incubations were carried out as described for the stereochemical studies with the chiral amines (Section 2.3.2.1), except that reactions were in 100 mM deuterated potassium phosphate buffer, pD 7.2, containing 15-20 μmol of unlabeled tyramine.

2.4 Kinetic Isotope Effects

2.4.1 Steady-state kinetics for benzylamine oxidation

2.4.1.1 Materials

Benzylamine was purchased from Sigma Chemical Company (St. Louis, MO). [α,α-^{2}H]benzylamine was synthesized by Dr. C. Swyngedouw in our laboratory by reduction of benzonitrile using $LiAl^2H_4$ in anhydrous tetrahydrofuran (Bardsley *et al.*, 1973). The sheep serum amine oxidase used for the kinetic studies was the enzyme prepared in Section 2.2.2.2.

2.4.1.2 Methods

Rucker and Goettlich-Riemann (1972) reported a K_m of 0.1 mM for benzylamine oxidation catalyzed by sheep serum amine oxidase. Therefore, six concentrations of substrate, ranging from 0.025 mM to 1.0 mM of either unlabeled or deuterated benzylamine, were used in the kinetic experiments. Initial rate measurements were carried out in a total volume of 0.99 mL containing 100 mM potassium phosphate buffer, pH 7.0, and substrate equilibrated to 25°C. Reaction was initiated by the addition of 10 μL of SSAO (0.012 units). The initial rate of reaction was estimated spectrophotometrically by monitoring the production of benzaldehyde, which results in an increase in absorbance at 250 nm. All kinetic experiments were done in duplicate.

The initial rate data obtained for the various substrate concentrations was fitted to the expression:

$$v = V_{max}[S] / (K_m + [S]) \qquad (2\text{-}1)$$

with a FORTRAN program based on the Cleland method (Cleland, 1979), where v is initial rate, V_{max} is maximal velocity, [S] is substrate concentration and K_m is Michaelis-Menten constant corresponding to the substrate concentration at half of the maximum velocity.

2.4.2 Steady-state kinetics for tyramine oxidation

2.4.2.1 Materials

Tyramine was purchased from Eastman Kodak Company (Rochester, NY). [α,α-^{2}H]Tyramine was synthesized by Dr. C. Swyngedouw in our laboratory by reduction of 4-(methoxyphenyl)acetonitrile using $LiAl^2H_4$ and subsequent deprotection of methoxyphenethylamine with HBr (Bardsley *et al.*, 1973, and Palcic *et al.*, 1983). Alcohol dehydrogenase (horse liver) and catalase (bovine liver) were obtained from Sigma Chemical Company (St. Louis, MO).

2.4.2.2 Methods

Based on the report of Palcic and Klinman (1983) that tyramine exhibited biphasic steady-state plots yielding K_m values of 1.3 mM and 52 mM for bovine plasma amine oxidase, six substrate concentrations ranging from 5 mM to 80 mM of unlabeled and deuterated tyramine were used for the sheep serum amine oxidase studies.

The rate of oxidation of tyramine to aldehyde catalyzed by SSAO was measured spectrophotometrically using a coupled enzyme system containing alcohol dehydrogenase (ADH) and NADH. In this coupled system, the 4-hydroxyphenylacetaldehyde produced by the amine oxidase is converted directly to 4-hydroxyphenethylalcohol (tyrosol) by alcohol dehydrogenase. NADH (molar absorptivity of 6.22×10^3 M^{-1} cm^{-1} at 340 nm) is converted to NAD^+ in the system, resulting in a decrease in absorbance at 340 nm.

Initial rate measurements were carried out in a total volume of 0.96 mL containing 100 mM potassium phosphate buffer, pH 7.0, and substrate equilibrated to 25°C. Reaction was initiated by the addition of 40 μL of SSAO (0.04 units). The initial rate data obtained for the various tyramine concentrations was fitted to Equation (2-1), as described in Section 2.4.1.2. All kinetic experiments were done in duplicate.

3. RESULTS AND DISCUSSION

3.1 Isolation of Porcine Plasma Amine Oxidase

A summary of the purification of porcine plasma amine oxidase is given in Table 3.1. The increase in enzyme purity at the various isolation steps is reflected in increasing specific activity (units/mg). The enzyme was purified 1,360 fold with an overall recovery of 25%.

All steps were carried out according to the literature (Falk *et al.*, 1983; Coleman *et al.*, 1989), except for the inclusion of AH-Sepharose chromatography, which had not been tried in the isolation of porcine plasma enzyme. The AH-Sepharose column was a new step introduced to modify the preparation, so serial elution with phosphate buffer of varying ionic strengths (45, 55, 75 and 100 mM) was attempted on AH-Sepharose. The resultant specific activity of 0.11 units/mg was an improvement compared to the 0.057 units/mg reported by Coleman *et al.* (1989) and equivalent to that of Falk *et al.* (1983), while the yield (25%) was much higher than that of the latter workers (5.3%).

The results of the gel electrophoresis at each purification step are shown in Figure 3.1. All gels were 11 % acrylamide. Approx. mol. wt. estimated from the gels of PPAO, which is a dimer, is 194,000 and this is in good agreement with values in literature (Falk *et al.*,1983). The final enzyme is highly purified as estimated by electrophoresis. This enzyme has been used by Ms. A. Coleman in our laboratory in stereochemical studies of the C-2 exchange process.

3.2 Isolation of Sheep Serum Amine Oxidase

A summary of the purification procedure is given in Table 3.2. The enzyme with the highest purity was purified about 24 fold. While the 40% saturated ammonium sulfate extraction was found to be the most effective purification step (6-fold increase in specific activity, compared with one after previous step), the calcium phosphate absorption step turned out to be disappointing. The concentrated enzyme solution after Step 6, the DE-52

Table 3.1 Purification of porcine plasma amine oxidase.

Purification Step	*Units*[a]	*Specific activity (units/mg)*	*Purification (fold)*	*Yield (%)*
Serum	60	8.1×10^{-5}	1	100
$(NH_4)_2SO_4$ fractionation (35-55%)	42	2.3×10^{-4}	3	70
DE-52 30 mM	17.2	3.2×10^{-4}	4	61
100 mM	19.4	6.7×10^{-4}	8	
Con A	34.2	5.9×10^{-3}	73	57
AH-Sepharose	24.9	1.6×10^{-2}	200	42
Hydroxyapatite	23.2	5.6×10^{-2}	690	38
Bio-Gel A 1.5 m	15.5	1.1×10^{-1}	1360	25

[a] 1 unit = 1 μmol product/min at 25°C

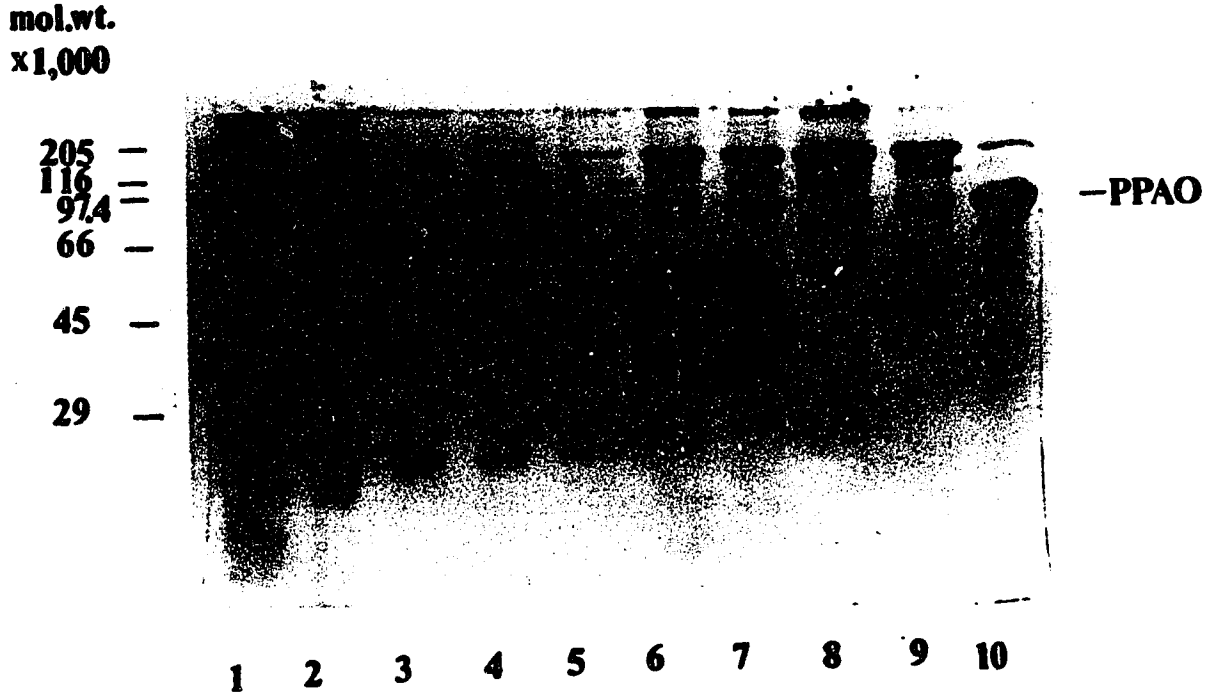


Figure 3.1. SDS-polyacrylamide gel electrophoresis of eluates after different purification steps in the porcine plasma amine oxidase isolation.

