ELECTROCHEMICAL BIODEVICES FOR THE STUDY OF BOVINE SERUM AMINE OXIDASE IN THE REACTIONS WITH SUBSTRATES AND HYDRAZINE INHIBITORS

KATRI PUNAKIVI

Docente guida: Prof. ENZO AGOSTINELLI
Dipartimento di Scienze Biochimiche “A. Rossi Fanelli”
Università degli Studi di Roma “La Sapienza”

Coordinatore: Prof. PAOLO SARTI
Dipartimento di Scienze Biochimiche “A. Rossi Fanelli”
Università degli Studi di Roma “La Sapienza”

Docenti esaminatori:
Prof. MAURIZIO PACI - Dipartimento di Scienze e Tecnologie Chimiche
Università degli Studi di Roma “Tor Vergata”

Prof. GIOVANNI ANTONINI - Dipartimento di Biologia
Università degli Studi “Roma tre”

Prof. NAZZARENO CAPITANIO - Dipartimento di Scienze Biomediche
Università degli Studi di Foggia
TABLE OF CONTENTS

Abstract .......................................................................................................................... 3
Preface .......................................................................................................................... 4
Abbreviations .................................................................................................................. 5
1. INTRODUCTION ........................................................................................................ 6
   1.1 AMINE OXIDASES .................................................................................................. 6
      1.1.1 Distribution of amine oxidases in vivo .......................................................... 6
   1.2 FAD-AMINE OXIDASES ...................................................................................... 7
   1.3 COPPER AMINE OXIDASES .............................................................................. 8
      1.3.1 Role of copper in amine oxidases ................................................................. 8
      1.3.2 Organic cofactor of amine oxidases .............................................................. 11
      1.3.3 Spectroscopic characteristics of copper amine oxidases ............................ 13
      1.3.4 Structure of copper amine oxidases and active site ................................... 14
      1.3.5 Reaction mechanism .................................................................................. 16
      1.3.5.1 Reductive half-reaction .......................................................................... 17
      1.3.5.2 Oxidative half-reaction .......................................................................... 18
      1.3.6 Inhibitors of amine oxidases ...................................................................... 19
         1.3.6.1 Product inhibition of the activity ......................................................... 21
   1.4 POLYAMINES ....................................................................................................... 21
      1.4.1 Methods for the polyamine determination .................................................. 25
         1.4.1.1 Thin-Layer Chromatography ............................................................... 25
         1.4.1.2 Gas Chromatography and Gas Chromatography-Mass Spectrometry ... 25
         1.4.1.3 High-Performance Liquid Chromatography ......................................... 25
         1.4.1.4 Electrophoretic techniques .................................................................. 26
         1.4.1.5 Radioimmunoassay and enzyme-linked immunosorbent assay .......... 26
   1.5 PHYSIOLOGICAL ROLE OF POLYAMINES, AMINE OXIDASES AND TUMORAL CELL STUDIES 26
   1.6 SENSORS AND BIOSENSORS ............................................................................ 28
      1.6.1 Conversion of the biological to physical signal ............................................ 28
      1.6.2 Biochemical transducers ............................................................................. 29
         1.6.2.1 Enzymes ............................................................................................... 29
         1.6.2.2 Microorganisms and vegetal or animal tissues ..................................... 29
         1.6.2.3 Immunoreceptors ................................................................................ 29
      1.6.3 Immobilization techniques ......................................................................... 29
         1.6.3.1 Physical immobilization ....................................................................... 29
         1.6.3.2 Chemical immobilization ................................................................... 30
            1.6.3.2.1 Immobilization with carbodiimide .............................................. 30
            1.6.3.2.2 Immobilization with glutaraldehyde ........................................... 30
            1.6.3.2.3 Immobilization with polyazetidine ............................................ 31
         1.6.3.3 Immobilization of microorganism, antigens, anticorps or tissues ....... 32
      1.6.4 Physico-chemical transducers .................................................................... 32
         1.6.4.1 Electrochemical sensors ...................................................................... 32
            1.6.4.1.1 Oxygen sensor ............................................................................. 33
            1.6.4.1.2 Hydrogen peroxide sensor ......................................................... 33
            1.6.4.1.3 Mediated electron transfer .......................................................... 33
            1.6.4.1.4 Direct electron transfer ............................................................... 33
         1.6.4.2 Potentiometric sensor ............................................................................ 33
         1.6.4.3 Other transducers ................................................................................ 34
      1.6.5 Theoretical aspects of enzyme sensors ......................................................... 34
      1.6.6 Interferences .................................................................................................. 35

2. AIMS OF THE WORK ............................................................................................. 36
Abstract
In this study electrochemical sensors based on bovine serum amine oxidase were developed to determine enzyme activity and concentration of its substrates; spermine, spermidine and benzylamine. Amine oxidases catalyze the oxidative deamination of primary aliphatic, aromatic amines and polyamines to corresponding aldehydes accompanied by two-electron reduction of molecular oxygen to hydrogen peroxide. These electroactive substances can be detected by amperometric sensors. The experimental measurements were performed by several configuration devices with free and immobilized enzyme. The determination of BSAO kinetic parameters resulted to be comparable with the values given in literature for free enzyme. The same kinetic characterization was performed for the BSAO based biosensor in order to evaluate the electroanalytical performances for its possible applications in real sample analysis. The results obtained show that this biodevice represents the first step toward a complete in vitro study of the catalytic properties of different molecular forms of BSAO, and its inhibitors.
Preface

This work has been carried out at the Department of Biochemistry at the University of Rome “La Sapienza” during the XVII cycle of Ph.D. program in Biochemistry.

I am very grateful to Professors Enzo Agostinelli and Claudio Botrè for the guidance of work and work facilities. I also thank Dr. Franco Mazzei for his co-tutoring and encouragement during this work. I thank Dr. Simona Montilla for her co-operation. I also want to thank all who have helped and advised me in my work at the University. Last but not least I want to thank Professor Amiconi for giving me the possibility to realize this Ph.D. study at the University of “La Sapienza”.

For the financial support I want to thank Centro of International Mobility in Helsinki and the Italian Ministry of Foreign Affairs.

Finally I warmly thank my family who enabled me to carry out this research and continued to give encouragement and support from a distance. I am grateful to Jaana and all my friends in Finland and Italy.
Abbreviations
AO  AO
AGAO Arthrobacter globiformis AO
BH y  Benzylhydrazine
BPAO Bovine plasma AO
BSAO Bovine serum AO
Bz  Benzylamine
Cad  Cadaverine
CD  Circular dichroism
CuAO Copper AO
DAO Diamine oxidase
ECAO Escherichia coli AO
EPR Electron paramagnetic resonance
FAD  Flavin-adenine dinucleotide
HPAO Hansenula polymorpha AO
IC  Inhibition concentration
LSAO Lentil seedling AO
MAO Monoamine oxidase
PAO Polyamine oxidase
PPAO Porcine plasma AO
PSAO Pea seedling AO
PAP Polymer polyazetidine
PH  2-hydrizinopyridine
PH y  Phenylhydrazine
Put  Putrescine
Spm  Spermine
Spd  Spermidine
TPQ  2,4,5-trihydroxyphenylalanyl quinone (Topaquinone)
1. INTRODUCTION

1.1 AMINE OXIDASES

Amine oxidases are ubiquitous extra cellular or cell surface enzymes occurring in almost all prokaryotic and eukaryotic organisms. The structural and biological functions differ depending on the source of the enzyme. Amine oxidases have a physiological role for instance, in nutrient metabolism, they remove biogenic amines from blood plasma, and regulate intracellular spermine and spermidine concentrations. They have been linked to congestive heart diseases and complications in diabetes. In prokaryotes, the AOs allow microorganisms to use amines as carbon and nitrogen sources, whereas in plants and mammals they are involved in the detoxification and regulation of fundamental cellular processes, such as tissue differentiation, cell growth and programmed cell death (Averill-Bates et al. 1993, Dove et al. 2000, Pietrangeli et al. 2000). In eukaryotics, AOs influence cell growth, signalling and development (Green et al. 2002).

AOs catalyze the oxidative deamination of primary aliphatic and aromatic amines followed by the production of corresponding aldehydes, hydrogen peroxide and ammonia (reaction 1); the secondary amines are catalyzed by “flavin”-amine oxidases (reaction 2):

\[
\begin{align*}
RCH_2NH_3^+ + O_2 + H_2O & \rightarrow RCHO + NH_4^+ + H_2O_2 \quad (1) \\
R_1CH_2NHCH_2R + O_2 + H_2O & \rightarrow R_1CHO + NH_2CH_2R + H_2O_2 \quad (2)
\end{align*}
\]

These enzymes, depending on their substrate specificity and catalytic reaction, can be divided into mono-, di- or polyamine oxidases, even though on the basis of prosthetic groups their classification is of major importance. According to the prosthetic groups the amine oxidases can be classified in two groups:

- “flavin” amine oxidases containing flavin-adenine dinucleotide as a cofactor (FAD-AOs - E.C. 1.4.3.4)
- copper containing amine oxidases (CuAOs - E.C. 1.4.3.6)

The last group contains, beside copper, an organic cofactor 2,4,5-trihydroxyphenylalanylquinone (TPQ). These enzymes catalyze two different catabolic processes: FAD-containing amine oxidases catalyze the retroconversion of the polyamines, with formation of “recyclable” products used for biosynthesis, while CuAOs catalyze a different catabolic pathway called terminal degradation with the formation of active metabolites not usable for biosynthesis (Teti et al. 2002).

1.1.1 Distribution of amine oxidases in vivo

AOs are purified from different microorganisms, plants and animals. They can be isolated e.g. from microorganisms such as Aspergillus niger, Micrococcus rubens, Penicillium chrysogenum, Arthrobacter globiformis and Escherichia Coli (Yamada et al. 1965, Okada et al. 1979, Parson et al. 1995, Wilce et al. 1997, Juda et al. 2001). CuAOs in Gram-positives are localized in cytoplasm, whereas CuAOs in Gram-negatives are found in periplasmics. AOs have been also purified from different vegetable sources e.g. Pisum sativum, Lens esculenta, Euphorbia characias, Avena sativa, Lathyrus cicela, secale cereale, Hordeum vulgare, Zea mays, Oryza sativa, Thea sinensis, Triticum aestivum and Vicia faba (Mondovi et al. 1989, Medda et al. 1995, Šebela et al. 2001). For example, Pea Seedling contains high quantities of CuAOs (Kumar et al. 1996). Features of CuAOs from vegetable and animal origins are similar. The aliphatic C3-C6 diamines are the main substrates for both microorganism and vegetable AOs whereas polyamines and aromatic or aliphatic monoamines are the main substrates for mammalian AOs (Moosavi-Nejad et al. 2001). Intracellular AOs, such as mitochondrial monoamine oxidase (MAO) and cytosolic polyamine oxidase (PAO), both found in almost all tissues, belong to FAD dependent amine
oxidases whereas serum enzymes and diamine oxidase in biological fluids are copper amine oxidases (Lyles 1996). The mammalian CuAOs have been isolated and characterized from serum, kidney, placenta or liver and the largest number of them are intracellular enzymes, even though they can also be extracellular (Table 1). The best characterized serum CuAOs are from bovine serum and swine serum, which are two enzymes with similar amino acidic sequence (Lindström, Pettersson 1973, Lindström, Pettersson 1978, Barker et al. 1979, Suzuki et al. 1983, Mondovì et al. 1989). Average weight for plant AOs is 140 kDs and for mammalian CuAOs, 170 kDs. They usually contain between 7 to 14% carbohydrates, except for bovine serum amine oxidase where the amount of carbohydrates correspond to 4.3% of molecular weight (Moosavi-Nejad et al. 2001, Calderone et al. 2003).

<table>
<thead>
<tr>
<th>Amine oxidase</th>
<th>Structure and molecular weight (kDa)</th>
<th>Cofactor</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine serum</td>
<td>Dimer, 170</td>
<td>Cu-TPQ</td>
<td>Aliphatic and aromatic primary amine groups</td>
</tr>
<tr>
<td>Swine serum</td>
<td>Dimer, 190</td>
<td>Cu-TPQ</td>
<td>Aliphatic and aromatic primary amine groups</td>
</tr>
<tr>
<td>Pig kidney</td>
<td>Dimer, 180</td>
<td>Cu-TPQ</td>
<td>C4-C7 aliphatic diamines and histamine</td>
</tr>
<tr>
<td>Human placenta</td>
<td>Monomer, 80</td>
<td>Cu-TPQ</td>
<td>Diamines and histamine</td>
</tr>
<tr>
<td>Rat liver</td>
<td>Monomer, 60</td>
<td>FAD</td>
<td>Secondary amino groups of spermine and spermidine</td>
</tr>
</tbody>
</table>

1.2 FAD-AMINE OXIDASES

Monoamine and polyamine oxidases catalyze the reaction to the secondary amine groups of free polyamines, such as spermine, spermidine, putrescine and their N-acetyl derivates (Morgan 1987, Lyles 1996).

MAO also has the ability to catalyze the tertiary amine group. On the basis of substrate specificity two different types of MAOs, MAO-A and MAO-B have been characterized. Both forms of MAO metabolize all the natural substrates, such as dopamine and tyramine. Tertiary amines seem to be specific substrates of MAO-A. MAO-A acts on hormones such as serotonin, adrenaline and noradrenaline and it is inhibited by clorgyline and moclobemide. MAO-B acts on β-phenetylamine and benzylamine and it is inhibited by selegiline (Grimsby et al. 1991, Silvestri et al. 2003).

MAO-A consists of 527 amino acids and MAO-B of 520. Though they have same sequences containing the peptide Ser-Gly-Gly-Cys-Tyr, both forms are made of similar but different polypeptides. They are formed of two identical subunits and each subunit is coupled with one FAD. This cofactor is bound to the protein through a thioether linkage with a cysteine. In humans both have about 70% sequence homology in the primary structure, which can be noted in three regions of polypeptides: in amino- and carboxyl-terminal regions and in active site (Wouters et al. 1999, Binda et al. 2002, Nagatsu 2004). In both MAO forms the overall tertiary structure is similar as well as active sites (Geha et al. 2002). In the region of
substrate specificity there are two types of binding sites, one responsible for interaction with amino group while the other is very hydrophobic. MAOs are hydrophobic molecules and react with lipophylic sites of substrate. The hydrophobicity plays an important role, particularly in MAO-B.

These two forms of MAO are present in most tissues, though in humans MAO-A is abundant in the brain, liver and placenta, MAO-B is well present in liver, lungs and intestine (Nagatsu 2004). The genes of MAO-A and MAO-B are located on the X chromosomes. They consist of 15 exons identically organized with intron. The results of 12th exon show 93.9% amino acid identity for both forms of MAO and that they were derived from the duplication of a common ancestral gene (Grimsby et al. 1991). The differences indicate that these genes have different transcriptional activity.