1. Standard (HMW)
2. Serum
3. & 4. Eluates of the DE-52 column
5. & 6. Eluates of the Con A column
7. Eluates of the AH-Sepharose column
8. & 9. Eluates of the Hydroxyapatite column
10. Eluates of the Bio-Gel A 1.5 m column

Table 3.2 Purification of sheep serum amine oxidase.

Purification Step		*Units*[a]	*Specific activity (units/mg)*	*Purification (fold)*	*Yield (%)*
Serum		139.0	5.5×10^{-4}	1	100
$(NH_4)_2SO_4$ fractionation (30-65%)		114.0	7.2×10^{-4}	1.3	82
$(NH_4)_2SO_4$ extraction (40%)		91.1	4.4×10^{-3}	8	66
Calcium phosphate gel adsorption		85.8	6.0×10^{-3}	11	62
$(NH_4)_2SO_4$ extraction (55%)		59.2	8.8×10^{-3}	16	43
DE-52 anion exchanger					
high activity fraction	1	19.8	1.3×10^{-2}	24	34
	2	23.0	1.0×10^{-2}	18	
low activity fraction		4.6	3.1×10^{-3}	6	

[a] 1 unit = 1 μmol product/min at 25°C

anion exchange chromatography, was stored at 4°C for 2 months without any significant loss of activity. Steps up to #5 (Section 2.2.2.2) were according to the literature (Rucker *et al.*, 1972). DE-52 chromatography and the subsequent chromatographic steps were introduced by us. The SDS-PAGE of the low activity DE-52 eluate (Figure 3.2) shows that the major contaminating proteins are albumin with a mol.wt. of approx. 66,000 and a 45,000 band. Hemoglobin (16,000) is also present in the sample and three high mol. wt. bands at approx. 97,000, 116,000 and 130,000 are evident. The minor band at mol. wt. approx. 97,000 is the enzyme, SSAO, based on the fact that the relative band intensity of mol. wt. 97,000 increased with improved specific activity of the enzyme solution. The low activity fraction was subjected to an additional series of chromatographic steps. The results of these steps are summarized in Table 3.3.

The Sephacryl S-300 SF (gel filtration) column was chosen to try to remove hemoglobin, albumin and the 45,000 dalton band, all of which have a lower mol. wt. than SSAO. The resolution of proteins was poor on the initial Sephacryl S-300 SF column and there was only a marginal increase in specific activity after this step. Chromatography on Blue Sepharose CL-6B was carried out on the combined fractions since this resin has been reported to adsorb albumin (Travis *et al.*, 1976). The gel in Figure 3.3, which shows sequential fractions from loading and washing of the Blue Sepharose column, demonstrates partial removal of both albumin and the 45,000 dalton band. However, this would not be a practical method of removing albumin. Con A remains the method of choice for removing albumin and hemoglobin, which are not glycosylated, from glycoproteins such as SSAO.

All fractions with activity (#27-35) were combined and applied to an AH-Sepharose column. AH-Sepharose was used because it is an affinity support for other amine oxidases, such as bovine (Svenson and Hynning, 1981) and porcine (Section 2.1) AO . These steps for the further purification of SSAO had not been attempted before elsewhere, and some proved to be unsatisfactory. For instance, the poor resolution attained from the first

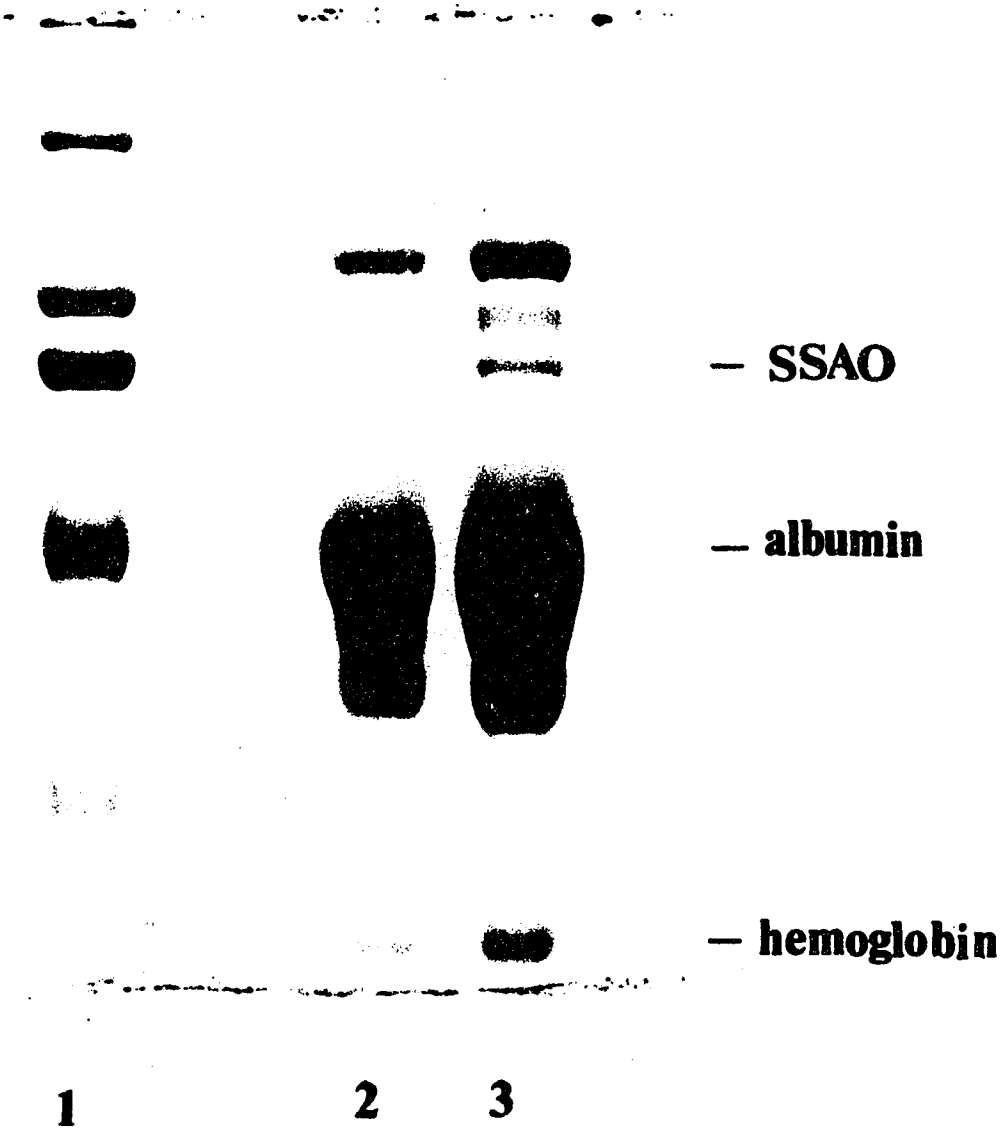


Figure 3.2. SDS-PAGE of low activity fraction from DE-52 of the sheep serum amine oxidase isolation.
1. Standard
2. & 3. Low activity fraction

Table 3.3 Further purification of SSAO.

Purification Step	*Units*	*Specific activity (units/mg)*	*Purification (fold)*	*Yield (%)*
DE-52 eluate (low activity fraction)	2.8	3.1×10^{-3}	1	100
Sephacryl S-300 SF #1	2.4	4.4×10^{-3}	1.4	86
Blue Sepharose CL-6B	1.8	1.1×10^{-2}	3.5	64
AH-Sepharose	1.2*	3.7×10^{-2}	12	43
Sephacryl S-300 SF #2	1.1	1.2×10^{-1}	39	29

* 1 mL (= 1 unit) of high activity fraction was added

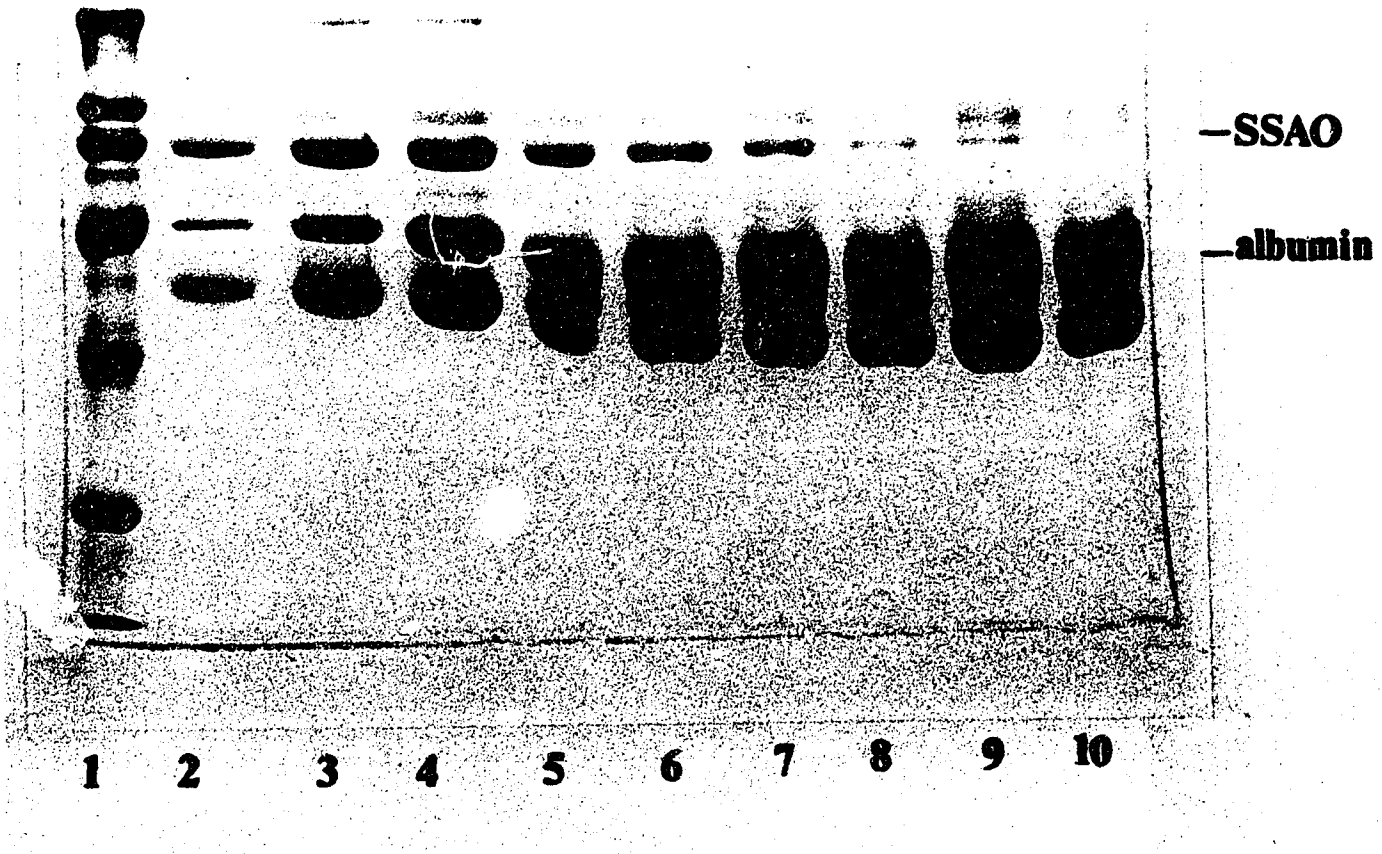


Figure 3.3. SDS-PAGE of Blue Sepharose eluates.
1. Standard
2. - 10. Fraction #27 → Fraction #35

Sephacryl S-300 column might be due to the low ionic strength (10 mM) of the buffer used. Eluates of the Blue Sepharose column at an early stage showed good resolution, while dimi[illegible] resolution towards the later stage of elution indicated that insufficient quantity of [illegible]harose CL-6B) was used (Figure 3.3). The cost of a larger column is pro[illegible]50 for 50 g of dry resin for the 2.5 x 50 cm column used in this preparation). [illegible] electrophoresis gels are included to illustrate the progress of the purification procedure. They are not of high quality due to an effort to make the SSAO band visible thereby resulting in considerable smearing of the major protein bands.

Though the AH-Sepharose column worked well, with a 3.4-fold increase in specific activity compared with the eluate from the Blue Sepharose column, the specific activity of the enzyme solution (0.037 units/mg) indicated that it might still contain a significant amount of contaminating protein. Therefore, the eluate of the AH-Sepharose column was rechromatographed onto the same Sephacryl S-300 SF column equilibrated with 100 mM buffer. The second gel filtration column gave an excellent resolution with a 3.2- fold purication. The high resolution of this column, compared with the first gel filtration column (10 mM buffer applied), might be due to the high ionic strength (100 mM) of the buffer. The Sephacryl gel contains carboxyl groups and thereby partial ionic interactions between the matrix and proteins can occur at low ionic strength. The specific activity of the final enzyme was 0.12 units/mg, which is the highest purification ever reported for SSAO.

Based on the results in this thesis a proposed scheme for the isolation of SSAO is: $(NH_4)_2SO_4$ fractionation (35-55%), then successive chromatography on DE-52 cellulose, Con A-Sepharose, hydroxyapatite and a Sephacryl S-300 SF column equilibrated with 100 mM potassium phosphate buffer, pH 7.4. It is suggested that the Con A-Sepharose column and hydroxyapatite column should be equilibrated and run as described for the porcine plasma amine oxidase, while the other steps are identical to those described for SSAO. An outline of the proposed isolation scheme for SSAO is given in Figure 3.5.

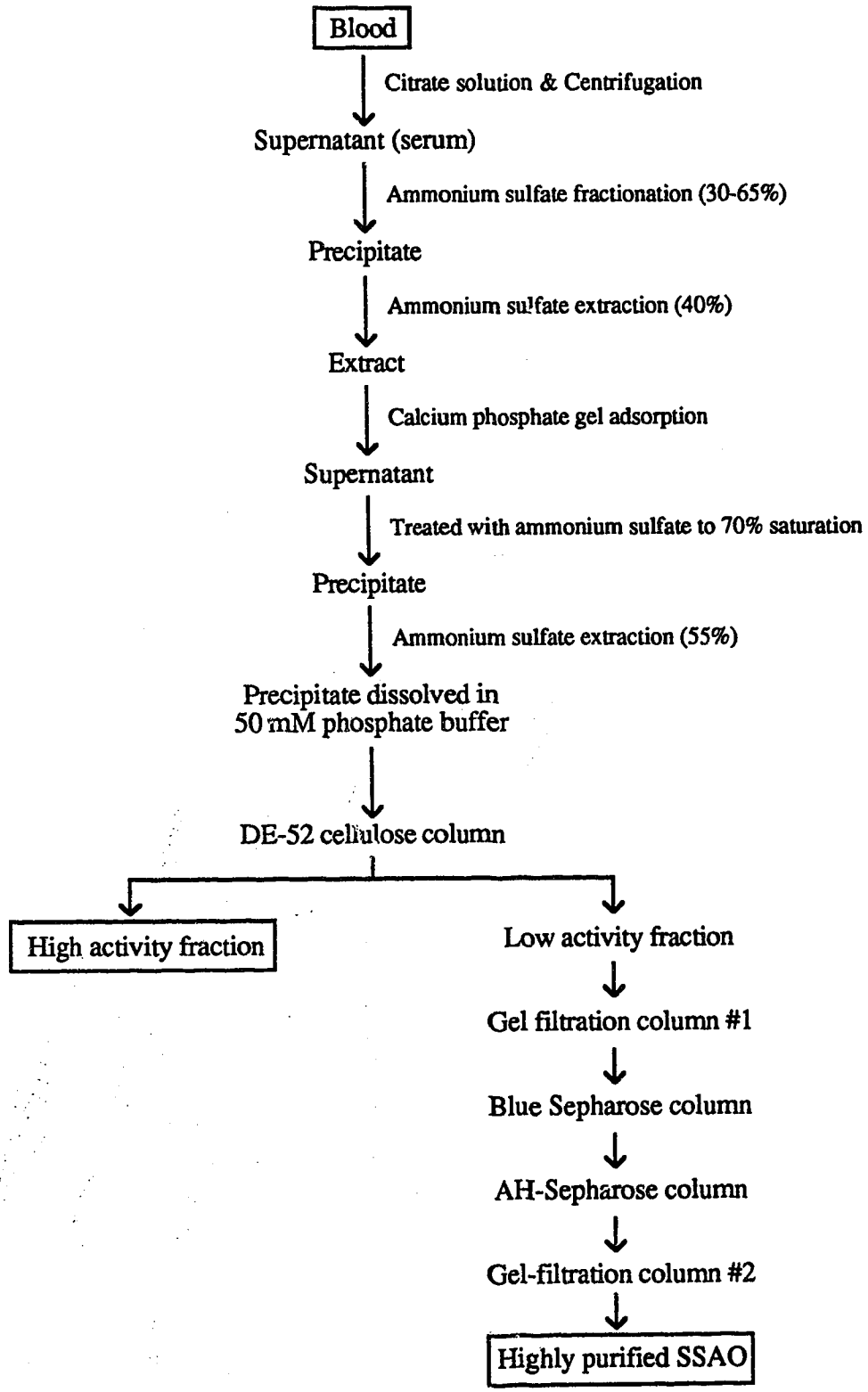


Figure 3.4. Outline of sheep serum amine oxidase isolation.