MAOs are involved in the metabolism of monoamine neurotransmitters and hence of interest in psychiatric and neurological disorders such as depression and Parkinson’s disease (Wouters et al. 1999, Binda et al. 2002, Silvestri et al. 2003, Nagatsu 2004). In fact monoamine oxidase inhibitors, were the first drugs to be used for the treatment of depression. The reversible inhibitors of MAO-A are specially used for depression treatment while the inhibitors of MAO-B are used in the therapy of Parkinson, particularly derivates of pyrroles and deprenyl. Recent studies have shown that high levels of MAO-B have been found to be connected with Alzheimer disease and to induce apoptosis in neuronal and kidney cells.

MAO and PAO share 20% identity in amino acidic sequence (Geha et al. 2002). The reaction products of PAO depend on the enzyme source (Martin-Tanguy 2001, Šebela et al. 2001). Spermidine is catalyzed to putrescine and spermine to spermidine by mammalian PAO with a formation of 3-aminopropanal. Plant and bacterial PAOs are more specific and oxidize only spermine and spermidine. Spermidine is oxidized to 4-aminobutanal, which spontaneously converts to pyrrole, which can be converted further to γ-aminobutyric acid. Spermine is oxidized to 1-(3-aminopropyl)-4-aminobutanal and converted further on to 1,5 diazabicyclononane. Other reaction products are hydrogen peroxide and diaminopropane, which can be catabolized to β-alanine and hence can not be used for polyamine synthesis.

1.3 COPPER AMINE OXIDASES

The other group of amine oxidases are called chinoproteins as they contain an organic cofactor topaquinone (TPQ) in the active site. Additionally they have two copper atoms as inorganic cofactors. In the chapters below the roles of inorganic and organic cofactors are discussed, followed by chapters on characterization, structure and reaction mechanism.

1.3.1 Role of copper in amine oxidases

In biological and abiological dioxygen activations copper active sites play an important role (Solomon et al. 2001). CuAOs have been identified with two magnetically independent cupric copper atoms as prosthetic groups by electron paramagnetic resonance (EPR) (Suzuki et al. 1983, Dooley, Coté 1985, Morpurgo et al. 1987). The role of copper ions in amine oxidases is not clear in the catalytic cycle, but it seems to have both a structural and functional role, by helping the protein to keep the tertiary structure and participating in the redox reaction, shuttling between Cu⁺ and Cu²⁺, during autocatalytic single turnover generating the active oxidized enzyme in a six-electron oxidation from precursor protein, as reported in the paragraph 1.3.2 (reaction 3) (Dooley 1999). Under anaerobic conditions the reaction reduces copper and catalyzes the post-translational oxidation of tyrosine to TPQ, probably affecting the redox potential of TPQ. In the mammalian AOs the copper seems not to have a redox role (Mondovi et al. 1989, Agostinelli et al. 1994, Moosavi-Nejad et al. 2001). It has also been proposed that metal acts as a Lewis acid and decreases the pKa of the water molecule and facilitates the proton transfer to O₂ (Agostinelli et al. 1997, 1998, De Matteis et al. 1999).
Precursor protein + 2O₂ + Cu⁺⁺ → E_{ox} + H₂O₂ (3)

Cu⁺⁺-semiquinolamine radical possibly binds oxygen and superoxide anion and though may function as an electron mediator in the electron transfer from reduced cofactor to molecular oxygen via the metal co-ordinated ligand orbitals (McGuirl, Dooley 1999). Intramolecular electron transfer to generate the Cu⁺⁺-semiquinone has been shown to be kinetically feasible in amine oxidases from both eukaryotic (pea seedling) and prokaryotic (*Arthrobacter P1*) sources (Kumar et al. 1996). In plant amine oxidases, copper has shown to be essential for faster a catalytic rate (Padiglia et al. 1999). Only the reoxidation step is abolished by the absence of one or two metal ions, whereas in the absence of catalytically competent reduced metal form, copper can not act as the site for molecular oxygen binding (Morpurgo et al. 1990, Agostinelli et al. 1994).

By electron paramagnetic resonance it could also be seen that the binding of copper affects the copper sites, but the two copper ions have different reactivity. The two copper sites do not appear to be identical since they are affected differently by the substrate. As a single copper is tightly bound, the other one can be either selectively removed or reinserted (Suzuki et al. 1986, Morpurgo et al. 1987, Mills, Klinman 2000).

The role of copper has been studied by copper binding inhibitors (such as azide, cyanide, EDTA or N,N-diethyldithiocarbamate (DDC) which slow down the catalytic reaction) in the presence or absence of benzylamine (Morpurgo et al. 1990, Agostinelli et al. 1994, 1997, 1998). Reactions with benzylamine, which is a reducing agent, have been studied to get more information concerning the metal’s role. The removal of copper in the presence of DDC and sodium dithionite in anaerobic conditions, results in the loss of colour and activity. The residual copper has usually been under 15%, ranging from 10–20% when as copper was removed in the presence of sodium dithionite and cyanide in anaerobic conditions, the residual copper content was less than 2% (Suzuki et al. 1983, Morpurgo et al. 1987, Mills, Klinmann 2000). The copper binding inhibitors were found to be competitive toward oxygen and uncompetitive toward amine (Morpurgo et al. 1990). The reduced state of TPQ, obtained in the presence of cyanide and sodium dithionite as above described, seems to facilitate the removal of Cu⁺⁺ ion from the enzyme. In the same way, it seems to facilitate the recovery of Cu⁺⁺ (Morpurgo et al. 1987). In the absence of metal, the reduced and oxidized forms of TPQ are unreactive toward phenylhydrazine (Mills, Klinman 2000).

In the apoenzyme, the reoxidation of TPQ by oxygen can be also achieved using Zn²⁺, Co²⁺ and Ni²⁺ as the metal cofactor (Suzuki et al. 1983, Agostinelli et al. 1994, Mills, Klinman 2000, Mills et al. 2002). These metal derivatives were prepared to get more information about the role of the metal. Native BSAO, Co²⁺ reconstituted, Ni²⁺ substituted and Ni²⁺ depleted BSAO showed similar bands, suggesting that TPQ displays positive CD peak at 370 nm and a negative peak at 440 nm. The native enzyme and the derivatives also have a similar optical absorption band (Suzuki et al. 1983, Pietrangeli et al. 2003). The dialysis of Cu-depleted BSAO against a solution of NiCl₂ gave pink with the Ni²⁺ substituted BSAO derivative at 484 nm. This band was reduced to 470 nm after nickel depletion. These bands disappeared when benzylamine was added, thus indicating TPQ reduction (Suzuki et al. 1983). By adding one equivalent molar of these metals, the reactivity towards phenylhydrazine is restored though showing a contemporary reaction with both TPQ and copper (Bossa et al. 1994). To obtain the cobalt re-constituted BSAO, the Cu-depleted BSAO
was dialyzed against a solution 2 mM of CoCl₂. Microcalorimetry measurements have indicated a similar conformational role for the copper and cobalt in BSAO (Agostinelli et al. 1994). This was not accepted by others, since a partial reduction of copper and formation of a radical was noted in EPR signal. The radical was also characterized with spectrophotometer. Three absorption bands were found in the region of 350-460 nm. The TPQ band at 480 nm was fully bleached indicating an equilibrium between Cu⁺-semiquinolamine and Cu²⁺-quinolamine. A radical of low intensity, 1-2% reduction of copper, was detected in BSAO and pig plasma AO (Dooley et al. 1991). In the presence of cyanide, in plant AOs, the equilibrium is shifted towards the formation of the reduced copper.

Enzyme activity decreases in half copper depleted and is abolished in fully copper depleted (Agostinelli et al. 1994). A difference in the co-ordinate ligand structure between native and half copper depleted BSAO has not been noted. The Cu-depleted BSAO by KCN and sodium dithionite lose up to 90% and 98% of catalytic activity in anaerobic conditions and the absorbance band at 480 nm. After titration by phenylhydrazine the band is slowed down to 434 nm. The addition of benzylamine into Cu-depleted BSAO in air decreased the residual TPQ band at 480 nm (Agostinelli et al. 1997). The copper removal decreased the benzylamine affinity for the protein (Agostinelli et al. 1998). The samples of Co²⁺-reconstituted amine oxidases are homogenous and contain a substantial proportion of molecules (50–70%), in which the carbonyl group is not available. In debates of the effect of residual copper in enzyme, Agostinelli et al. (1998) showed, that the reactivity of carbonyl group reagents is fully restored by Co²⁺-reconstituted BSAO and that Kₘ values for several substrates are different than the values of native BSAO. This was shown by making 50% of copper incompetent for catalysis and hence one subunit was inactive. Additionally it was shown that the small radical signal in EPR spectrum of BSAO is not temperature-dependent (Pietrangeli et al. 2000). Specific activity of Co²⁺ substituted enzyme was reduced to about 40% of the native amine oxidase value, but it does not substantially impair the enzymatic activity (Morpurgo et al. 1990, Padiglia et al. 2001). The residual copper does not affect the activity of cobalt dialyzed samples (Agostinelli et al. 1998). Cobalt has lower affinity for the enzyme than copper, though it is catalytically competent and most likely occupies the same site as copper, but it may have different co-ordination, which excludes essential step of semiquinone radical formation. The metal exchange does not occur in the re-oxidized enzyme (Agostinelli et al. 1998). A linear relationship has been found between re-incorporated copper and recovered native properties in fully copper depleted BSAO (Table 2) (Agostinelli et al. 1994, 1998, Mills, Klinman 2000). Both studies had similar results and the residue copper was found to be ~35% and they also exhibit the characteristic absorption band at 486 nm and the removal did not affect the thermal stability as shown in differential scanning calorimetry studies (Agostinelli et al. 1994).
### Table 2. Copper depleted and recovered BSAO.

<table>
<thead>
<tr>
<th></th>
<th>Native</th>
<th>Half Cu-depleted</th>
<th>Fully depleted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu/mol protein</td>
<td>~2.0 ± 0.1</td>
<td>1 ± 0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Carbonyl/mol protein</td>
<td>1</td>
<td>0.19 – 0.22</td>
<td>0.06</td>
</tr>
<tr>
<td>Reduced Specific activity</td>
<td></td>
<td>16 – 19%</td>
<td>2%</td>
</tr>
<tr>
<td>Recovered with Cu^{2+}</td>
<td>87%</td>
<td>87%, 79%</td>
<td></td>
</tr>
<tr>
<td>Reactivated</td>
<td>62%</td>
<td>68%</td>
<td></td>
</tr>
<tr>
<td>Recovered with Co^{2+}</td>
<td>40%</td>
<td>27%, 1.1 cobalt atom/dimer</td>
<td>15%, 2.1 cobalt atom/dimer</td>
</tr>
<tr>
<td>Carbonyl/mol protein, Recovered with Co^{2+}</td>
<td>66%</td>
<td>62%</td>
<td></td>
</tr>
</tbody>
</table>

### 1.3.2 Organic cofactor of amine oxidases

Beside catalyzing the amine oxidation, copper amine oxidases synthetize their own cofactor. The organic cofactor was previously identified as the pyridoxal phosphate and then pyrroquinoline quinone, but in the 1990 the cofactor was identified as topaquinone (TPQ), an oxidized tyrosine residue, which is covalently bound to the active site of the enzyme through standard peptide bonds (Janes et al. 1990, Morpurgo et al. 1992). Janes et al. (1990) characterized the pentapeptide as having the following sequence: Leu-Asn-hydroxydopa-Asp-Tyr. Their study with mass spectrometry, ultraviolet-visible spectroscopy, and proton nuclear magnetic resonance showed two carboxylate groups in the pentapeptide, which can be accounted for in the peptide itself, indicating that there are not any other carboxyl groups in the cofactor. Plant AOs have two TPQs and two active sites per dimeric molecule that can catalyze the oxidation at the same time, while mammalian AOs, have two TPQs but one active site or two dependent active sites (Moosavi-Nejad et al. 2001). TPQ is present in all CuAOs except in lysyl oxidase (Dawkes, Phillips 2001). According to the study of Mu et al. (1992) TPQ is not dissociable from its cognate enzyme since it is derived by post-translational process from a tyrosine residue in the active site of protein itself. It has been observed that in both Hansenula polymorpha and Arthrobacter globiformis, the TPQ biogenesis takes place in the enzyme active site through a copper dependent, self-processing mechanism (Cai, Klinman 1994, Choi et al. 1995). The centrality of the role played by copper in the TPQ biogenesis expressing an amine oxidase enzyme from Hansenula polymorpha in a Saccaromyces Cerevisiae mutant lacking the capability to express a similar enzyme has been shown. Both the wild type enzyme and a mutant enzyme containing an altered amino acidic sequence surrounding the tyrosine residue could undergo tyrosine oxidation, whereas altering the copper binding side residues meant that the copper could no longer be fixed and the enzyme failed to produce TPQ. In 1994 Tanizawa’s group reached the same conclusion isolating and studying a copper-depleted, TPQ deficient forms of two different Arthrobacter globiformis amine oxidases. Hence, it was demonstrated that TPQ production can be performed in vitro by just adding copper to the reaction in the presence of molecular oxygen, the starting point being the formation of the tyrosine-copper complex by means of the tyrosine hydroxyl group. Role of oxygen in the TPQ biogenesis of HPAO has been explored using N_3^- as a probe (Schwartz et al. 2001). Azide inhibits catalysis in HPAO and it has been observed that, during
TPQ biogenesis, it inhibits the tyrosine-copper complex formation that is a crucial starting point for the spontaneous oxidation of the aromatic amino acid, displaying an equatorial water molecule from copper (McGuirl et al. 1997). This inhibition results non competitive with the respect to oxygen to kinetic studies. By using N\textsubscript{3}\textsuperscript{-}, the mechanism previously suggested has been once more confirmed (Schwartz et al. 2001). These studies assess for the hypothesis according to which O\textsubscript{2} prebinds to copper containing AOs in a non-metal site (Schwartz et al. 2000) and that only after that, it reacts directly with the reduced copper form during biogenesis. N\textsubscript{3}\textsuperscript{-} shows a competitive inhibition respect to O\textsubscript{2} in PPAO. It could be explained assessing either that even though copper containing AOs have the same catalytic mechanism, differences exist among these enzymes or that the competitive inhibition of N\textsubscript{3}\textsuperscript{-} observed in PPAO is simply due the difficulty in distinguishing weak non-competitive from competitive inhibition. The stoichiometry of TPQ biogenesis has been studied by Ruggiero and Dooley (1999). Several mechanisms requiring 3 to 1.5 mol O\textsubscript{2} per 3 to 0 mol H\textsubscript{2}O\textsubscript{2} with one mol TPQ produced respect to one H\textsubscript{2}O\textsubscript{2} have been presented. The final reaction was stabilized to 2 mol of O\textsubscript{2} per TPQ formed indicating that one dioxygen is reduced by four electrons and the second is reduced by two electrons to produce H\textsubscript{2}O\textsubscript{2}. Using X-ray crystallography, Tanizawa and coworkers (Kim et al. 2002) recently provided the first structural evidence for TPQ biogenesis intermediates. It was assumed the apo AGAO structure, where Histidines 431,433,592 and the hydroxyl group of the tyrosine 382 (the precursor of TPQ) are arranged in a tetrahedral geometry, was the starting point of the process. Cu\textsuperscript{2+} enters the active site locating itself in the center of a trigonal pyramid. Tyrosine has been observed to be one of its ligands even before the oxygen binding to the active site. It is a very weak interaction, perhaps due to the withdrawing effect exerted by copper upon the tyrosine phenol ring electrons.