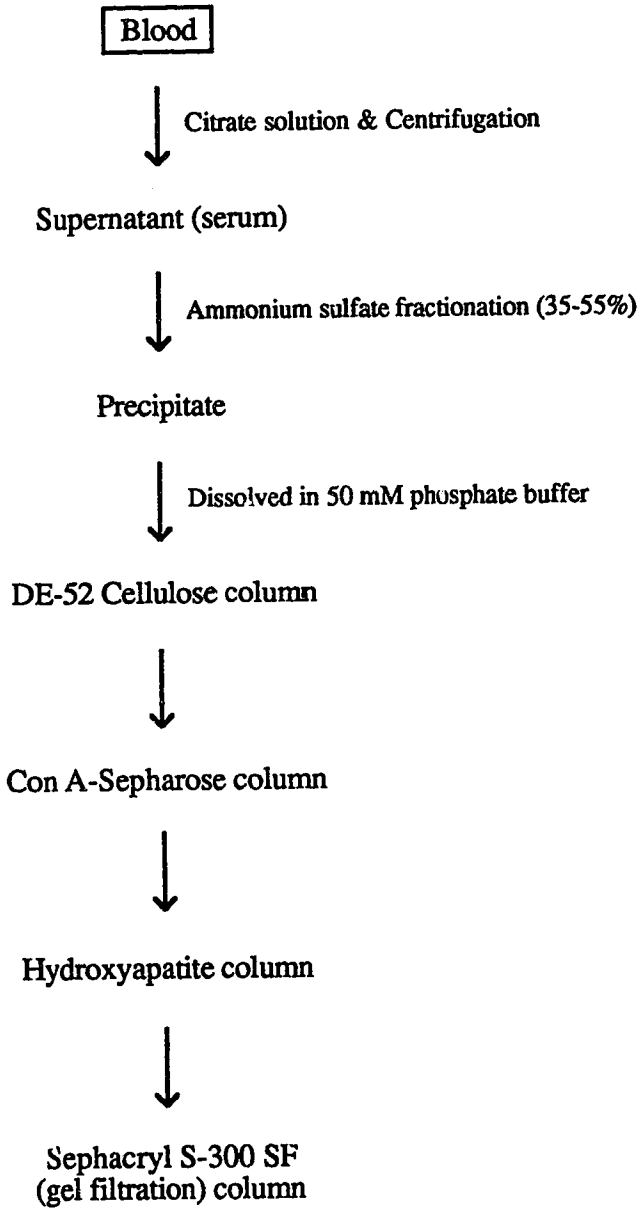


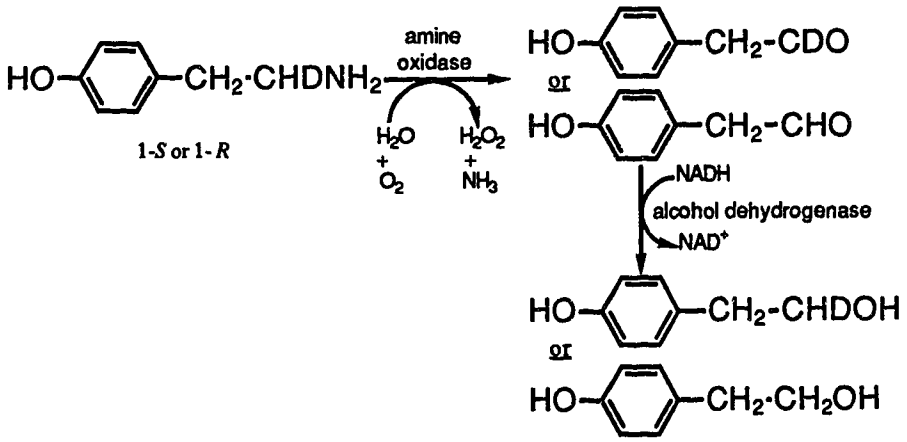
Figure 3.5. The proposed isolation scheme for SSAO.

3.3 Stereochemistry of Sheep Serum Amine Oxidase Reactions

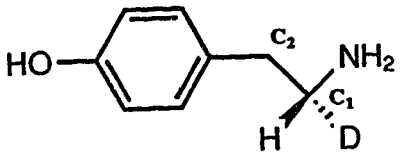
3.3.1 Oxidation of monodeuterated tyramines

Figures 3.6 and 3.7 show that the ^1H NMR spectra of stereospecifically deuterated tyramines isolated after incubation of L-tyrosine in ^2H_2O and DL-[2-^{2}H]tyrosine in H_2O, respectively, with tyrosine decarboxylase. The clean doublets at 2.89 ppm show that the deuteration was complete within the limits of sensitivity of the ^1H NMR spectra (≥98%) and the absolute configuration of the chiral tyramines are assigned based on the established stereospecificity of the tyrosine decarboxylase reaction with retention of configuration (Palcic and Floss, 1986).

Stereochemical studies with both chiral amines (1*S*- or 1*R*-tyramine) and sheep serum amine oxidase were carried out in a coupled manner such that the *p*-hydroxyphenyl acetaldehyde produced was reduced directly to *p*-hydroxyphenethyl alcohol by the action of alcohol dehydrogenase (ADH) and NADH.



Scheme 3-1



(1R)-[^{2}H]Tyramine

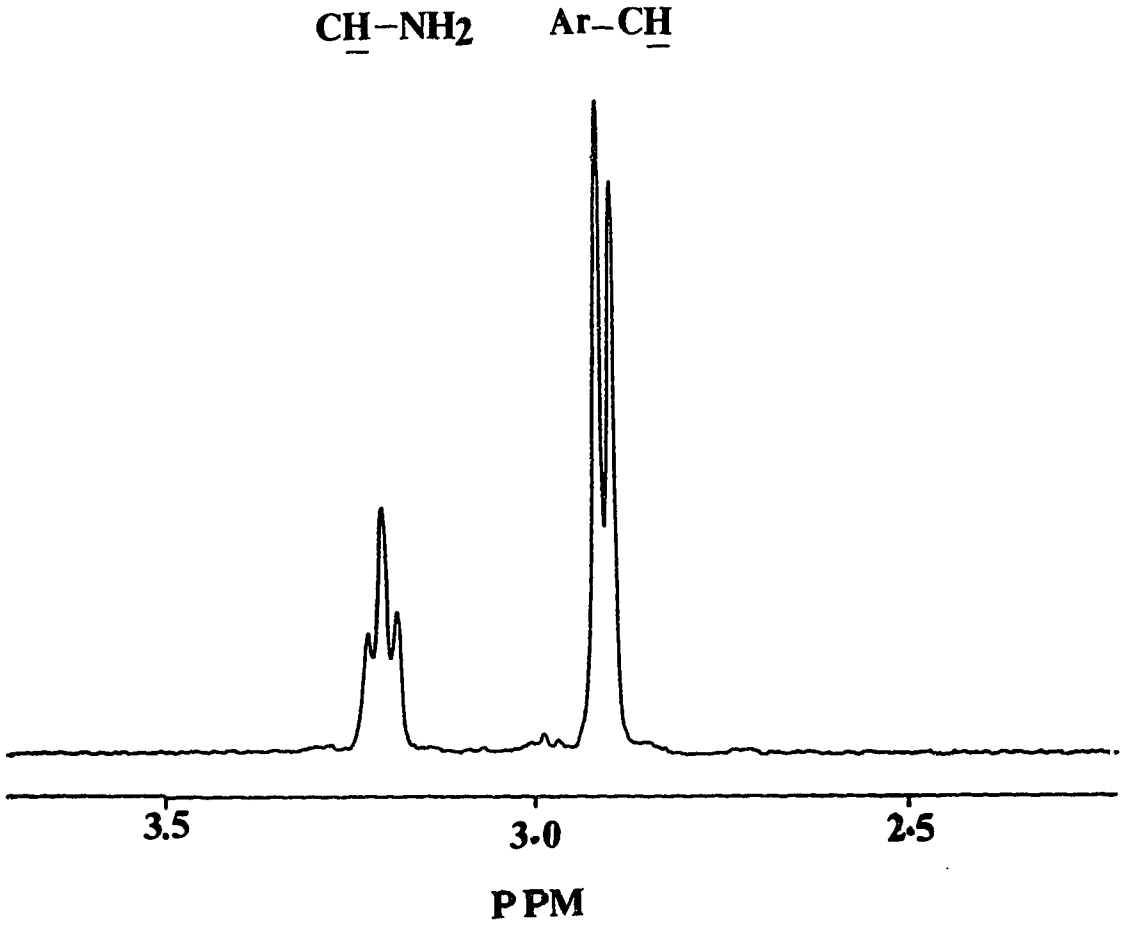
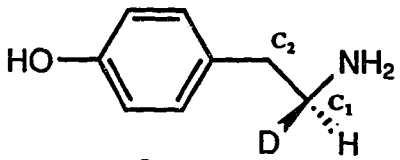


Figure 3.6. Partial 360 MHz ^{1}H NMR spectrum of (1*R*)-[^{2}H]tyramine derived from incubation of tyrosine decarboxylase with L-tyrosine in ^2H_2O.



(1S)-[^{2}H]Tyramine

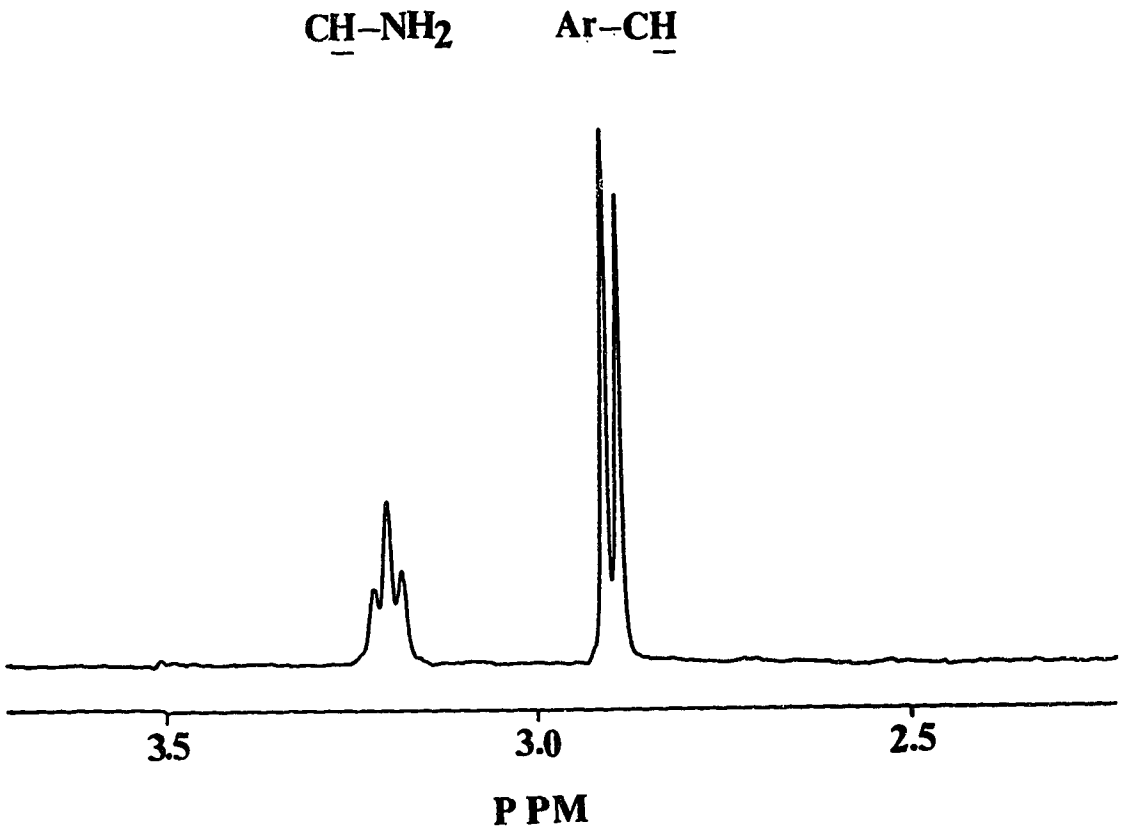


Figure 3.7. Partial 360 MHz ^{1}H NMR spectrum of (1*S*)-[^{2}H]tyramine derived from incubation of tyrosine decarboxylase with DL-[2-^{2}H]tyrosine in H_2O.

Figure 3.8 shows the ^1H NMR spectrum of *p*-hydroxyphenethyl alcohol (tyrosol) isolated from incubations with (1*S*)-[^2H]tyramine and sheep serum amine oxidase after the reaction was >90% complete. The spectral pattern shows a mixture of two alcohol products, one fully protonated and the other monodeuterated, with the latter being the major product. The pattern for this mixture is two overlapping triplets for the C-1 proton signals near 3.7 ppm and a doublet superimposed on a triplet for the C-2 protons near 2.8 ppm. The signals labeled N near 4.0 ppm are due to residual NADH, while that labeled X near 2.8 ppm is due to an unidentified impurity.

The minor triplet at 3.74 ppm arises from the product alcohol that is fully protonated at C-1. The major triplet at 3.72 ppm is from tyrosol that is monodeuterated at C-1. This signal is shifted about 0.02 ppm to higher field due to an α-deuterium isotope effect (Hansen, 1983). The signals for the C-2 protons are a triplet at 2.79 ppm exhibited by the minor product (fully protonated tyrosol). For the major product (monodeuterated tyrosol), a doublet for the C-2 protons is at 2.78 ppm, shifted upfield due to a ß-deuterium isotope effect. In this case, 89% of the deuterium of the original tyramine was retained in the product alcohols and 11% was lost, indicating that oxidation of the *S*-amine occurred in a partially nonstereospecific fashion.

The spectrum of the *p*-hydroxyphenethyl alcohol obtained from incubation of (1*R*)-[^2H]tyramine with sheep serum amine oxidase is shown in Figure 3.9. This sample also consists of a mixture of monodeuterated and fully protonated alcohols, with the monodeuterated product predominating. In this instance, 72% of the deuterium was retained in the product, indicating that reaction occurred in a partially nonstereospecific manner. These stereochemical studies demonstrate that sheep serum amine oxidase exhibits the rare mirror-image bindings and catalysis reported for bovine plasma amine oxidase (Summers *et al.*, 1979; Farnum *et al.*, 1986).

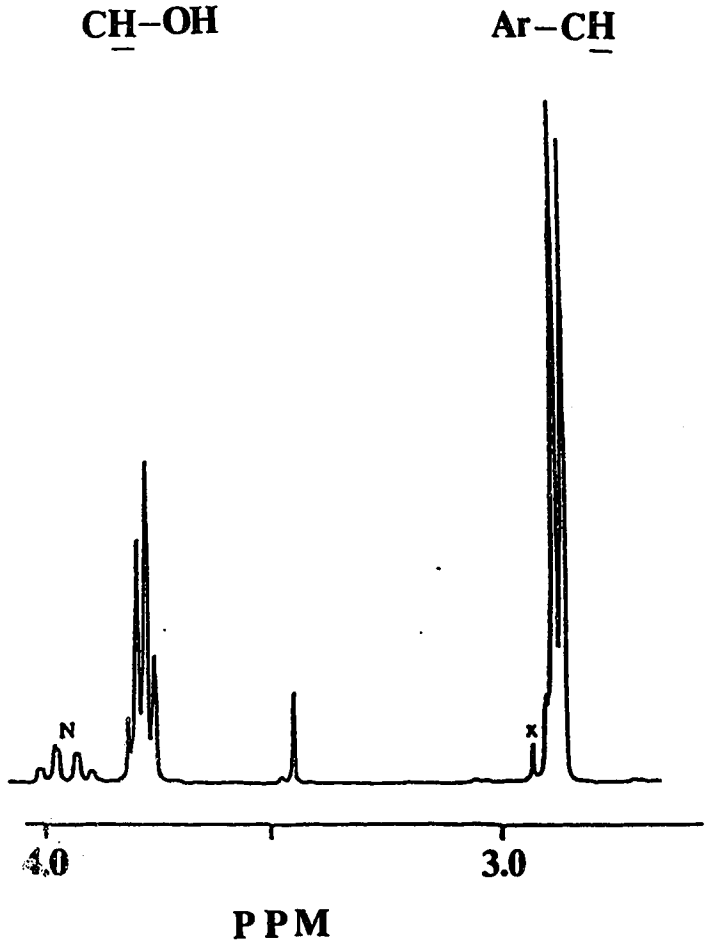


Figure 3.8. The ^{1}H NMR spectrum of alcohol derived from coupled incubation of (1*S*)-[^{2}H]tyramine and sheep serum amine oxidase with NADH and alcohol dehydrogenase.

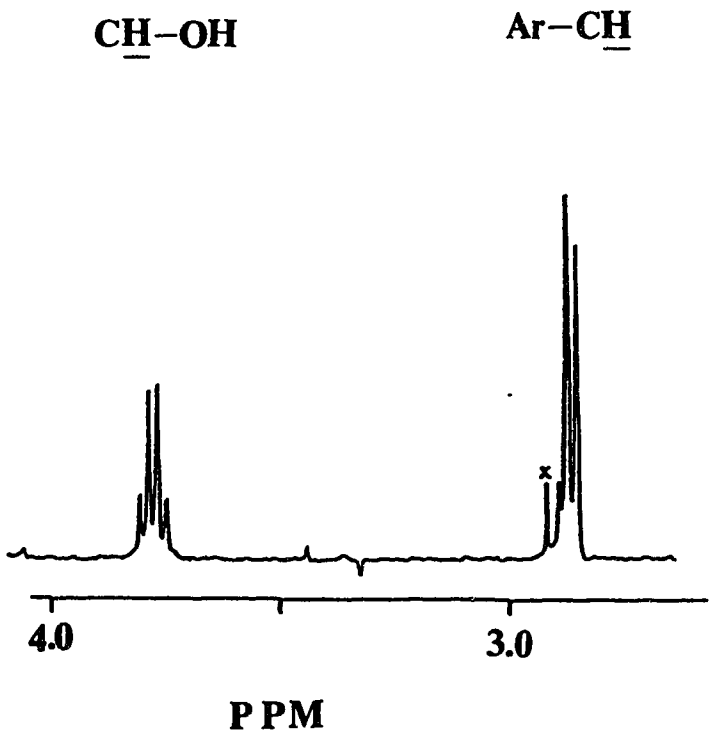
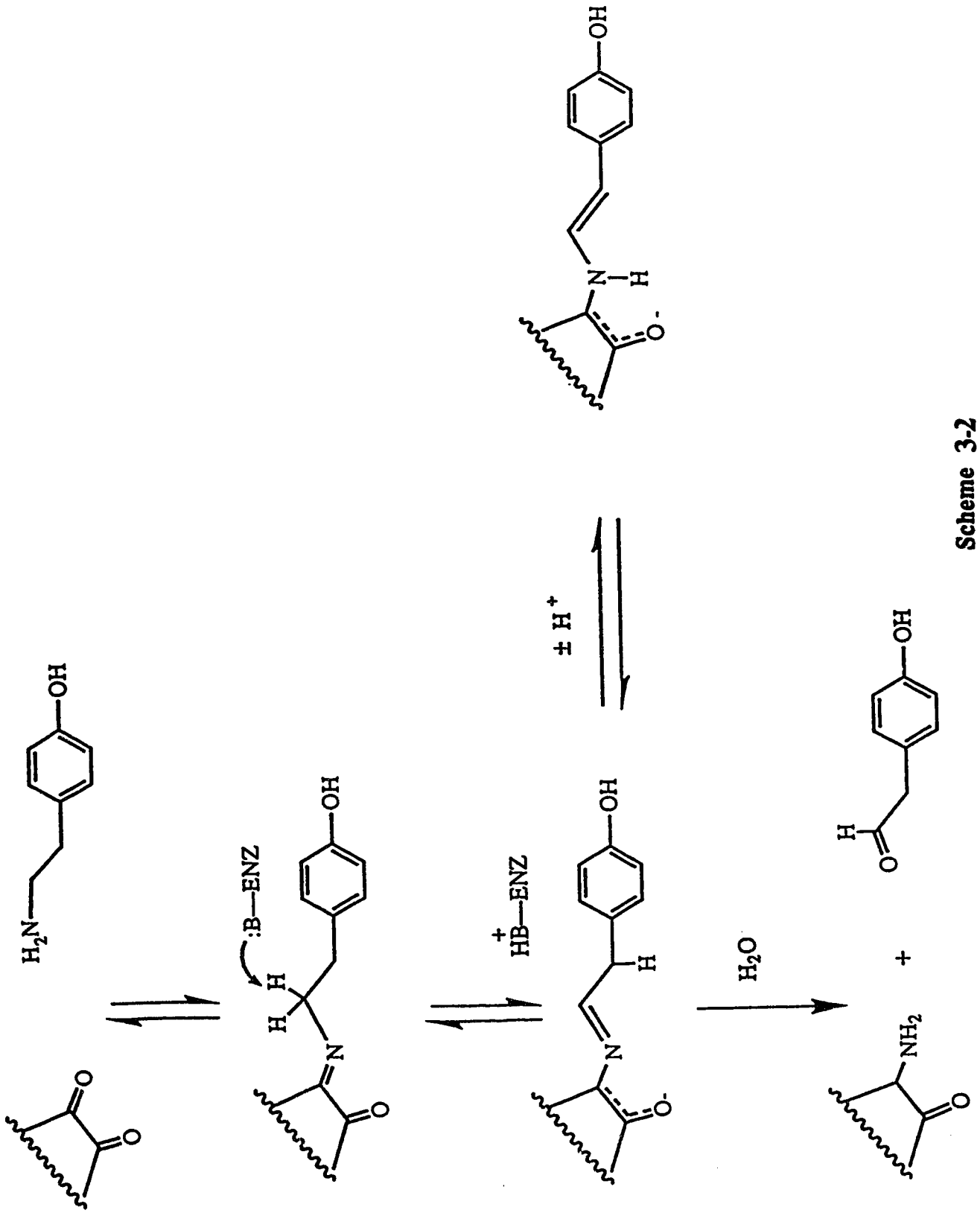