Copper co-ordination to tyrosine in AGAO crystals was not the consequence of the copper reduction and it was not somehow concerned with it. This interaction becomes a ligand to metal charge transfer interaction, as the copper get closer to the C4 tyrosine deprotonated hydroxil group. Infact, a direct attack of oxygen to the C3 of the tyrosine ring takes place, in the meanwhile C4 hydroxil group deprotonates and the copper moves toward the tyrosine residue (Dove et al. 2000, Schwartz et al. 2000). The oxygen molecule localized between copper and C3 of the tyrosine ring breaks down and the C3 position is further oxidized through a reprotonation and subsequent dehydration. The dopa quinone formed then makes a 180\degree rotation around the C\textsubscript{\beta}-C\textsubscript{\gamma} bond so that C2 position of TPQ can be attacked by either a water molecule or by a hydroxil group generated in the surrounding medium. His592 orientation changes during TPQ biogenesis. This seems to be critical to the changes in the copper co-ordination structure during the process. The changing in the structure from square-pyramidal to trigonal-bypiramidal of copper is supposed to produce the movement of its axial water ligand into an equatorial position, which should increase its acidic character. As a consequence a nucleophilic attack to C2 of dopa quinone ring is energetically allowed yielding Topa. The final step needs another O\textsubscript{2} molecule to enter the active site. It rapidly oxides TPQ producing hydrogen peroxide. Copper atom then binds another water molecule and returns to a square pyramidal geometry. The replacing of copper with cobalt, nickel or zinc seems to disable the biogenesis due to the incapacity of oxygen to activate (Dawkes, Phillips 2001). Niculescu and coworkers (2000) have also demonstrated the possibility of replacing the oxygen in the catalytic mechanism by chronoamperometric determination for histamine, when copper free enzymes were immobilized on graphite electrodes and these enzymes responded to be active.

Another interesting model for TPQ biogenesis is offered by oxidative half-reaction of amine oxidases where the ammonia in the reductive first half reaction is transferred to the C5 position of topaquinone, resulting hydrolysis and, to release of aldehyde from Schiff base complex (Williams, Klinman 2000, Murray et al. 2001). The formed aminoquinol has to be re-oxidized by dioxygen to regenerate TPQ.
1.3.3 Spectroscopic characteristics of copper amine oxidases

Amine oxidases copper ions and the organic carbonyl group exhibit a characteristic pink color at 480 nm, with an extinction coefficient ($\varepsilon$) 3500 M$^{-1}$cm$^{-1}$, due to $\pi \rightarrow \pi^*$ transition of TPQ-anion in $p$-chinonic form (Figure 1).

The dialysis of native enzyme against a solution of sodium dithionite ($10^{-3}$ M) and potassium cyanide ($10^{-2}$ M) in phosphate buffer (0.1 M, pH 7.2) reduces TPQ, covalently binds it, and removes the metal and hence leads to a loss of the visible band at 480 nm. The band at 480 nm can be restored by treatment with a solution 2 mM of CuSO$_4$, CuCl$_2$, or other metal salts, as mentioned in the previous paragraph. The presence of metals is not directly responsible for the band formation, but they are essential for the re-oxidation of TPQ and the band formed by the TPQ.

Circular Dichroism (CD) studies have shown d-d transition to Cu$^{2+}$ at the wavelength superior of 500 nm, while the peak at 480/500 nm the optical spectra shows the low intensity of the transition due to the organic cofactor. The positive extremum in the CD band can be seen at 370 and 650 nm and a negative extremum at 450 nm, showing a negative shoulder at 525 nm. The CD spectra, along with EPR spectra at 35 GHz, have shown the copper chromophore to be close to the active site (Suzuki et al. 1983). The EPR studies of various CuAOs have shown similar characteristics of copper ions as in complexes with low molecular weight and symmetrical axe. Also in the BSAO, has been shown similar characteristics even though the two copper ions have different reactivity. A signal of copper in EPR indicating the axial symmetry of Cu$^{2+}$ radical at $g$ 2.001 and a hyperfine constant ($A_{hf}$) at about 336 mT has been observed (Figure 2). Addition of the substrate makes variations in EPR of copper due to conformational changes.

Figure 1. Optical spectra of native BSAO.
1.3.4 Structure of copper amine oxidases and active site

The AOs crystal structure has been analyzed from pea seedling, yeast Hansenula polymorpha, Escherichia coli and Arthrobacter globiformis (Figure 3) (Parsons et al. 1995, Tipping, McPherson 1995, Wilce et al. 1997, Wilmot et al. 1997, Su, Klinman 1998, Murray et al. 1999, Dove et al. 2000). Recently, the preliminary crystal structure of BSAO as the first mammalian AO has also been investigated. The structure resulted to be very similar to that of ECAO (Caldenore et al. 2003). The structures show homology between prokaryotic and eukaryotic enzymes and through SDS-PAGE electrophoresis the subunits have been seen to be equal.

CuAOSs are generally soluble glycoproteins composed of two identical subunits, which seem to be linked together by one or more disulphide bridges and a large number of non-covalent interactions (Mondovi et al. 1989). The enzymes have 33 completely conserved residues of which 10 are found within 41 aligned residues at the C-terminal tails, the region

Figure 2. EPR spectra of native BSAO, BSAO (130 µM) in potassium buffer (0.1M, pH 7.2), spermine added up to 5 mM concentration, 10 GHz, temperature 100 K (Pietrangeli et al. 2000).

Figure 3. The structure of ECAO homodimer, where one monomer is colored by domains (main figure). a) active site arrangement of ECAO in oxidized resting state, b) active site of reduced ECAO with nitric oxide. Copper ions are shown as green spheres.
missing from the original lentil sequence (Tipping, McPherson 1995). Between the two subunits, a solvent filled cavity has also been found, which may be the route for the diffusion of Cu ions, O₂ molecules, H₂O₂ molecules and/or NH₄⁺ ions between the molecular surface and the active site (Wilce et al. 1997). Per an enzyme molecule, there are two well-separated Cu⁺⁺ atoms and TPQ cofactor at a consensus sequence occurring per active site (Barker et al. 1979, Mondovi et al. 1989, Moosavi-Nejad et al. 2001, Pietrangeli et al. 2003). Although the copper and TPQ are not bound to each other, they are hydrogen connected by a water molecule and O₂ of TPQ with O5 close to the base Asp383 in active site: this is known as off-copper conformation (Dawkes, Phillips 2001). In addition to copper atoms, the presence two other metal ions, which are distant from the active site, have been found. These other ions have been found to be Mn²⁺ or Ca²⁺ (Parson et al. 1995, Murray et al. 1999, Di Paolo et al. 2002).

The crystal structure of different amine oxidases varies in the dimensions and electrostatic properties of the active site channel (Shepard et al. 2002). The shape of amine oxidase dimer is similar of mushroom cap with four domains in square-pyramidal arrangement, though some amine oxidases miss the D1 domain. For example, in pea seedling amine oxidase three domains have been characterized in the dimensions 110*65*45 Å (Wilce et al. 1997, Wilmot et al. 1997). In ECAO the D1 (α/β) domain consists of 85 residues and normally forms the stalk of the mushroom. The resting domains D2 and D3 are formed by about 100 homologous amino acids in structure and amino acid sequence and sited on the outer edge of the mushroom form. From D4 domain, which is consists of 440 amino acid residue β-sandwich at the C terminus containing the active site, also extends two β ribbon arms to embrace the D4 domain of other subunit (Wilmot et al. 1997). The function of these arms is unclear but Wilce et al. (1997) suggest that besides stabilizing the enzyme they have an effect on the substrate access to the active site. Their study has revealed a channel originated on the molecular surface near the junction of domains D3 and D4, which seems to be for the movement of reactants and reaction products to and from the active site. The molecular surrounding the channel entrance is predominantly negative and is associated with the side chains of domains D3 and D4. The internal surface of the channel is lined by residues, which become more hydrophobic with the approach of the active site. This internal surface regulates the access to the channel and the accessibility to the TPQ (Murray et al. 2001, Shepard et al. 2002).

The two copper ions, located in the active site bound to nitrogen and oxygen, are coordinated by imidazole groups of three histidine (His524, His526, His689) side chains and two water ligands of which one is bound to the symmetric axe and the other one to the equatorial axe, even though in reduced form of ECAO was observed only a water molecule bound to the axial position of copper ion (Barker et al. 1979, Wilce et al. 1997, Wilmot et al. 1997, Dawkes, Phillips 2001, Mills et al. 2002). The BSAO differs from ECAO in the domains D1 and D2 and in the orientation of TPQ, otherwise the structure is same (Calderone et al. 2003). Although the study done in 1991 by Janes and Klinman suggests that the BSAO contains two active sites per dimer, in HPAO a chain of immobilized water molecules have been identified, starting from the axial copper ligand, ending with the fifth water, which is hydrogen bind to a cluster of solvent molecules in the other solvent-accessible cavity (Green et al. 2002 and references therein). The distance of this other solvent-accessible cavity in HPAO is relatively large leading to H-bonding chain, which has extensive and conserved nature suggesting it is essential to the protein structure. In the inactive site the copper is coordinated by phenolic oxygen of TPQ instead of water molecules. Electron paramagnetic studies of different native amine oxidases have shown typical copper spectra, which are similar to those complexes where the metal is present at low molecular weight with symmetric axes (Barker et al. 1979, Suzuki et al. 1983, Dooley, Coté 1985). In these studies a change in the symmetry of the region surrounding copper has been observed after adding substrate and equatorial coordination of anions. The plane TPQ ring is 4-5 Å from copper.
TPQ can also bind the copper through its O4 oxygen at a distance of 2.4 Å, when it rotates toward the copper site in a manner similar to precursor tyrosine. This form is always catalytically inactive (Dove et al. 2000). The study by Murray et al. 2001 showed that Tyr369 bound by hydrogen bond to TPQ O4 is important for maintaining the conformation of TPQ. When the substrate reacts at C=O5 of TPQ, a rotation of ~180° in the TPQ orientation in Cβ-Cγ bond towards the copper occurs (Dawkes, Phillips 2001, Green et al. 2002). TPQ is very mobile and can rotate freely and also flip into direct contact with the copper. This is called on-copper conformation.

Concerning the three dimensional structure of the active site, it has been noticed that enzyme reacts with the whole substrate molecule and not just with the amine group. The amine group interacts in its non protonated form with the protonated group on the active site through hydrophobic electrostatic interaction (Murray et al. 1999). Another negatively charged group located 6–9 Å from the active site binds the second amine group, which seems to be in its protonated form. Hydrophobic region is propably between the active sites to bind hydrocarburic chain of amine.

Even though structurally similar CuAOs have functional variance in the active site, LSAO and BSAO have one amino acid identity percentage around 25%. But almost 75% of these identical amino acids belong to LSAO catalytic domain 4 (Moosavi-Nejad et al. 2001). This could hence explain the similar activities in AOs. Studies demonstrate that structural differences between plant and mammalian AOs affect on their behaviour at different temperature. For example this could have an effect in their physiological role in different organisms. Very interestingly, the occurrence of temperature-induced conformational changes has been observed both in lentil seedling amine oxidases and BSAO. BSAO, in fact, showed nonlinear-Arrhenius behaviour with increasing temperature (Befani et al. 1989, Stevanato et al. 1995). Differential scanning colorimetric profiles for BSAO revealed three distinct temperature transitions (Giartosio et al. 1988) and five energetic domains (Agostinelli et al. 1994). LSAO, itself, reveals similar differential scanning colorimetric (DSC) profile, (Moosavi-Nejad et al. 2001) showing two faces, one from 17 to 37°C and the other from 42 to 57°C, the activation energy of LSAO being higher below 37°C. As both BSAO and LSAO show similar non linear Arrhenius plot shapes with a jump in them it can be assumed that conformational changes from one stable form to another take place in both enzymes with the increasing of temperature; each form possessing a catalytic activity. At the same time it can be observed that BSAO activity is independent of temperature at the mammal physiological conditions. In plants, on the contrary, physiological temperature ranges from 0 to 50°C (Raven et al. 1986), it can help modulating AOs physiological role in those organisms.