Figure 3.9. The ^{1}H NMR spectrum of alcohol derived from coupled incubation of (1*R*)-[^{2}H]tyramine and sheep serum amine oxidase with NADH and alcohol dehydrogenase.

A completely nonstereospecific reaction arising from equal flux through two catalytically competent modes, defined as pro-*S* or pro-*R* abstraction of hydrogen at C-1, would give a 1:1 mixture of monodeuterated and fully protonated alcohols. Any deviations from a ratio of 50/50 indicate preferred reaction through either the pro-*R* or pro-*S* abstraction mode. Preferred reaction through a mode can arise from a kinetic isotope effect (KIE) where the isotope effect results in a reduction in the rate of C-D bond cleavage relative to C-H bond cleavage. If a KIE of 6 is observed, the rate of reaction is 6-fold greater for C-H bond cleavage vs C-D bond cleavage. This would give products enriched in deuterium, i.e. 86% deuterated and 14% protonated. Differences in the deuterium content of the samples derived from (1*R*)- or (1*S*)-[^{2}H]tyramines arise from differences in the isotope effects in each mode.The KIE's estimated from the observed product ratios for the sheep serum amine oxidase are 8.1 for the *S* mode and 2.6 for the *R* mode. The reduced KIE for (1*R*)-tyramine indicates that a step other than C-H bond cleavage is partially rate-limiting in the *R* mode, which reduces the observed isotope effect.

An aminotransferase mechanism has been proposed for the copper amine oxidases, with initial Schiff base formation between the cofactor carbonyl and substrate amine (Scheme 3-2) (Farnum and Klinman, 1986). This is followed by base-catalyzed proton abstraction from C-1 of substrate such that the cofactor functions as an electron sink during catalysis, analogous to pyridoxal phosphate. Imine hydrolysis yields product aldehyde and an amino quinol as the reduced cofactor species (Hartman *et al.*, 1988).

For bovine plasma enzyme, the reduction in the observed isotope effect for the *R* mode has been attributed to a partially rate-limiting conformational change prior to imine hydrolysis. This reorientation does not occur in the *S* mode, hence the isotope effect is more fully expressed for these substrates.



Scheme 3-2

A summary of the stereochemical studies is shown in Table 3.4. A different KIE can be expressed for V_{max} (saturated substrate levels) or V_{max}/K_m conditions (sub-saturating substrate concentrations). If there is a difference in KIE for V_{max} and V_{max}/K_m, and the reaction goes from V_{max} to V_{max}/K_m control, there will be a difference in deuterium content in products isolated as a function of reaction progress. However, Table 3.4 shows that the amount of deuterium in products is constant for all tyrosols isolated after 50% reaction. This suggests that the KIE is the same for V_{max} and V_{max}/K_m or that the K_m for tyramine is high and reaction was under V_{max}/K_m control throughout.

3.3.2 Solvent exchange into product

Previous studies have reported that a solvent exchange pathway exists off the main catalytic pathway for several amine oxidases (Coleman *et al.*, 1989; Farnum *et al.*, 1986). This is attributed to reversible enamine formation after C-1 proton abstraction, thereby allowing for exchange of solvent into C-2 of product aldehydes (Scheme 3-2).

Figure 3.10 shows the spectrum of the alcohols obtained by incubation of sheep serum amine oxidase with fully protonated tyramine in ^2H_2O. Solvent is incorporated into products, evidenced by the overlapping triplets near 2.7 ppm and a doublet superimposed on a triplet near 3.7 ppm. The amount of deuterium incorporated into C-2 of products in duplicate experiments was 92 and 94% when reaction went to completion (>90%). The C-1 proton abstraction in Scheme 3-2 should be regarded as an irreversible step since the spectrum (Figure 3.10) indicates that no deuterium in ^2H_2O was incorporated into C-1 of products. Studies by Coleman *et al.* (1989) showed no solvent exchange in the case of the pro-*S* specific amine oxidases such as porcine kidney amine oxidase and chick pea amine oxidase. The absence of exchange can be attributed to kinetic or steric constraints for these enzymes. Kinetically, imine hydrolysis and solvent exchange via enamine formation are competing reactions (Scheme 3-2). Enzymes for which the rate of hydrolysis is rapid relative to enamine formation would function without an exchange

Table 3.4. Characteristics of ^1H NMR spectra of alcohols derived from coupled incubations of chiral amines with SSAO.

	Alcohols from S-tyramine		*Alcohols from R-tyramine*	
	diprotonated	*monodeuterated*	*diprotonated*	*monodeuterated*
Chemical shifts at				
C-1	3.74	3.72	3.73	3.71
C-2	2.79	2.78	2.79	2.78
Deuterium (%) at				
50% conversion		91		74
>90% conversion		87		70
KIE		8.1		2.6

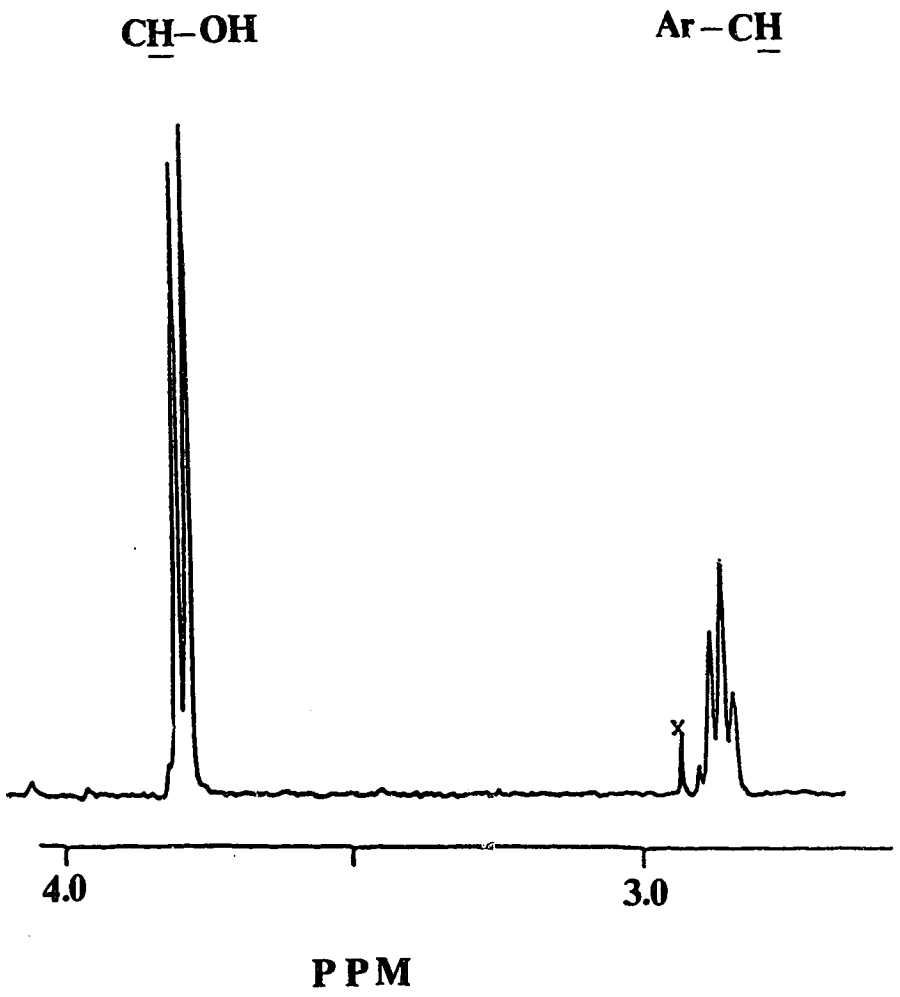


Figure 3.10. The ^{1}H NMR spectrum of alcohol derived from coupled incubation of sheep serum amine oxidase and tyramine in $^{2}H_2O$.

process. Alternatively, if enamine formation requires proton transfer to a residue of the enzyme active site, lack of a correctly positioned or unionized base to accept the proton would also preclude solvent exchange.