1.3.5 Reaction mechanism

AOs function by a ping-pong enzyme-substitution mechanism, which is double displacement or transaminase type mechanism. The catalytic cycle of the copper amine oxidases consists of two half-reactions: well studied reductive half-reaction, which is independent of copper and less clear oxidative half-reaction.

\[
\text{Reductive site } E_{\text{ox}} + R-\text{CH}_2\text{NH}_3^+ \rightarrow E_{\text{red}}-\text{NH}_3^+ + R-\text{CHO} \quad (4)
\]

\[
\text{Oxidative site } E_{\text{red}}-\text{NH}_3^+ + O_2 + H_2O \rightarrow E_{\text{ox}} + NH_4^+ + H_2O_2 \quad (5)
\]
1.3.5.1 Reductive half-reaction

In the figure 4 is presented the reductive half-reaction of the bovine plasma AO. In the reductive half-reaction the initial step is the substrate binding to the active site in protonated form, giving though a carbinolamine intermediate (3 in the figure 4). The amino group of the substrate is moved in the deprotonated state, which is required to facilitate the nucleophilic addition to TPQ<sub>ox</sub>. In anaerobic conditions substrate recognizes the active site of free enzyme and forms electrostatic forces and Van der Waals bonding. The stereochemistry of proton abstraction seems to vary depending on the enzyme source, suggesting that AOs have different structures of active sites in relation to the substrate specificity. The formation of Schiff base leads to activating the C-1 proton of the substrate and water removal and to a covalent bonding between the substrate and TPQ at C-5 carbonyl building the first intermediate, substrate Schiff base (5 in the figure 4), which can be trapped by reduction with NaCNBH₃ (Hartmann, Klinman 1987, Bushnell et al. 1990). It has been proposed that an electrostatic stabilization of the formed Schiff base is provided by the low pKₐ of the C-4 hydroxy group, and that base-catalyzed proton abstraction then gives a carbanion transition intermediate (6 in the figure 4) (Hartmann, Klinman 1991). This formation of carbanion and electron transfer to TPQ is followed by a large increase of pKₐ of the C-4 hydroxy group, leading to proton transfer from the active-site base to the oxynion at C-4 (6 → 7). The imine complex of the Schiff base (7 in the figure 4), which is not anymore electrostatically stabilized, undergoes a rapid hydrolysis and forms the product aldehyde (7 → 11). This intermediate (7) has been difficult to isolate due to this rapid rate of hydrolysis, but it was confirmed as its quinonoid tautomer by using benzyamines with electron-releasing substituents in rapid-scanning stopped-flow studies (Hartmann et al. 1993). The tautomerization from substrate Schiff base to product Schiff base, of a proton at the C-1 position of the substrate, to form an intermediate and then release the aldehyde, leaves the enzyme in an aminoquinol form, which is the reduced form of TPQ (Anthony 1996, Wilmot et al. 1997, Agostinelli et al. 1998, Mills, Klinman 2000, Dawkes, Phillips 2001, Padiglia et al. 2001, Mure et al. 2002). The key step of this kind of mechanism is usually the proton abstraction from C-1 of the substrate, Anthony (1996) proposes that also the steps 2 → 3 (proton transfer from the nitrogen to the oxygen), 3 → 5 (removal of the water from the resulting carbinolamine) and 7 → 11 (later hydrolysis of the Schiff product base) require the involvement of an active-site base. Upon the TPQ reduction the protonation of oxygens at C-2 and C-4 of the TPQ-ring is also excepted. Particularly the TPQ reduction follows, to form the neutral aminoquinol TPQ<sub>red</sub> with copper hydroxide. The aminoquinol of the enzyme and Cu<sup>2+</sup> are quickly in equilibrium with the semiquinone form of the enzyme and Cu', though over 80% is in aminoquinol-Cu<sup>2+</sup> form in anaerobic state at room temperature (Dooley et al. 1991).
Figure 4. The reductive half-reaction of amine oxidases.

The resulting reduced form of the cofactor (the aminooquinol) is then re-oxidized by O₂ to produce hydrogen peroxide in the oxidative half-reaction.

1.3.5.2 Oxidative half-reaction

The mechanism of oxidative half-reaction is, however, not clear as the reductive half-reaction and it may be possible that there are debates of more than one possible pathways depending of the enzyme source.

The reconstitution of the oxidized enzyme starts from the aminoquinol form of the reductive half-reaction (Figure 5). The mechanism is dioxygen dependent and in the first step oxygen interacts with reduced TPQ to form hydrogen peroxide and iminoquinone. Since the electron transfer between aminoquinol TPQ_red and Cu-aminooquinol is faster than catalysis, the site for O₂ is Cu⁺-semiquinone followed by a rapid formation of Cu²⁺-superoxide complex e.g. in ECAO and AGAO (Dawkes, Phillips 2001). It has been proposed that oxygen binds to another, non metal-site, of enzyme. Su and Klinman in 1998 showed, using BSAO, that the first electron transfer to oxygen seems to be rate determining, and that it is from TPQ_red and not from copper in Cu²⁺-aminooquinol form. The re-oxidation of BSAO by dioxygen was studied by Bellelli et al. (2000), and occurs at least in two steps in agreement with the study by Su and Klinman. All this raised a question if copper is needed as an oxygen-binding site and the role of copper would rather be stabilizing the electrostatics of oxygen during its reduction. Hence, it is doubtful whether copper is needed and whether Cu⁺-semiquinone, being irrelevant in the pathway.

In the second step the re-oxidized enzyme is converted to the native form of enzyme (Agostinelli et al. 1998, Mills, Klinman 2000, Dawkes, Phillips 2001). In the oxidative half-reaction (k_cat/K_m for O₂) hydrogen peroxide and ammonia are released as a result of hydrolysis of the iminoquinone, when reduced TPQ reacts with dioxygen regenerating TPQ_ox (Dawkes, Phillips 2001, Mure et al. 2002, Mills et al. 2002). The oxidized TPQ appears as a pink color at 480 nm varying between 472-500 nm due to different charge delocalization of TPQ_ox in different AOs (Dawkes, Phillips 2001, Mure et al. 2002). The hydrogen bounded
oxygen of TPQ-O2 and TPQ-O4 through the hydroxyl of Tyr369 and water molecule, provide the transfer of the two hydrogens from TPQred which are needed to generate hydrogen peroxide from oxygen. The dioxygen seems to be fully reduced to peroxide and ready to release. In the substrate channel aldehyde is again trapped interacting with Asp383, and the aspartate activates a water molecule for nucleophilic attack on TPQ-C5. This water is thought to be responsible for the release of ammonia and regeneration of the TPQ for the next catalytic cycle (Brazeau et al. 2004).

![Diagram](image)

*Figure 5. The oxidative half-cycle in BSAO (Su, Klinman 1998).*

1.3.6 Inhibitors of amine oxidases

The inhibitors of amine oxidases can be divided into two classes:

1) ligands able to react with carbonyl groups, such as hydrazines and semicarbazide
2) copper ligands such as anions N\(^3\), CN\(^-\), SCN\(^-\), DDC as described previously in paragraphs 1.3.1 and 1.3.2

With different inhibitors have been studied the reactivity of two subunits. The simplest competitive (binding selectivity based on active-site fit) and non-competitive inhibitors, which are used to study the reactivity of subunits, are arylhydrazines, arylhydrazides and semicarbazides (Morpurgo et al. 1988, 1990, 1992). These reactions are stoichiometric and the enzyme derivatives are catalytically inactive. Semicarbazide is an irreversible inhibitor of serum amine oxidases. Consequently name semicarbazide sensible amine oxidases (SSAO) have been accepted.

By titration with phenylhydrazine (PHy) and substrate one or two organic carbonyl cofactors can be identified, one cofactor per protein subunit though the reactivity of two cofactors is different (Agostinelli et al. 1994). According to the hypothesis of Janes and Klinmann (1991), there are two mols of PHy per dimer in BSAO. Two cofactors are capable of binding inhibitors covalently. The reaction of phenylhydrazine, as other hydrazines, is stoichiometric with amine oxidases. The aromatic phenylhydrazine adduct form a band at 447 nm and benzylhydrazine at 405 nm, while the aliphatic hydrazine adducts form a band at 350-360 nm (Morpurgo et al. 1992, Schwarz et al. 2001). The molecules, substrates and hydrazines, that reduce organic cofactor can induce conformational change in protein that inhibits the reaction of the second factor by a mechanism-based hydrazine-transfer reaction, forming hydrazone or azo derivates while hydrazides only slow down the covalent binding between the inhibitor and protein (Morpurgo et al. 1989, 1992). The hydrazines inhibit the formation of stable Schiff bases or they act as pseudo substrates (Bossa et al. 1994). Phenylhydrazine reacts 1:1 protein dimer and is also bound by most 1:1 enzyme-hydrazide adducts forming a ternary derivatives. The reactivity and adduct conformation are affected by DDC. With DDC one copper ion could be removed without the loss of enzymatic activity, but
in the presence of DCC the reaction is affected by stabilizing benzaldehyde hydrazone form (Morpurgo et al. 1987, 1989).

Aromatic hydrazides (NH2NCO(CH2)nC6H4R) generally bind covalently to the organic cofactor and have a band at 350-400 nm. It has been noted that the reaction is very fast when the substituted R is hydrophobic. It has therefore been thought to be a hydrophobic region close to organic cofactor and that this region is responsible for inhibitor reactions. (Morpurgo et al. 1992). The 1:1 or 1:2 reaction of BSAO with hydrazides, forms an adduct which can be seen spectrophotometrically at 350–360 nm though with phenylacetic hydrazide the band was shifted to 395 nm (Morpurgo et al. 1988, 1992). The intensity of the optical band is high for benzohydrazides (360 nm), semicarbazides (365 nm) and acetohydrazides. Aromatic hydrazides are covalently bound by BSAO. Excess hydrazide makes the reaction faster but doesn’t change the intensity. Ortho-benzohydrazides are unreactive whereas excess of acetohydrazide only reacts slower. Hydrazides slow down the reaction, but do not inhibit it completely. In the case of BSAO it was found out a fast reaction of one molecule per dimer, followed by a slow reaction of 0.2-0.3 molecules (Pietrangeli et al. 2003). DDC reacts with copper, when the organic cofactor is bound to hydrazide. However, in the presence of DDC the reaction of hydrazide is slower in BSAO (Morpurgo et al. 1987, 1988). The peak caused by hydrazide in BSAO is bleached to 330 nm in the presence of DDC and the DDC peak is shifted from 370 nm to 350 nm (Morpurgo et al. 1987).

The study by De Biase et al. 1996 found out that the reactivity of BSAO was between one and two sites per dimer and in the pig plasma the reactivity depends of the inhibitor (De Biase et al. 1996, Pietrangeli et al. 2003). BSAO reacts 1:1 with PHy and also with 2-hydrizinopyridine (PH) (Morpurgo et al. 1992, De Matteis et al. 1999). In the pig plasma AO has been reported to bind PH in the ratio two per dimer (Collison et al. 1989). This could be explained by a need for a larger conformational change that impairs the reaction of the second site. In plant CuAOs the metal chelators inhibit non-competitively and different amine oxidases have different sensitivity (Medda et al. 1995). Besides of PHy, also PH reacts irreversibly with TPQ in ECAO in the pH range 5.5-9.4 (Saysell et al. 2002). PH adduct has also shown that PSAO contains two TPQ cofactors per dimer as well as in LSAO, two TPQs could be shown in ECAO, but with longer reaction times. This suggests that the TPQ reactivity and environment is different depending on the amine oxidase source. Tranycypromine (TCP) binds reversibly ECAO, which can be seen in the 300–500 nm. The adduct formation of TCP is analogous to substrate Schiff base formation.

The effect of other inhibitors has also been studied by using 4-(2-naphthyloxy)-2-butyn-1-amine, 1,4-diamino-2-chloro-2-butene, 1,6-diamino-2,4-hexadiyne and 2-chloro-5-phthalimidopentyamine which all contain a functional group that can produce mechanism based inactivation (Shepard et al. 2002). These inhibitors are called suicide inhibitors, since they affect reaction mechanism by turnover dependent conversion to electrophilic products capable of covalent binding. These inhibitors do not just display binding selectivity on active site: the electrophilic product is also capable of accessing active-site nucleophile. In some cases the inactivation seems to appear from TPQ modification by precluding reoxidation or other, from the alkylation of protein residues and hence block the access of substrate. 4-(2-naphthyloxy)-2-butyn-1-amine was found to inhibit all the enzymes except PSAO. 1,4-diamino-2-chloro-2-butene and 1,6-diamino-2,4-hexadiyne were found to decrease the activity of all the enzymes, but 1,6-diamino-2,4-hexadiyne affects more to PSAO, PPLO and EPAO. 2-chloro-5-phthalimidopentyamine inhibited only BPAO and EPAO. The dynamics of “gate” residues, the dimensions or shapes of the substrate channel and binding pocket and orientation of residues have an important role in the inhibition in the TPQ accessibility. The advantage with these inhibitors is that selectivity can arise from the variations in the initial non-covalent binding of the inhibitors with the enzymes or in the ensuing chemistry.
1.3.6.1 Product inhibition of the activity

As the CuAOs seem to have different physiological roles, their activity in the proportion of produced hydrogen peroxide in the presence of excess oxygen has been studied (Padiglia et al. 1999, Pietrangeli et al. 2000). In the case of BSAO it has been noted a loss of catalytic activity, up to 80% inactivation, when the incubation time and substrate concentration are increased, particularly when spermine is the substrate. In anaerobic conditions with catalase inactivation of the activity has not been noted and hence it has been concluded that the inactivating agent is only the hydrogen peroxide produced in the catalytic reaction. The result has been confirmed in the assessment of substrate by adding hydrogen peroxide. It is considered a toxic product due to the high reactivity of hydrogen peroxide and it has been thought that inactivation by hydrogen peroxide possibly may be part of autoregulatory mechanism in vivo and is relevant to cell adhesion and redox signalling (Yegutkin et al. 2003).

1.4 POLYAMINES

The substrates of copper amine oxidases are polyamines such as spermine and spermidine and diamines putrescine and cadaverine (Figure 6).

Figure 6. Structures of putrescine, spermidine and spermine.

Polyamines are simple cationic and aliphatic molecules with a low molecular mass, and organic bases which can be called biogenic amines since they induce biological activity (Table 3). They are found ubiquitously in virus, bacteria, microorganisms, plants, humans and animals. Their polycationic nature allows them to interact in physiological pH with negatively charged molecules such as DNA, RNA, proteins, membranes, phospholipids and polysaccharides and hence can be involved in the conformational modification of DNA. Polyamines and their intracellular levels have an important role in cell growth and development and in maintaining cell life, since cells need polyamines to duplication. The intracellular levels of polyamines are regulated by biosynthesis, degradation and uptake of amines (Williams 1997, Aziz et al. 1998, Bouchereau et al. 1999, Martin-Tanguy 2001, Teti et al. 2002).

In mammals and fungi, the biosynthesis is strongly regulated by two enzymes. First, by ornithine decarboxylase (ODC, 1 in the figure 7) which converts ornithine to putrescine in
rate-limiting step. Ornithine, an amino acid which is not incorporated in proteins, is the precursor of polyamines. The second enzyme is S-adenosylmethionine decarboxylase (2 in the figure 7), catalyzing decarboxylation of S-adenosylmethionine. Putrescine is converted to spermine and spermidine by two other enzymes, spermidine synthase (3 in the figure 7) and spermine synthase (4 in the figure 7). The biological half-lives of ornithine decarboxylase and S-adenosylmethionine decarboxylase (5-60 min) are among the shortest known for mammalian enzymes, and so allow a rapid change in the polyamine levels in cells.

Figure 7. Polyamine synthesis. (http://www.biol.lu.se/zoophysiol/Cellprolif/Research/research_area_1.html).

The polyamine degradation pathway is strongly regulated by spermidine/spermine N\(^1\)-acetyl transferase and amine oxidases (Figure 8). Normally, cells can synthesize all the polyamines they need. In addition to regulation of polyamine levels by biosynthesis and degradation, they are equipped with an efficient transport system for utilization of exogenously derived polyamines. The spontaneous degradation of spermine and spermidine, after the oxidation to aldehyde, can differently evolve oxidation by aldehyde dehydrogenase to form spermine and putrescine with a production of acrolein or oxidation by alcohol dehydrogenase to produce alcohol. Spermine and putrescine can be re-utilised in the interconversion cycle (Seiler 2004).
Figure 8. Polyamine interconversion cycle and polyamine transport pathways of cells.
Table 3. Chemical properties of polyamines.

<table>
<thead>
<tr>
<th>Synonyms</th>
<th>Spermine</th>
<th>Spermidine</th>
<th>Putrescine</th>
<th>Cadaverine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1,4-Butanediame; N,N'-Bis(3-aminopropyl)-1,4-butanediame; 1,4-Butanediame; N,N'-bis(3-aminopropyl)-1,4-diaminobutane; Diaminopropyldiamine</td>
<td>N-(3-aminopropyl)-1,4-butanediame; N-(3-Aminopropyl)-1,4-diaminebutane; N-(3-Aminopropyl)-1,4-butandiamine; 1,5,10-Triazadecane; 1,4-diaminobutane; N-(3-aminopropyl)-</td>
<td>1,4-Butylenediame; 1,4-Diamino-n-butane; 1,4-diaminobutane; 1,4-butanediame; tetramethylenediamine</td>
<td>1,5-pentanediame; pentamethylenediamine; Biodex1-; 1,5-Diaminopentane</td>
</tr>
<tr>
<td>Molecular formula</td>
<td>C₁₀H₂₆N₄</td>
<td>C₇H₁₉N₃</td>
<td>C₄H₁₂N₂</td>
<td>C₅H₁₄N₂</td>
</tr>
<tr>
<td>Molecular weight (g/mol)</td>
<td>202.34</td>
<td>145.25</td>
<td>88.15</td>
<td>102.18</td>
</tr>
<tr>
<td>Boiling point (°C)</td>
<td>308.4±0.0</td>
<td>246.6±8.0</td>
<td>159.0±8.0</td>
<td>179.0±8.0</td>
</tr>
<tr>
<td>Vapour pression at 25°C</td>
<td>6.83E-4 Torr</td>
<td>0.0269 Torr</td>
<td>2.55 Torr</td>
<td>0.961 Torr</td>
</tr>
<tr>
<td>Flash point (°C)</td>
<td>175.6±40.7</td>
<td>118.1±39.7</td>
<td>51.7±0.0</td>
<td>76.5±44.7</td>
</tr>
<tr>
<td>Solubility in water</td>
<td>Very soluble</td>
<td>Very soluble</td>
<td>Very soluble</td>
<td>Very soluble</td>
</tr>
<tr>
<td>pKa</td>
<td>10.86±0.19</td>
<td>10.51±0.19</td>
<td>10.68±0.20</td>
<td>10.85±0.20</td>
</tr>
</tbody>
</table>
1.4.1 Methods for the polyamine determination

Traditionally biogenic amines have been determined with chromatographic methods:

- Thin-Layer Chromatography (TLC)
- Gas Chromatography (GC)
- Gas Chromatography coupled to Mass Spectrometry (GC-MS)
- High-Performance Liquid Chromatography (HPLC)
- Electrophoretic techniques
- Radioimmunoassay (RIA)
- Enzyme-linked immunosorbent assay (ELISA)

The chromatographic methods are normally performed in at least two steps: separation of other amino groups, often by extraction with perchloric acid and derivatization. This because direct detection of polyamines is difficult as they do not have native adsorption and fluorescence in the same wavelength as interfering compounds (Sadain, Koropchak 1999, Teti et al. 2002). The advantages of chromatographic methods are the low limits they can detect and the ability to separate the biogenic amines.

1.4.1.1 Thin-Layer Chromatography

Thin-Layer chromatography is based on the determination of fluorescent compounds (Teti et al. 2002). The primary and secondary amino groups are derivatized with dansyl chloride to give fluorescent emission (Madhubala 1998, Shalaby 1999). The preferred material for TLC-plates is silica due to their high performance. Samples are applied on the plates and separated by ascending chromatography in filter-paper-lined chromatographic tank with different developing solvents. The quantitative estimation can be performed by in situ scanning or fluorescence or conventional fluorimetry after eluation. The sensitivity range is from $10^{-12}$ mol to $100*10^{-12}$ mol. Thus, this method can be used for preliminary studies of polyamine identification and semiquantitation.

1.4.1.2 Gas Chromatography and Gas Chromatography-Mass Spectrometry

The first gas chromatography used was with flame ionization detection (FID), which was just enough sensitive for analyzing polyamines in urine. Improvements were obtained by using trifluoroacetylated, resulting in better sensitivity and perform analysis of amines in blood. Gas chromatographic techniques have been developed over the years and with several derivatization methods and changing packet columns to capillary, rapid analysis has been gained with good sensitivity, robustness and reproducibility (Choi et al. 2000, Teti et al. 2002). The separation capacity of GC can be coupled to sensitive and specific mass-spectrometry. For the CG-MS methods have been developed various derivatization and Choi et al. (2000) have even developed a method to block the remaining active hydrogen atoms and thus, achieving even better separation of polyamines. Fernandes and Ferreira (2000) have developed a method for simultaneous and quantitative determination of the most relevant diamines and polyamines.

1.4.1.3 High-Performance Liquid Chromatography

There are many HPLC techniques presented in the literature for the detection of polyamines in different biological fluids and food samples. Techniques with ion-exchange and reversed-phase chromatographic methods based on the fluorescence or
spectrophotometric detection, with possibly pre- and post-column modes, have been developed (Oguri et al. 1997, Sadain, Koropchak 1999, Eliassen et al. 2002, Lange, Wittmann 2002, Teti et al. 2002). Derivatization methods for the fluorimetric detection is usually used for dansyl chloride, fluorescamine or o-phthaldehyde (OPA). The derivates for UV-Vis spectrophotometry are usually prepared with benzoyl, dabsyl or tosyl chloride. The disadvantages of derivatization are long analysis time, tedious sample preparation, and possible instability of the derivate, interferences of by-products and indeterminate errors. However, the accuracy and sensibility as well as derivatization methods have been improved over the years and made the use of HPLC popular in polyamine assays. The possibility for automation has made the use of HPLC easy.

1.4.1.4 Electrophoretic techniques

Electrophoretic techniques are commonly considered to be paper and high-voltage and capillary electrophoresis (CE) (Teti et al. 2002, Rokka et al. 2004). Capillary electrophoresis has become an important separation method of biological fluids over the decade. The method is based on the charge differentiations in the species based on the electrophoretic mobility under the influence of an applied electric field. The electrophoretic mobility is directly related to the net charge of specie and inversely related to its hydrodynamic mass. These methods have need of little sample volume, are applicable to water-soluble, non-volatile and high-molecular mass species. These methods can be modified by pH, electrolyte and derivatization to obtain better sensitivity. However, CE is less sensitive than GC or HPLC. To resolve the problems of modification, indirect techniques have been developed, which do not need derivatization. The native analytes can be indirectly detected in the capillary with the use of run buffer containing a background electrolyte. “In-capillary” and “on-column” derivatization have also been developed, there separation and derivatization are carried out simultaneously in a separation capillary tube with run buffer containing derivatization reagent. Capillary zone electrophoresis has been reported to be most convenient for the quantitative determination of polyamines (Oguri 2000). Other varieties of CE are micellar electrokinetic capillary electrophoresis, capillary electrochromatography, capillary isoelectric focusing, capillary gelphoresis and capillary isotacophoresis.

1.4.1.5 Radioimmunoassay and enzyme-linked immunosorbent assay

RIA or ELISA are other techniques which can be considered since they allow multiple analysis in parallel (Teti et al. 2002). These methods do not need sample pre-treatment and are fast, though they have modest specificity. Cross-reactivity could interfere and the methods are unable to obtain antibodies against putrescine. The presentation of monoclonal antibodies against spermine and spermidine allow free polyamines with higher sensitivity to be detected.

1.5 Physiological role of polyamines, amine oxidases and tumoral cell studies

The physiological role of polyamines and amine oxidases is correlated by the role of substrates and reaction products.

In microorganisms containing spermine and putrescine, the conditions of growth varies according to the concentrations and in function to essential factors such as pH and medium where they are located.

In generally gram-negative bacteria present higher concentrations of polyamines and specially in E.coli and Haemophilus parainfluenzae putrescine and/or spermine are essential factors for cell growth. Much interest was evolved when it was noted that some viruses can also contain diamines and polyamines.
As mentioned before, polyamines are practically present in all cells and have an important role in molecular signalling in plants, response to stress, cell differentiation and growth, development and in maintaining cell life and their intracellular levels are regulated by biosynthesis and degradation even though their biological activity is not well defined. In plants the hydrogen peroxide produced in deamination of polyamines seems to be utilized in cell wall synthesis and in cicatrization of scars. The toxicity of amines is also observed when exposed to stress and in the processes of yellowing and aging.

Sensible and reproducible methods and inhibitor studies, mostly by inhibiting the synthesis and reverting the inhibitory effect, have made it possible to identify and determine of polyamines in many organisms, tissues and biological fluids such as urine, plasma, hair, saliva and thus made able the study of pathogenesis of many diseases and cellular processes involved in pathogenesis such as infections, psoriasis, *polycythemia rubra vera*, *systemic lupus erythematosus*, uremia, chronic nephritis, liver cirrhosis, cystic fibrosis, diabetes, muscular dystrophy, Alzheimer’s disease and cancer (Teti *et al.* 2002). The studies have shown the relation of polyamine metabolism and vital physiological processes in mammals, e.g. circadian rhythm, age, sex, menstrual cycle and reproductive system.

A higher activity of amine oxidases has been measured in pregnant women, implying a role of polyamines in the placenta. Elevated concentrations of polyamines have been noted in tumor cells, as well as in the metabolism of food products (Halász *et al.* 1994, Wang, Bachrach 2000). In many cell systems the differentiative process seems to be inhibited and the role of polyamines is of an interest in differentiation of tumor cell lines, such as colon-, carcinoma-, human leukemia- and B16 melanoma cells. Cells are equipped with specific transport systems, which allow the take up of polyamines from environment. The polyamine transport is greater in tumor cells than in normal cells (Aziz *et al.* 1998). Polyamine transporter has a broad tolerance towards a wide range of polyamine conjugates in three types of mammalian cells (Cullis *et al.* 1999).

High concentrations of polyamines can be found in active proliferating cells. Even though the role of polyamines in cell division is not well understood, the role of polyamines in the proliferation of mammal cells and the polyamine regulation are of interest as a chemotherapy target in prevention of carcinomagenesis by blocking the tumor growth with polyamine synthesis inhibitors and analogous. However, the mechanism how polyamines exert the tumorigenic effect may be very different depending of the tumor. The involvement of polyamines and ODC in tumor growth is well known in many studies on polyamine metabolism in human neoplastic lesions, e.g. in carcinoma of breast, esophageus, stomach, colon and liver (Cullis *et al.* 1999, Teti *et al.* 2002).

In this contest amine oxidases could have a fundamental role in regulation and modification of cellular processes such as tissue differentiation, proliferation, tumor cell growth and programmed cellular death (apoptosis) (Averill-Bates *et al.* 1993, Agostinelli *et al.* 1994, Sharmin *et al.* 2001, Teti *et al.* 2002). Hydrogen peroxide, also produced in the catalytic reaction with AOs, in suitable concentrations is able to impair proliferation and cell growth and to regulate gene expression and cellular signaling. A study by Calabrini *et al.* (2002) showed the cytotoxicity induced by hydrogen peroxide on human colon carcinoma cells as future anticancer agents. Cytotoxicity of aldehydes has been observed since they inhibit on nucleic acid and protein synthesis (Averill-Bates *et al.* 1993, Agostinelli *et al.* 1994). The study by Sharmin *et al.* (2001) showed the acrolein to be the most potent inhibitor in mouse mammary carcinoma and leukemia cells, showed by nearly parallel cytotoxicity of spermine, spermidine and 3-aminopropanal with the amount of acrolein produced from each component. The CuAOs from vegetable origins seem to be utilizable in cardiac anaphylactic controlling. BSAO has been shown to protect rat hearts from injuries induced by free radicals. The study of specific inhibitors of copper-enzymes are specially useful to formulate anti-fibiotics and for the treatment of Parkinson’s disease (Teti *et al.* 2002, Silvestrini *et al.* 2003).
1.6 SENSORS AND BIOSENSORS

In the last 20-30 years there has been an increasing number of applications of biochemical devices in different fields of research; such as food, agricultural, environmental and medical, in order to obtain sensitive, simpler and faster analytical methods for the analysis in real samples.

In order to solve the problems bound to the analysis in real samples, researchers focused their attention on the development of biosensors. The modern concept of biosensor was introduced in 1962 by Clark and Lyons, when the glucose oxidase was entrapped onto a dialysis membrane and coupled with an oxygen electrode for the glucose analysis by measuring the decrease of oxygen consumed in the reaction. In the 1967 Updike and Hicks developed the first example of “functional biosensor” with the glucose oxidase immobilized on a support for the determination of the glucose concentration.

The general mechanism on which all enzyme based electrochemical sensors are based on is simple and depends on the interaction between the analyte present in the sample and the biochemical component resulting in the formation or consumption of an electro active specie leading to an output signal, which is evaluated by the electrochemical transducers.

1.6.1 Conversion of the biological to physical signal

Biosensors are self-contained integrated devices, which are capable of providing specific quantitative and semi-quantitative analytical information. It is a device made up of an immobilized biochemical component, typically enzymes or binding proteins and of a physico-chemical transducer of the electrical signal with an electronic amplifier (Figure 9) (Thèvenot et al. 2001). Biorecognition system can also be nucleic acids, bacteria, cells and even whole tissues. An electrochemical, optical, mass or thermal transducer can be chosen depending upon the parameters measured. The characteristics of biosensors are defined by the sensitivity, selectivity, reliability, reproducibility, life time, stability, response time and sample output.

Figure 9. Scheme of biosensor.
1.6.2 Biochemical transducers

1.6.2.1 Enzymes

The advantage of enzymes is their high purity and the possibility to characterize them easily. The disadvantage of enzymes might be their stability and need of a cofactor.

1.6.2.2 Microorganisms and vegetal or animal tissues

The advantage of these is that the enzymes are maintained in their natural environment and that gives the possibility to use them as commercial enzymes. However, they often are less sensitive.

1.6.2.3 Immunoreceptors

In this case the reaction between nucleic acids and antibodies is used. This kind of biosensors provide selective interactions to form a thermodynamically stable complex by given ligand.

1.6.3 Immobilization techniques

To construct a biosensor the enzyme has to be immobilized on the electrode surface. The immobilization of biochemical component shows fundamental characteristics, since the component is kept in very close distance from the transducer to create optimal electrochemical conditions. The immobilization technique can have affect e.g. to stability of sensor, kinetic parameters. Several techniques can be used for immobilization. All the conventional methods such as adsorption, covalent binding, cross-linking or entrapping can be used (Thèvenot et al. 2001, Mello, Kubota 2002).

1.6.3.1 Physical immobilization

Enzymes can be entrapped in the pores of fibres or gels. The support matrix of physical immobilization is often polyacrylamide gel, amide, dialysis membrane or acetylcellulose.