The nonstereospecificity of SSAO results from mirror-image binding and catalysis, which is a rare phenomenon in enzymology. There seems to be a correlation between the stereochemical course of proton abstraction at C-1 and a solvent exchange pathway into C-2 of products for all enzymes in the class. The pro-*R* specific and nonstereospecific enzymes have maintained the solvent wash-in pathway, while it has not been detected for any pro-*S* enzyme to date. More surprisingly, the modal preference for the nonstereospecific enzymes is uniform; the kinetic isotope effects for the pro-*R* abstraction mode are reduced relative to the pro-*S* mode for all nonstereospecific amine oxidases studied to date.

3.4 Kinetic Isotope Effects

The stereochemical studies with tyramine and sheep serum amine oxidase (SSAO) described in Section 3.3 demonstrated that two kinetically competent binding modes exist in the enzyme. One mode results in abstraction of the pro-*R* hydrogen of substrate, the other pro-*S* hydrogen abstraction. Furthermore, there is a difference in the kinetic isotope effect (KIE) for these modes, 8.1 for the *S* mode and 2.6 for the *R* mode. An isotope effect can be observed on V_{max} with substrate saturating and V_{max}/K_m with sub-saturating substrate concentrations. If a different KIE exists for V_{max} and V_{max}/K_m and the stereochemical studies are carried out starting with saturating substrate under V_{max} condition and shifted to V_{max}/K_m, the deuterium content in product can vary as a function of reaction extent.

To estimate the magnitude of KIE's, a series of steady-state kinetic measurements were carried out. The overall reaction catalyzed by SSAO occurs in two steps, as shown in Scheme 3-3 (Farnum *et al.*, 1986). This is a minimal kinetic scheme modeled on the bovine plasma amine oxidase reaction:

$$E_{ox}+RCH_2NH_3^+ \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_{ox}\cdot RCH_2NH_3^+ \underset{k_4}{\overset{k_3^*}{\rightleftharpoons}} E_{red}-N^+H=CHR \xrightarrow[H_2O]{k_5} E_{red}-NH_3^+(RCHO) \quad (3\text{-}1)$$

$$E_{red}-NH_3^+(RCHO) \xrightarrow[O_2]{k_6} E_{ox} + RCHO + NH_4^+ + H_2O_2 \quad (3\text{-}2)$$

Scheme 3-3

In the first step, after the formation of an E•S complex, proton abstraction and imine hydrolysis yield the aldehyde product and reduced enzyme (Eqn 3-1). This is followed by enzyme reoxidation by molecular oxygen, which gives H_2O_2 in the second step (Eqn 3-2).

Introduction of deuterium into substrate brings about unique perturbation of a single step in catalysis, k_3^*. For Scheme 3-3 above, the kinetic parameters and isotope effects on kinetic parameters are given as follows (Palcic *et al.*, 1983; Cleland, 1975):

$$V_{max} = \frac{k_3k_5k_6[E_t]}{k_3k_5 + k_3k_6 + k_6(k_4+k_5)} \quad (3\text{-}3)$$

$$^DV_{max} = \frac{(V_{max})_H}{(V_{max})_D} = \frac{^Dk + k_{3H}/k_5 + k_{3H}/k_6 + {}^DK_{eq}k_{4H}/k_5}{1 + k_{3H}/k_5 + k_{3H}/k_6 + k_{4H}/k_5} \quad (3\text{-}4)$$

$$V_{max}/K_m = \frac{k_1k_3k_5[E_t]}{k_2(k_4+k_5) + k_3k_5} \quad (3\text{-}5)$$

$$^DV_{max}/K_m = \frac{(V_{max}/K_m)_H}{(V_{max}/K_m)_D} = \frac{^Dk + k_{3H}/k_2 + {}^DK_{eq}k_{4H}/k_5}{1 + k_{3H}/k_2 + k_{4H}/k_5} \quad (3\text{-}6)$$

where $^DK_{eq} = \frac{(K_{eq})_H}{(K_{eq})_D} = \frac{k_{3H}k_{4D}}{k_{3D}k_{4H}}$ and $^Dk = \frac{k_{3H}}{k_{3D}}$

It can be seen that V_{max}/K_m includes all steps up to the first irreversible step, while V_{max} includes all steps after E_{ox}•S complex formation. ^Dk, the intrinsic isotope effect, is the full isotope effect originating from the single isotopically sensitive step of catalysis, exclusive of all effects from isotopically insensitive steps. An isotope effect is expressed as a decreased rate with deuterated substrates only if C-H bond cleavage is rate-limiting. Usually, in enzyme reactions bond cleavage is not fully rate-limiting, rather other slow steps such as product release, conformational changes, or reoxidation of the enzyme will reduce the magnitude of the observed isotope effect (Northrop, 1976).

The primary deuterium isotope effect for benzylamine oxidation catalyzed by sheep serum amine oxidase was determined by comparing reaction rates with unlabeled and dideuterated benzylamine (Figure 3.11). The kinetic paramaters obtained by computer analysis of this data using a program based on the Cleland method (Cleland, 1979) are listed in Table 3.5. It was shown that replacement of the hydrogen atoms at the α-carbon of benzylamine by deuterium decreased the steady-state maximum velocity by a factor of 1.44. The K_m was increased by a factor of 2.25, giving an isotope effect on V_{max}/K_m of 3.35.

The primary deuterium isotope effect for tyramine oxidation was obtained by comparing the reaction rates with unlabeled and dideuterated tyramine (Figure 3.12). The reaction was biphasic, therefore only the high substrate concentration points are shown. The data for low substrate concentrations showed considerable scatter. The kinetic parameters obtained by analysis of this data are also listed in Table 3.5. In this instance the kinetic isotope effect on V_{max} is 3.14 and there is no isotope effect on V_{max}/K_m. Because V_{max} and V_{max}/K_m are dependent on different components of the reaction mechanism (Eqn 3-4 and 3-6), the interpretation of isotope effects on V_{max} and V_{max}/K_m must also be different. Eqn 3-4 indicates that an isotope effect on V_{max} is governed by the forward ratios of catalysis, k_{3H}/k_5 and k_{3H}/k_6, while Eqn 3-6 shows that an isotope effect on V_{max}/K_m is influenced by the forward commitment to catalysis,

k_{3H}/k_2. Thus, $^{D}V_{max}$ of 1.44 and $^{D}V_{max}/K_m$ of 3.35 for benzylamine indicate that reoxidation of the enzyme is slow relative to the isotope-sensitive step (i.e. C-H bond cleavage), thereby abolishing the expression of the isotope effect by causing k_{3H}/k_6 to become large (Northrop, 1981). $^{D}V_{max}$ of 3.14 and $^{D}V_{max}/K_m$ of 0.9 (approx. 1.0) for tyramine suggest that imine hydrolysis and enzyme reoxidation are fast relative to C-H bond cleavage and that the slow step must be at dissociation of E•S complex suppressing the expression of the isotope effect by causing k_{3H}/k_2 to become large (Northrop, 1975).

The kinetic study of tyramine oxidation was initiated to try to account for the invariance in the deuterium content of the products isolated in the stereochemical studies. Two models were proposed. In the first, the K_m for tyramine is high and therefore the stereochemical studies were under V_{max}/K_m conditions throughout. Alternatively, the KIE on V_{max} and $V_{max}K_m$ is the same. The K_m's obtained in the kinetic study are 23 mM for unlabeled tyramine and 6.6 mM for dideuterated tyramine. Assuming the K_m for the monodeuterated tyramines will be in this range, the stereochemical study with initial substrate concentrations of 15 to 20 mM will be mainly under V_{max}/K_m control since substrate concentrations are well below saturating (10 x Km).

However, the KIE on V_{max}/K_m obtained in the kinetic study is approx. 1. For our nonstereospecific reaction an isotope effect of 1 would yield only products that retain 50 % deuterium. This contrasts with experimental values obtained of 89 % deuterium retention for the *S*- amine and 72 % for the *R*- amine. There is no straightforward explanation for this discrepancy. It can be noted that the kinetic experiments are initial rate measurements with negligible depletion of substrate while in the stereochemical study substrate concentration is continually being reduced. Estimates of the K_m for both (1*R*)- and (1*S*)-[^{2}H]tyramine should be obtained as well as estimates of $^{D}V_{max}$ and $^{D}V_{max}/K_m$ for these substrates. This study is

currently prohibitive with the limited supplies of chiral compound but will be carried out in the future.

Table 3.5 Kinetic parameters for the oxidation of benzylamine and tyramine.