Three principal methods are used:

* Entrapment in natural or synthetic gel
* Microincapsulation in semipermeable polymers
* Physical adsorption on solid matrix

The immobilization procedure of adsorption relies on the non-specific physical interaction of the enzyme solution on the pores of support matrix, performed by the gravity or with help of slight external pressure to facilitate the entrance of the enzyme solution on the support. Adsorption is simply performed by incubating the enzyme (up to 1 g protein/ g matrix) and adsorbent in suitable conditions (pH, ionic strength, temperature) before washing out the excess enzyme. A major advantage of adsorption is that no reagents and only a minimum of activation steps are usually required. Adsorption is cheap and tends to be less disruptive for the enzymatic protein than chemical immobilization, the binding being mainly by hydrogen bonds, multiple salt linkages and Van der Waal's forces. In this respect, the method is similar to biological membranes in vivo.
1.6.3.2 Chemical immobilization

In this kind of immobilization, the enzyme is chemically bound to a support matrix, which is usually of nylon, cellulose, polyvinylalcohol, collagen or acetate cellulose. This immobilization can be performed by two methods:

* Covalent binding
* Cross-linking

1.6.3.2.1 Immobilization with carbodiimide

This immobilization technique is based on the reaction between the amino groups present in the enzymatic protein and the carbodiimide solution (e.g. 1-ethyl-3(3-dimethylaminopropyl)carbodiimide) which leads to an amide linkage between carboxyl groups of the membrane and amino groups of the enzyme (Figure 10) (Mascini, Mazzei 1987, Botrè et al. 1992).

![Figure 10. Reaction with carbodiimide and enzyme](image)

1.6.3.2.2 Immobilization with glutaraldehyde

Another agent often used for immobilization is bi-functional glutaraldehyde with two aldehyde groups, which react with ammonia groups of enzyme and bovine serum albumin (BSA) forming a link between the enzyme and BSA (Figure 11) (Guilbault, Lubrano 1973).

![Figure 11. Scheme of reaction between an enzyme and BSA.](image)
1.6.3.2.3 Immobilization with polyacetidine

There are also methods which utilize both the physico-chemical immobilization. Poliazetidine Prepolymer PAP reacts with several functional groups such as carboxylic, -NH₂ and -OH, and can also react with itself forming a polymer, enabling a physico-chemical immobilization to be realized (Figure 12). In fact the enzyme is covalently bound with the polymer, as well as physically entrapped in the polymeric matrix (Mascini, Mazzei 1987).

During the last ten enzyme-modified carbon paste electrodes years have been studied and developed where the method is based on carbon powder mixed with a pasting liquid. This method makes to use of paste possible since it can easily be modified by enzymes, cofactors and mediators (Gorton 1995).

**Figure 12. Scheme of the immobilization with PAP.**
1.6.3.3 Immobilization of microorganism, antigens, anticorps or tissues

The immobilization of microorganisms is usually based on physical entrapment of microbes on dialysis membrane, gel or a pore membrane (Arica et al. 1993, Valuev et al. 1993).

Biological material can be immobilized directly on the electrode surface, e.g. anticorps can be immobilized by thermal treatment on the surface of pyrolytic graphite giving a good reactivity of the material for a link with anti-corps. Anti-corps can also be immobilized chemically or physically (Babacan et al. 2000, Qian et al. 2000).

The immobilization of antigens is more delicate due to their nature and in the consequence the most convenient method is physical immobilization.

The immobilization of tissues is very simple: a tissue layer is fixed on the outside of the electrode surface (Mazzei et al. 1994, Mazzei et al. 1995).

1.6.4 Physico-chemical transducers

1.6.4.1 Electrochemical sensors

A particular subclass of sensors is represented by the electrochemical sensors or electrodes (Thévenot et al. 2001, Mello, Kubota 2002). The most common electrochemical sensors used are amperometric sensors (Figure 13). The formation of one or more electroactive species generates an electrical signal or variation of a pre-existing signal. This signal is proportional to the concentration or activity of the chemical species to be determined.

![Figure 13. Scheme of an amperometric sensor.](image)

Amperometric sensors are generally divided into three generations according to the principle of the electron transfer (Chaubey, Malhotra 2002, Mello, Kubota 2002). A first generation type of amperometric biosensors are based on the detection of electroactive species such as oxygen or hydrogen peroxide without addition of electron transfer mediators by measuring the change of current on the working electrode. The two other types of electrochemical sensors are based on an electrochemical detection of a mediator (electron acceptor or donor) or on a direct electron transfer between the enzyme and the electrode.
1.6.4.1.1 Oxygen sensor

Oxygen sensor is made up of a platinum or gold electrode with a reference electrode of silver/silver chloride. A potential of ca. -700 mV is applied to the working electrode, compared to the reference electrode. At this potential oxygen is reduced at the cathode and silver is oxidized on the anode (Reactions 6 and 7).

\[ \text{O}_2 + 4\text{H}^+ + 4e^- \rightarrow 2\text{H}_2\text{O} \quad (6) \]

\[ \text{Ag} \rightarrow \text{Ag}^+ + 1e^- \quad (7) \]

1.6.4.1.2 Hydrogen peroxide sensor

Hydrogen peroxide sensor is constructed as the oxygen sensor, a platinum electrode as an anode and a cathode of silver/silver chloride with an applied potential of ca. 700 mV (Reactions 8 and 9).

\[ \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}^+ + 2e^- \quad (8) \]

\[ \frac{1}{2} \text{O}_2 + 2\text{H}^+ + 2e^- \rightarrow \text{H}_2\text{O} \quad (9) \]

1.6.4.1.3 Mediated electron transfer

The use of mediators is based on the possibility for detecting the electron transfer between the redox cofactor and the electrode with the aid of a redox mediator. In this way lower working potentials can be used respect to the measurements of substrates or products directly. This method also enables the interferences to be reduced (Chaubey, Malhotra 2002). The first mediator used in 1984 was ferrocene, it is one of the most common mediators in use.

1.6.4.1.4 Direct electron transfer

In the direct electron transfer the electrons are directly transferred from the enzyme to electrode surface. Normally the protein molecule is too large and the transfer from enzyme’s redox center to electrodes surface can not be easily achieved. Direct electron transfer can be promoted e.g. by 4,4'-bipyridine (Smolander 1995). The natural oxidation mechanism of the enzyme effects how the enzyme communicates with the electrode. Hence the direct electron transfer is rare.

1.6.4.2 Potentiometric sensor

In a potentiometric sensor, the logarithms of concentration of active specie is measured as a change of potential at the working electrode respect to a reference electrode or between two reference electrodes separated by a permselective membrane without a significant current flow (Mello, Kubota 2002). Examples of potentiometric sensors are change measurements of pH and ion concentration when coupled to ion selective electrode. These biosensors are based on the immobilization of biologically active compound on a membrane, on the surface of a transducer.
1.6.4.3 Other transducers

There is less use of transducers such as conductometric biosensors, which measure the change of conductance between a pair of metal electrodes as a consequence of a biological compound when microorganisms metabolize uncharged substrates (Sukeerthi, Contractor 1994). The disadvantage of these sensors is that they are usually non specific and have a poor response.

Ion selective field-effect transistors are used to determine ion concentrations and become a biosensor when coupled to a biologically active compound (Campanella, Mazzei 1988).

Piezoelectric sensors use different kind of crystals under electric field applied from out. The crystal has its typical resonance and the change of frequency is caused by the absorbance of the molecule on the crystal surface which is proportional to the variation of materials mass (Wu 1999).

There are various kinds of optical transducers. Some measure the change of absorbance, light reflectance as a result of wavelength dye which comes of pH change or oxidation of the dye. The others measure luminescence, a method often used with enzymes that produce or use adenosine triphosphate (ATP) (Choi 2004).

Thermal transducers measure the heat production of enzyme catalyzed reaction by thermistors and is directly proportional to the analyte concentration (Xie 1999).

1.6.5 Theoretical aspects of enzyme sensors

As well known, enzymatic proteins are able to act as catalysts in several biochemical reactions. The characterization of their kinetic properties are based on the determination of $K_m$ and $V_{max}$. Generally these kinetic studies are carried out with the enzyme free in solution. In the case of the enzymatic immobilization, the catalytic properties are markable modified, in fact in this case several steps have to be taken into account (Figure 14), that can be summarized as follow:

1. Transport of the substrate from the solution to enzymatic membrane
2. Diffusion of the substrate through the enzymatic layer and contemporary transformation to the reaction products
3. Migration of the products towards the sensor
4. Transformation of the molecular recognition signal to a physical signal by a transducer.

Figure 14. Schematical presentation of the theoretical aspects of the oxidase based biosensors.
The classical method to determine the kinetic analysis is schematized:

$$E + S \xrightarrow{k_{-1}} ES \xrightarrow{k_2} E + P$$

$$v = \frac{d[P]}{dt} = \frac{d[S]}{dt} = V_{max} \frac{[S]}{K_m + [S]}$$

with \(K_m = \frac{(k_{-1} + k_2)}{k_{-1}}\) and \(V_{max} = k_2 [E_0]\) for \([S] >> K_m\)

When \(\frac{[S]}{K_m} \to 0\), \(V \to (V_{max}/K_m) [S]\) (first order reaction respect to \([S]\))

When \(\frac{[S]}{K_m} >> 0\), \(V \to V_{max}\) (zero order reaction respect to \([S]\)).

In the kinetics, the products concentration on the outside of the sensor is not considered since the diffusion become inside the biocatalytic layer. The temperature, \(V_{max}\) and \(K_m\) are considered constants through the enzymatic layer and it is assumed that the transduction of the signal doesn’t consume substrates or products of reaction.

Hence it is clear that in the case of the immobilized enzymes, the kinetic behaviour changes. Particularly the kinetic is effected by partition and diffusion. The partitioning behaviour is described by the electrostatic partition coefficient Lambda, where positively charged matrix repels positive ions and attract negative ions resulting in partitioning into or out of the microenvironment. The diffusion effect comes simply from the need of the substrate to pass the matrix surface to gain the immobilized enzyme in the matrix. This usually has two parts, the external diffusion to the enzyme surface and the internal diffusion through the matrix.

### 1.6.6 Interferences

A general problem in the real matrixes is the possible effect of other substances found in the analyte. These other substances can be electroactive or capable of adsorbing onto the electro surface and hence influence the signal measured. This problem can partly be resolved by applying a permeable membrane to the analyte on the electron surface. This membrane increases the selectivity and the stability of the electrode. By adjusting the membrane’s charge, porosity, polarity and hydrophobic or lipophilic characteristics, the transport and diffusion capacity of the membrane can be affected. In the table 4 examples of membranes used to limit interferences can be seen. The best way to avoid the interferences would be to have a system with such a working potential where other electrochemically active substances do not appear.

<table>
<thead>
<tr>
<th>Transport mechanism</th>
<th>Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimension exclusion</td>
<td>Cellulose acetate, cellulose acetate hydrolized with alkals</td>
</tr>
<tr>
<td>Charge exclusion</td>
<td>Naphion, polyvinylpiridine, polyesthersulfonico acid</td>
</tr>
<tr>
<td>Mixed control</td>
<td>Naphion-cellulose acetate, polyvinylpiridine-cellulose acetate</td>
</tr>
</tbody>
</table>
Amine oxidases have been applied to biosensor technology, for the analysis of amines in food matrices and clinical applications (Hasebe et al. 1997, Carsol, Mascini 1999, Niwa et al. 2000, Nagy et al. 2002). Amine oxidases can be used for several purposes, e.g. for analytical applications in clinical medicine, in the pharmaceutical and food industry as well as in agricultural and environment analyses.

2. AIMS OF THE WORK

The aim of this study was to construct a biosensor or electrochemical device to determinate the catalytic activity of BSAO as well as to determine the concentrations of biogenic amines, spermine, spermidine and of synthetic substrate benzylamine by the catalyzed reaction.

\[
\begin{align*}
\text{NH}_2\text{(CH}_2\text{)}_3\text{NH(CH}_2\text{)}_4\text{NH(CH}_2\text{)}_3\text{NH}_2 + 2\text{O}_2 + 2\text{H}^+ & \rightarrow \text{CHO(CH}_2\text{)}_2\text{NH(CH}_2\text{)}_4 \\
\text{NH(CH}_2\text{)}_2\text{CHO + 2NH}_3 + 2\text{H}_2\text{O}_2 & \rightarrow \text{NH}_2\text{(CH}_2\text{)}_4\text{NH}_2 + 2\text{CH}=\text{CHCHO} \\
\text{amine oxidase} & \beta\text{-elimination}
\end{align*}
\]

The electroanalytical properties of the electrochemical devices were tested through several experiments with enzyme free in solution, as well as with immobilized enzyme. Further studies were performed in order to characterize the enzyme kinetics in absence and presence of BSAO inhibitors i.e. phenylhydrazine (a classical inhibitor) and benzylhydrazine (a pseudo-substrate). The additional aim was also to optimize the biosensor performances for the analysis of biogenic amines. A further step was to apply the biosensor for the determination of biogenic amines in human cell cultures.
3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Enzyme purification

BSAO was purified from bovine serum according to the method by Turini et al. (1982) modified with two additional steps on chromatographic columns of Q-sepharose at pH 7.5 and 6.8 and subsequently were also added two ionic exchanges.

* Ionic exchange with ‘CM-Cellulose’ column to separate heme-groups. These groups absorb at 410 nm and are revealable in the optical spectra of BSAO as second band.

* Ionic exchange of ‘AE-Agarose’ resin which allows the separation of ceruloplasmin, a blu cuproprotein (Wang et al. 1994).

In the table 5 are reported the chromatographic steps, the protein concentrations determined at 280 nm and, the specific activity at 250 nm determined with benzylamine.

Table 5. Volume, concentrations and specific activity corresponding following steps with second fling down with (NH₄)₂SO₄ (55%).

<table>
<thead>
<tr>
<th>State</th>
<th>Volume (ml)</th>
<th>Conc. (mg/ml)</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM-cellulose</td>
<td>520</td>
<td>12.5</td>
<td>0.016</td>
</tr>
<tr>
<td>AE-agarose</td>
<td>520</td>
<td>12.5</td>
<td>0.016</td>
</tr>
<tr>
<td>Q-sepharose pH 7.5</td>
<td>320</td>
<td>1.2</td>
<td>0.11</td>
</tr>
<tr>
<td>Concanavalina A-sepharose</td>
<td>60</td>
<td>1.4</td>
<td>0.21</td>
</tr>
<tr>
<td>Q-sepharose pH 6.8</td>
<td>45</td>
<td>0.9</td>
<td>0.30</td>
</tr>
</tbody>
</table>

3.1.2 Reactants

The enzyme substrates spermine, spermidine, benzylamine, putrescine and cadaverine were purchased from Sigma Chemical Co. (St.Louis, MO, USA). Phenylhydrazine and benzylhydrazine were purchased from Fluka.

Glutaraldehyde, bovine serum albumin and carbodiimide were purchased from Sigma Chemical Co. (St.Louis, MO, USA). Nylon (6.6) Biodyne C and Ultrabind membranes were purchased from Pall (Italy).

All the solutions were prepared with reagents of analytical grade and using bidistilled water.

3.2 ANALYTICAL METHODS

3.2.1 Enzyme activity

The activity of BSAO was measured by monitoring spectrophotometrically the formation of benzaldehyde at 250 nm (ε = 12,500 M⁻¹ cm⁻¹) and resulted to be 0.23 UI/mg (UI is defined as µmol of benzylamine oxidized in a minute). The protein concentration was determined spectrophotometrically at the absorption band 280 nm with the absorption coefficient of 1.74 Lg⁻¹ cm⁻¹ at 25°C, using a “Perkin Elmer Lambda 9 UV/VIS NIR” or “Perkin Elmer Lambda 11 UV/VIS”.


3.2.2 Titration of organic cofactor by phenylhydrazine

The determination of organic cofactor was performed with titration with phenylhydrazine. The enzyme reacts with hydrazine and forms a covalent binding which leads to an absorption band at 447 nm \( (\varepsilon = 41500 \text{ M}^{-1} \text{ cm}^{-1}) \), as indicated in the figure 15. This determination has shown a carbonyl group per enzyme mol and to be very useful to study the stoichiometric and velocity of the reaction.

![Figure 15. Blue line, optical spectra of native BSAO, red line after addition of equivalent of PHy, green line after two equivalents of PHy.](image)

3.2.3 Electrophoresis of BSAO

The characterization of purified BSAO and the molecular weight have been defined by electrophoresis on 7.5% polyacrylamide gel adding 0.1% of sodium dodecylsulphate (SDS, Molecular Weight Markers by Sigma), as denaturating agent and, in the presence or absence of \( \beta \)-mercaptoethanol (5%). Electrophoresis strips were performed on the slab gel between two glasses at the temperature of 4°C. After the run, ca. an hour, the coloration has been performed with a solution of Blue Comassie (0.1%) in a mixture of acetylic acid (12%) and methanol (50%).

With this method it has been possible to confirm that BSAO is formed of two similar subunits, bound with a disulfide bridge. Through SDS-PAGE (Figure 16), it has been observed one migration band with molecular weight ca. 170 kDa. In the presence of \( \beta \)-mercaptoethanol appear two bands which corrispond ca. 85 kDa.

![Figure 16. Electrophoresis on 7.5% polyacrylamide gel with 0.1% of sodium dodecylsulphate (SDS) as denaturating agent and in the presence of \( \beta \)-mercaptoethanol (5%). 1) BSAO, 5 \( \mu \)l = 7 \( \mu \)g. under reduced conditions 2) BSAO, 15 \( \mu \)l under non-reducing conditions, 3) molecular mass markers.](image)
3.3 ENZYME IMMOBILISATION

To evaluate the immobilization technique and the effect of the support were performed four different types of immobilizations on different membranes. The immobilization of BSAO was made on four ways:

1) Immobilization on a nylon (6.6) membrane with free carboxyl groups on the surface (porosity 0.2 μm, diameter 0.8 cm) by physico-chemical binding with prepolymer polyazetidine (PAP) (12% in water) or by chemical covalent bonding with carbodiimide.

Ca. 1 mg of lyophilized enzyme was solubilized in 25 µl of PAP to obtain a density of 0.135 mg/cm². The membrane was left for 24 hours at room temperature and washed with phosphate buffer (0.1 M, pH 7.0) before use.

The carbodiimide was in NaH₂PO₄ buffer (0.5 M, pH 4.7) and mixed to obtain a final concentration 0.1 mol/l. The carboxylic support membrane was immersed in the carbodiimide solution and left for 40 minutes under magnetic agitation in room temperature. The membrane was washed with bicarbonate buffer (0.04 M, pH 7.0). On the surface of the activated membrane was homogeneously layed 1 mg of enzyme in 20 µl bicarbonate buffer (0.04 M, pH 7.0). The membrane was left for 24 hours in humid at 4°C. The membrane was washed with bicarbonato buffer, to eliminate residues of the chemical reaction on the membrane, before use. (Mascini, Mazzei 1987, Botrè et al. 1992).

2) Immobilization on a dialysis membrane (cutoff 12000 Da) by using PAP. Ca. 1 mg of lyophilized enzyme was solubilized in 10 µl of PAP and corresponded a density of 0.135 mg/cm². The membrane was left for 24 hours at rooms temperature and washed with phosphate buffer (0.1 M, pH 7.0) before use.

3) Immobilization with glutaraldehyde and bovine serum albumin was performed on the dialysis membrane. Ca. 1 mg of lyophilized enzyme was solubilized in 10 µl of bovine serum albumin (15% p/v) in phosphate buffer (0.1 M, pH 7.0). The solution was deposited on the dialysis membrane and 5 µl of glutaraldehyde (25% in water) was add to achieve homogenous solution on the membrane. The membrane was left for an hour in a room temperature and washed with distilled water for 15 minutes and then 15 minutes with glycine buffer (0.1 M) to wash away the excess of glutaraldehyde.

4) Immobilization on Pall Ultrabind membrane. The enzyme (100 µl) was deposited on the Ultrabind membrane and left to react on the membrane surface for 30 minutes.

The evaluation of ideal conditions for the experiments was made by studying the electroanalytical fundamentals of biosensor considering the life time of enzymatic layer (as the enzyme activity in the function of time).

3.4 PREPARATION OF ACETATE CELLULOSE MEMBRANE

The acetate cellulose membrane was used to eliminate other possible electroactive interferences present in the sample. This method uses molecular exclusion technique.

A solution of 40 ml tetrahydrofuran, 60 ml acetone 3.96 g acetate cellulose and 80 mg polyvinylacetate was prepared and casted on glass plate to obtain 200 or 400 µm layer. After the solvent evaporation the membrane was detached from the glass by immersing in the water and dried before use.
3.5 ELECTROCHEMICAL MEASUREMENTS

3.5.1 Apparatus

Amperometric measurements were performed in the batch with a Universal Lever – potentiostat. Current-voltage profiles were recorded by hand or Amel.

3.5.2 Electrodes

As the electrodes were used platinum (anode) as working electrode and silver/silver chloride (cathode) electrode as a reference. A difference of potential of 700 mV was applied between the electrodes for the hydrogen peroxide measurements. At this difference of potential value, hydrogen peroxide, produced from the BSAO catalyzed reaction, is oxidized on the platinum electrode and the current measured by the amperometer is directly proportional to the H₂O₂ present in the sample. At a potential of -700 mV was measured the oxygen consumed in the reaction.

3.5.3 Sensors assembly

The screw cap was assembled by placing on the outer body of the electrode a sequence of two different membranes, according to the following order:

1) An inner membrane permeable to the inorganic species, detected by the sensor, to eliminate interferences deriving from other electroactive substances eventually present in the sample. For oxygen, it was used a teflon membrane and for hydrogen peroxide an acetyl cellulose membrane (cutoff PM 100 Da)

2) An outer dialysis membrane

An O-ring was assembled to keep the membranes unmoved.

3.5.4 Biosensors assembly

The screw cap was assembled as the sensor screw cap by placing on the outer body of the electrode a sequence of different membranes (Figure 17), according to the following order:

1) An inner membrane permeable to the inorganic species
2) Biocatalytic membrane with immobilized BSAO
3) An outer dialysis membrane, to prevent microbial and proteolitic attack to the enzymes and/or their leaking from the membrane

A: amperometric sensor
B: selective membrane
C: biocatalytic membrane
D: dialysis membrane
E: O-ring

Figure 17. Model of the biosensor.
Between the determinations the biosensor was kept in a phosphate buffer solution (0.1 M, pH 7.0), while for the longer conservation the biosensor was disassembled and the biocatalytic membrane was kept in a dry-state under -15°C.

### 3.5.5 Experimental measurements in batch

All the measurements with free enzyme were carried out in a glass cell, thermostated at 37 degrees by forced water circulation, ensuring a uniform magnetic stirring at a constant rate (250 rpm). The assays were performed in 2.5 ml of sodium phosphate buffer or HEPES solution (both 0.1 M, pH 7.0) containing BSAO 3 mU/ml. The electrode cap was filled with internal solution of KCl in KH2PO4, 0.01 M, pH 6.6 and connected to the electrode tip. The amperometric measurements were carried out by connecting the electrode to an amperometric detector (Figure 18).

The measurements with enzymatic membrane of BSAO immobilized were performed the same way as experiments with free enzyme in solution. At the end of the measurement the screw cap was washed and new buffer solution was immersed into the glass cell.

![Figure 18. Model of measurements in batch.](image_url)

At the beginning of the measurement the electrodes were pre-treated at a fixed potential until a stable background current was obtained. After stabilization of the current, increasing quantities of different substrate stock solutions were added and the increase (or decrease in the case of oxygen) of the current, proportional to the H2O2 produced (or oxygen consumed) by the oxidative activity of BSAO. The variation of the current was recorded with interval of one minute. The calibration curve can be easily derived by reporting the increase (or decrease) of current as a function of the concentration of added substrate.

The kinetic parameters, Michaelis-Menten constant (Km) and maximum velocity (Vmax), were determined from Michaelis-Menten and Lineweaver-Burk by Sigma Plot Enzyme kinetic Module 1.1 from SPSS Inc.

The experiments with inhibitors and free BSAO in solution were performed by incubating with the inhibitor during the stabilization of the signal and after stabilization of the current the increasing amounts of stock solutions were added.
3.5.6 Experimental measurements with sensor “Dry”

The experiments were performed with an integrated cell. The cell was made of a rubber cylinder around the electrode (Figure 19) and the model is defined as “dry” sensor due to the reduction of sample volume. The sensor was assembled as mentioned in the paragraphs 3.5.3 and the amperometric measurements were carried out in a volume of 0.4 ml with 0.1 M phosphate buffer at pH 7.0. BSAO was used 3 mU/ml. Different concentrations of several substrate stock solutions were added with an interval of one minute and the variation of current was recorded.

A: Electrochemical sensor
B: O-ring
C: polymeric sample cell
D: cellulose acetate membrane + dialysis membrane

Figure 19. Model of sensor “dry”.
4. RESULTS

The experiments were performed by following determination:

1) BSAO free in solution
   a) Characterization of main electroanalytical features with the natural substrates of BSAO
   b) Electrochemical determination of kinetic parameters: $K_m$ and $V_{max}$, relative to the activity of BSAO
   c) Evaluation of the effect of inhibitors: Phenzylhydrazine and Benzylhydrazine

2) BSAO immobilized
   a) Characterization of the immobilization techniques for the realization of BSAO based biosensor
   b) Electrochemical determination of kinetic parameters: $K_m$ and $V_{max}$, relative to the activity of BSAO
   c) Effect of ionic strength

4.1 BSAO FREE IN SOLUTION

4.1.1 Characterization of electro-analytical features of free BSAO with its substrates

In the first experiment setup was evaluated the difference between hydrogen peroxide and oxygen electrode in the monitoring of the BSAO catalytic activity (Table 6). In the experiments with hydrogen peroxide electrode the linearity range was from 1.25 to 200 µM with a correlation coefficient 0.9984 while the slope was 0.08 for spermine. The linearity range of spermidine was 10 times higher and the range of benzylamine was 20 times higher respect to spermine showing less sensibility to these two substrates. From the experimental data has been stressed out the following results:
   a) BSAO showed higher affinity to spermine than to other substrates
   b) The hydrogen peroxide electrode showed a better sensibility and lower detection limits respect to oxygen electrode

Table 6. Experimental results obtained by hydrogen peroxide and oxygen electrode.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>H$_2$O$_2$</th>
<th>O$_2$</th>
<th>H$_2$O$_2$</th>
<th>O$_2$</th>
<th>H$_2$O$_2$</th>
<th>O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range (µM)</td>
<td>1.25-200</td>
<td>12-197</td>
<td>12.5-1500</td>
<td>388-1235</td>
<td>25-2000</td>
<td>567-1440</td>
</tr>
<tr>
<td>Slope $\Delta nA/\Delta µM$</td>
<td>0.08</td>
<td>0.09</td>
<td>0.008</td>
<td>0.01</td>
<td>0.002</td>
<td>0.01</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.9984</td>
<td>0.9996</td>
<td>0.9942</td>
<td>0.9970</td>
<td>0.9901</td>
<td>0.9972</td>
</tr>
<tr>
<td>Detection limit (µM)</td>
<td>1.25</td>
<td>6.0</td>
<td>12.5</td>
<td>180.0</td>
<td>25.0</td>
<td>300.0</td>
</tr>
</tbody>
</table>

4.1.2 Determination of kinetic parameters: $K_m$ and $V_{max}$

In order to evaluate the potential application of electrochemical method for the study of the BSAO kinetic, it has been determined the main enzymatic kinetic parameters, constant of Michaelis-Menten and maximum velocity. The determinations of kinetic parameters were performed by hydrogen peroxide electrode. The performance has been as described in the
Experimental

44

materials and methods. The BSAO has been used in free form with spermine, spermidine and benzylamine as substrates. First, the values were determined by Michaelis-Menten equation:

\[
\frac{v}{v_{\text{max}}} = \frac{c_s}{K_m} = \frac{c_s}{1 + \frac{c_s}{K_m}}
\]

\[
[c_s] = K_m \quad \text{when: } v = \frac{v_{\text{max}}}{2}
\]

When the concentration of active sites is high enough respect to the substrate concentration the reaction order passes from first order to zero order and comes to stationary state and the maximum velocity. The results have been elaborated also by Lineweaver-Burk method to obtain \(V_{\text{max}}\) and \(K_m\):

\[
\frac{v_{\text{max}}}{v} = \frac{K_m + c_s}{c_s}
\]

Equation Lineweaver-Burk:

\[
\frac{l}{v} = \frac{l}{c_s v_{\text{max}}} + \frac{l}{v_{\text{max}}}
\]

In the figures 20-25 are shown the results obtained for the determination of \(K_m\) and \(V_{\text{max}}\), in the presence of spermine, spermidine and benzylamine with the methods by Michaelis-Menten and Lineweaver-Burk.
Figure 20. Determination of $K_m$ for spermine with Michaelis-Menten.

Figure 21. Determination of $K_m$ for spermine with Lineweaver-Burk.

Figure 22. Determination of $K_m$ for spermidine with Michaelis-Menten.
Table 7 summarizes the main enzymatic kinetic parameters, constant of Michaelis-Menten and maximum velocity obtained by elaboration of the experimental data with specific software. Data obtained are in the same range as those reported in the literature and though confirmed the suitability of system, it was obtained a $K_m$ value of 30 µM for spermine, 270 for spermidine and 1550 for benzylamine. In addition was determined the maximum velocity.
to these substrates, which resulted to be in the presence of 3 mU/ml of BSAO, 1.6 µM/min for spermine and spermidine and half of the value for benzylamine.