	Benzylamine		*Tyramine*	
	Unlabeled	*Dideutero*	*Unlabeled*	*Dideutero*
K_m (mM)	0.12	0.27	23.0	6.6
V_{max} (nmole/min)	7.5	5.2	16.2	5.1
V_{max}/K_m (nmole/min•mM)	64.8	19.4	0.70	0.78
KIE on V_{max}	1.44		3.14	
KIE on V_{max}/K_m	3.35		0.90	

KIE on $V_{max} = (V_{max})_H/(V_{max})_D$

KIE on $V_{max}/K_m = (V_{max}/K_m)_H/(V_{max}/K_m)_D$

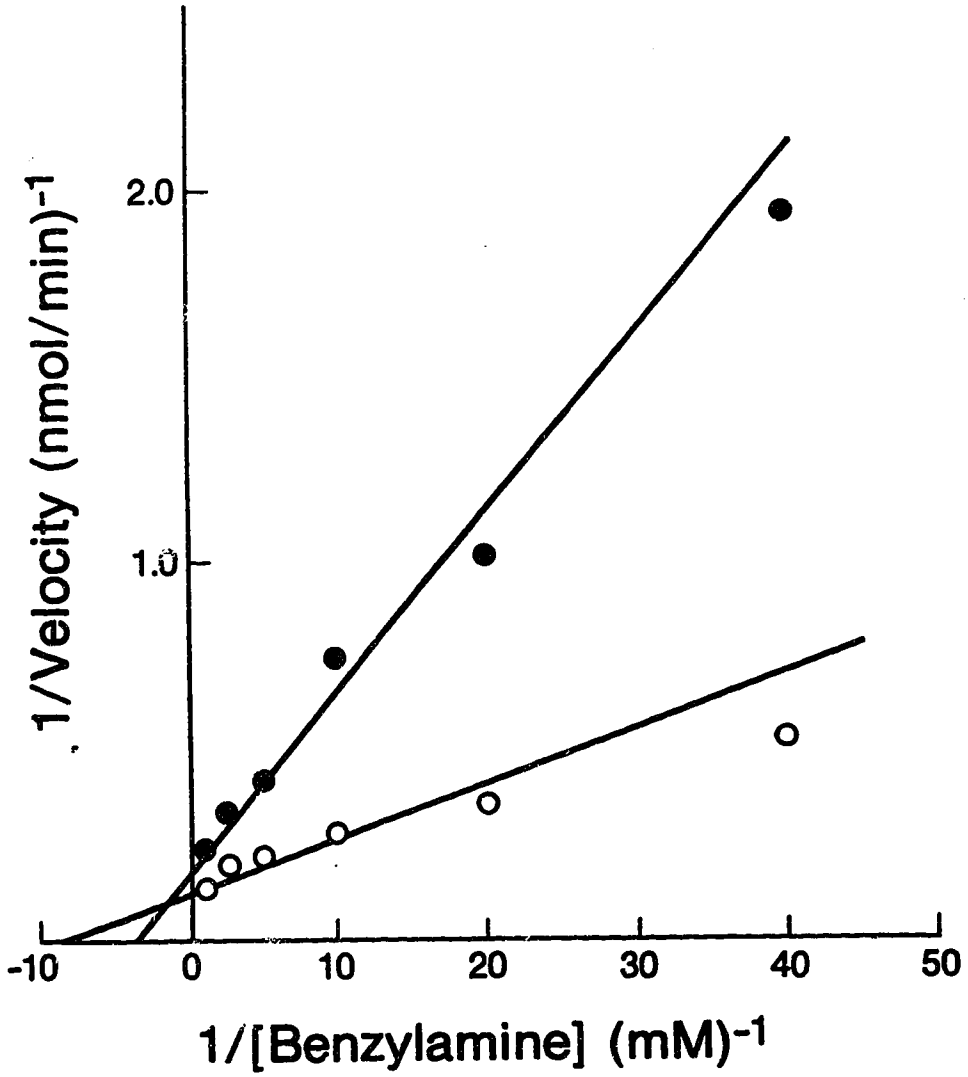


Figure 3.11. **Double-reciprocal plot of initial rate data for the oxidation of benzylamine by sheep serum amine oxidase (0.012 units) in 100 mM potassium phosphate buffer, pH 7.0, at 25°C.**The initial rates were measured by monitoring benzaldehyde formation at 250 nm. Open circles are for unlabeled benzylamine and closed circles are for dideuterated benzylamine.

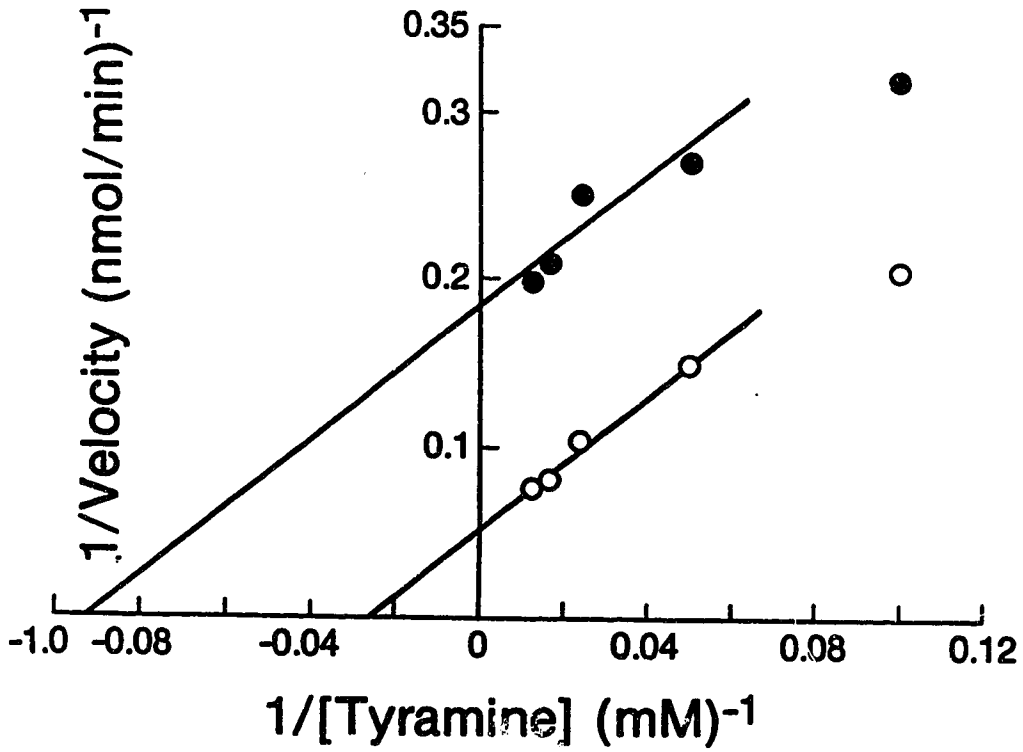


Figure 3.12. Double-reciprocal plot of initial rate data for the oxidation of tyramine by sheep serum amine oxidase (0.04 units) in a coupled system containing alcohol dehydrogenase and NADH in 100 mM potassium phosphate buffer, pH 7.0, at 25°C. The initial rates were measured by monitoring decrease in absorbance at 340 nm. Open circles are for unlabeled tyramine and closed circles are for dideuterated tyramine.

4. SUMMARY AND CONCLUSIONS

Porcine plasma amine oxidase was isolated from pig blood by applying ammonium sulfate fractionation (35-55%), chromatographic procedures using resins such as DE-52 cellulose, Con A-Sepharose, AH-Sepharose, hydroxyapatite, and finally a gel filtration colmn. The final enzyme, with a specific activity of 0.11 units/mg, was more than 90% pure as estimated by SDS-PAGE.

Sheep serum amine oxidase was isolated from sheep blood and purified by application of ammonium sulfate fractionation (30-65%), calcium phosphate gel adsorption, ammonium sulfate extraction (40 and 55%), a DE-52 anion exchanger, gel filtration (twice), Blue Sepharose CL-6B, and AH-Sepharose chromatography. The specific activity of the final enzyme was 0.12 units/mg.

The investigation carried out in this work showed that chiral tyramines were oxidized with a net nonstereospecific proton abstraction from C-1 by the sheep serum amine oxidase. The enzyme exhibited rare mirror-image binding (with differential flux) through two opposite and stereospecific reaction modes. Differential kinetic isotope effects were observed for each mode, 8.1 for the *S* mode and 2.6 for the *R* mode. The reduced KIE for (1*R*)-tyramine indicated that a step other than C-H bond cleavage is partially rate-limiting in the *R* mode, thus reducing the observed isotope effect.

The deuterium isotope effects on V_{max} and V_{max}/K_m with benzylamine and tyramine substrates were estimated in steady-state kinetic experiments. $^{D}V_{max}$ of 1.44 and $^{D}V_{max}/K_m$ of 3.35 for benzylamine indicate that reoxidation of the enzyme (SSAO) is slow relative to C-H bond cleavage, while $^{D}V_{max}$ of 3.14 and $^{D}V_{max}/K_m$ of 0.9 for tyramine show that the slow step must be at dissociation of E•S complex.

Although the above research revealed a great deal about this enzyme (SSAO), further studies are necessary on the stereochemistry of SSAO with other substrates, such as benzylamine, since bovine enzyme is stereospecific with benzylamine, but nonstereo-specific with dopamine and tyramine. Other areas deserving exploration include stereo-

chemistry of wash-in, amino acid sequence of active site, nature of the cofactor, and determination of intrinsic isotope effect.

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