*Table 7. Principal kinetic parameters (K<sub>m</sub> and V<sub>max</sub>) with free BSAO (3 mU/ml) in sodium phosphate buffer 0.1 M, pH 7.0, T 37.0°C.*

<table>
<thead>
<tr>
<th></th>
<th>Spermine</th>
<th>Spermidine</th>
<th>Benzylamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range (µM)</td>
<td>1.25-200</td>
<td>12.5-1500</td>
<td>25-2000</td>
</tr>
<tr>
<td>Slope (∆nA/∆µM)</td>
<td>0.080</td>
<td>0.008</td>
<td>0.002</td>
</tr>
<tr>
<td>Correlation Coefficient</td>
<td>0.998</td>
<td>0.994</td>
<td>0.990</td>
</tr>
<tr>
<td>Lower detection limit (µM)</td>
<td>0.62</td>
<td>6.25</td>
<td>12.5</td>
</tr>
<tr>
<td>K&lt;sub&gt;m&lt;/sub&gt; (µM)</td>
<td>30</td>
<td>270</td>
<td>1550</td>
</tr>
<tr>
<td>K&lt;sub&gt;m&lt;/sub&gt; (µM) data in the literature</td>
<td>20-30</td>
<td>225-310</td>
<td>1500-2260</td>
</tr>
<tr>
<td>V&lt;sub&gt;max&lt;/sub&gt; (µM/min)</td>
<td>1.6</td>
<td>1.6</td>
<td>0.8</td>
</tr>
</tbody>
</table>

The same experiments were performed with HEPES (0.1 M, pH 7.0) buffer solution. The results are shown in the figures 26-31.

*Figure 26. Determination of K<sub>m</sub> for spermine with Michaelis-Menten.*
Figure 27. Determination of $K_m$ for spermine with Lineweaver-Burk.

Figure 28. Determination of $K_m$ for spermidine with Michaelis-Menten.

Figure 29. Determination of $K_m$ for spermidine with Lineweaver-Burk.
Figure 30. Determination of $K_m$ for benzylamine with Michaelis-Menten.

Figure 31. Determination of $K_m$ for benzylamine with Lineweaver-Burk.

The results obtained with HEPES buffer solution are summarized in the table 8. HEPES as a buffer increased the affinity of the substrates.

Table 8. Principal kinetic parameters ($K_m$ and $V_{max}$) with free BSAO (3 mU/ml) in HEPES buffer 0.1 M, pH 7.0, $T$ 37.0°C.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (µM/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spermine</td>
<td>16.0</td>
<td>3.5</td>
</tr>
<tr>
<td>Spermidine</td>
<td>120.0</td>
<td>2.9</td>
</tr>
<tr>
<td>Benzylamine</td>
<td>565.0</td>
<td>0.8</td>
</tr>
</tbody>
</table>
4.1.3 Determination of inhibition effect on the catalytic activity of BSAO by Phenylhydrazine and Benzylhydrazine

In order to quantify the inhibition effects by both phenylhydrazine and benzylhydrazine and to evaluate the sensibility of these electrochemical methods, have been performed a set of experiments with free enzyme at the concentration of 73 nM. Experiments were carried out in the same conditions previously reported and deriving the calibration curves by reporting the increase of current as a function of the concentration of added substrate (spermine) obtained in presence of different increasing concentrations of inhibitors and for different incubation times.

The results obtained in the experiments performed with phenylhydrazine confirmed the irreversible inhibition of PHy as the lost of enzymatic activity. The experimental results (Table 9 and figure 32), show an increase of the inhibition degree as a function of the increase of PHy concentration and the incubation time. It could be observed the inhibition concentration of 50% at different PHy concentrations by incubating different times. In the table 9 is shown the 50% of BSAO inhibition, obtained at 50 nM PHy by incubation of 20 minutes, or at 75 nM by incubating 10 minutes. Doubling the concentration from 50 to 100 nM the IC50 was obtained directly while at the same concentration by incubating 20 minutes could be obtained nearly 90% inhibition.

Table 9. Inhibition of BSAO in function of PHy concentration and time.

<table>
<thead>
<tr>
<th>Inhibition % in function of concentration</th>
<th>Incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>%, PHy 25 nM</td>
<td>-</td>
</tr>
<tr>
<td>%, PHy 50 nM</td>
<td>12.0</td>
</tr>
<tr>
<td>%, PHy 75 nM</td>
<td>17.3</td>
</tr>
<tr>
<td>%, PHy 100 nM</td>
<td>36.4</td>
</tr>
<tr>
<td>%, PHy 150 nM</td>
<td>49.4</td>
</tr>
</tbody>
</table>

Figure 32. Inhibition with phenylhydrazine.
The experiments with benzylhydrazine as a pseudo-substrate show a different kind of response (forms intermediates). It was observed that in the presence of BH\(\text{y}\) the inhibition increased as a function of BH\(\text{y}\) concentration (Table 10 and figure 33). BH\(\text{y}\) reacts with the mixing time and at longer incubation times we observed a recovery of the enzymatic activity. For example inhibition concentration of 50\% was obtained with 15 nM BH\(\text{y}\) by incubating 10 minutes and at 25 nM of BH\(\text{y}\) was obtained by incubating 15 minutes while at 100 nM of BH\(\text{y}\) was obtained a 100\% inhibition practically with all incubation times.

**Table 10. Inhibition of BSAO in function of BH\(\text{y}\) concentration and time.**

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>-</th>
<th>5’</th>
<th>10’</th>
<th>15’</th>
<th>20’</th>
</tr>
</thead>
<tbody>
<tr>
<td>%, BH(\text{y}) 15 nM</td>
<td>54.5</td>
<td>52.0</td>
<td>50.9</td>
<td>46.0</td>
<td>33.5</td>
</tr>
<tr>
<td>%, BH(\text{y}) 25 nM</td>
<td>71.4</td>
<td>63.3</td>
<td>59.0</td>
<td>50.5</td>
<td>48.7</td>
</tr>
<tr>
<td>%, BH(\text{y}) 50 nM</td>
<td>93.0</td>
<td>90.1</td>
<td>87.8</td>
<td>83.9</td>
<td>76.3</td>
</tr>
<tr>
<td>%, BH(\text{y}) 75 nM</td>
<td>98.6</td>
<td>96.5</td>
<td>96.0</td>
<td>93.4</td>
<td>90.8</td>
</tr>
<tr>
<td>%, BH(\text{y}) 100 nM</td>
<td>100</td>
<td>99.7</td>
<td>99.1</td>
<td>98.2</td>
<td>97.6</td>
</tr>
</tbody>
</table>

**Figure 33. Inhibition with benzylhydrazine.**
4.1.4 Results with integrated cell sensor “Dry”

The results of experiments performed with hydrogen peroxide sensor coupled with BSAO free in solution in the “dry” sensor configuration, are shown in the table 11 and confirm the trend showed in the experiments reported in the table 6.

Table 11. Experimental results obtained with sensor “Dry”.

<table>
<thead>
<tr>
<th></th>
<th>Spermine</th>
<th>Spermidine</th>
<th>Benzylamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range (µM)</td>
<td>15-500</td>
<td>60-500</td>
<td>120-500</td>
</tr>
<tr>
<td>Slope (ΔnA/ΔµM)</td>
<td>0.015</td>
<td>0.004</td>
<td>0.0009</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.9980</td>
<td>0.9908</td>
<td>0.9857</td>
</tr>
<tr>
<td>Detection limit (µM)</td>
<td>6</td>
<td>30</td>
<td>60</td>
</tr>
</tbody>
</table>

4.2 BSAO IMMOBILIZED

4.2.1 Characterization of different physico-chemical immobilization techniques

From preliminary experiments arise that the BSAO catalytic activity is heavily affected by the different immobilization procedure. In order to evaluate the influence of different immobilization procedures, on the biosensors performances, four different synthetic membranes and alternative methods of immobilization have been tested and compared. The main physico-chemical parameters and the lifetime study of the considered biosensors are reported in the table 12 and figure 34.

These results clearly indicate that membranes 1-3 show comparable technical features. As far as the lifetime is concerned, the activity of membrane 4 decreases of about 20% in the first 30 days. All the other considered membranes show a sharper decrease of activity. Particularly, membrane 1 and 3 shows a steep decrease (about 60%) during the first month. A better behaviour is presented by membrane 2 where the decrease of the response is about 50% after 30 days.

Apart from the lifetime of operation, another relevant advantage shown by the Ultrabind membranes lies in the fact that they require a surface concentration of enzyme markedly lower than the other membranes (i.e. 0.15 U/cm², compared to concentrations in the order of 0.5-0.6 U/cm²), to ensure the same performance. It is self evident that, apart from simple economic considerations, an excessively high enzyme content on the membrane can be responsible for a lowering of the overall electrode performance, as a consequence of the easier occlusion of the pores. Moreover, also the immobilization process becomes more difficult, and the differences recorded among the experimental features of individual sensors, even though prepared according to the same procedure, become more evident.

On the basis of these results it can be concluded that the physico-chemical immobilization of BSAO on Ultrabind membranes presents several advantages in comparison to the immobilization on other types of membranes; this procedure can therefore be suggested as a general, alternative method of immobilization whenever the preparation of biocatalytic membranes, to be employed as the catalytic layer of enzyme based electrochemical biosensors, is requested.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range (µM)</td>
<td>20-280</td>
<td>25-200</td>
<td>50-200</td>
<td>50-430</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.998</td>
<td>0.997</td>
<td>0.997</td>
<td>0.990</td>
</tr>
<tr>
<td>Lower detection limit (µM)</td>
<td>10</td>
<td>10.5</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Number of assays</td>
<td>45</td>
<td>50</td>
<td>35</td>
<td>300</td>
</tr>
</tbody>
</table>

Figure 34. Characteristics of different biosensors.
4.2.2 Determination of kinetic parameters: $K_m$ and $V_{max}$

The same experimental measurements above presented have been performed with an amperometric BSAO based biosensor, which have not been found to be realized before. As immobilization method has been chosen Ultrabind, method number 4. The experiments have been performed in phosphate buffer and HEPES.

In the table 13 are shown the main enzymatic kinetic parameters, including the values of the constant of Michaelis-Menten and of the maximum velocity for the three enzyme substrates. Interestingly immobilized BSAO showed highest affinity towards benzylamine with a value 423 mM. However, spermine showed highest sensibility with a slope value 53.4 $\Delta nA/\Delta mM$. The $K_m$ apparents (apparent values) and linearity ranges were higher than the $K_m$ of free enzyme.

<table>
<thead>
<tr>
<th></th>
<th>Spermine</th>
<th>Spermidine</th>
<th>Benzylamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range (µM)</td>
<td>50-430</td>
<td>99-549</td>
<td>99-549</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.990</td>
<td>0.987</td>
<td>0.991</td>
</tr>
<tr>
<td>Slope ($\Delta nA/\Delta mM$)</td>
<td>53.4</td>
<td>42.2</td>
<td>44.4</td>
</tr>
<tr>
<td>Lower detection limit (µM)</td>
<td>25</td>
<td>49</td>
<td>49</td>
</tr>
<tr>
<td>$K_m$ (µM)</td>
<td>892</td>
<td>1475</td>
<td>423</td>
</tr>
<tr>
<td>$V_{max}$ (µM/min)</td>
<td>22.6</td>
<td>33.9</td>
<td>17.2</td>
</tr>
</tbody>
</table>

The experiments were also performed with HEPES (0.1 M, pH 7.0) as buffer and $K_m$ apparent was ca. 10 times less than with phosphate buffer showing hence higher affinity of BSAO towards substrates in the presence of HEPES.

4.2.3 Effect of ionic strength on biosensor experiments

The calibration of biosensor was performed with all 0.1 M, 0.05 M and 0.025 M sodium phosphate buffer (P.B.) and HEPES (pH 7.0). The buffers at 0.1 M and 0.05 M gave similar results whereas 0.025 M buffers showed better response and affinity (Figure 35).
4.3 PRELIMINARY CELL EXPERIMENTS

It has been applied the use of amperometric sensor “dry” on tumoral cell line of adenocarcinoma both, wild type and multi drug resistant to study the effect of hydrogen peroxide and aldehydes produced in the reaction with BSAO.

4.3.1 Preparation of adenocarcinoma colon cell lines (LoVo)

A human colon adenocarcinoma cell line wild type (LoVo WT) was isolated from a metastatic nodule and its multi drug resistant variant (LoVo DX) were used. Cell lines were grown in monolayer in Ham’s F12 medium (GIBCO BRL/Life Technologies, Paisley, UK) supplemented with 10% FBS (Hyclone, Cramlington, UK), 1% L-glutamine (GIBCO BRL/Life Technologies), 1% penicillin (50 U/ml) – streptomycin (50 µg/ml) (GIBCO BRL/Life Technologies) and 1% vitamins (GIBCO BRL/Life Technologies) in a humidified atmosphere of 5% CO₂ in a water-jacketed incubator at 37°C. The pleiotropic multi drug resistant cell line LoVo DX was selected for resistance to doxorubicin (Adriblastina, Pharmacia and Upjohn, Milan, Italy). LoVo DX cells were also resistant to etoposide and vincristine.

Cells were harvested with 10 mM EDTA in phosphate buffer with add of 0.25% trypsin solution in phosphate buffer, washed and centrifugated (2 min, 1,500g) and then resuspended in buffer solution (0.1 M, pH 7.2).

The cells were homogenized (10% w/v) with an ultrasound apparatus Ultra-Turrax (4 times 20 seconds at 10 kHz and 30 W) in ice bath and then centrifugated. The supernatants obtained from these two cell lines were diluted with phosphate buffer (0.1 M, pH 7.2) to achieve a concentration 200,000 cells/ml.

4.3.2 Experiments to determine polyamine content

The experiments were performed in configuration “dry”. A potential of +700 mV was applied between the electrodes for the determination of hydrogen peroxide at 37°C. Noted and increasing aliquots of cell culture supernatant solution descript above were added to a volume of 250 µL phosphate buffer (0.1 M, pH 7.2). The experiments without the addition of BSAO were performed to reveal the interferences caused by the polyamines in cell homogenates.
Ones the electrochemical signal had reached stable state different quantities of supernatants and free BSAO (in final concentration 6 µM) and followed the change of current in picoampers, proportional to added supernatant concentration.

### 4.3.3 Preliminary results

As preliminary results can be said that in the cell homogenates with BSAO is produced hydrogen peroxide, which can be determined amperometrically (Figure 36). The research is in progress and the obtained experimental results are hence presented as preliminary results together with the demands for further studies.

![Figure 36. Calibration curves of LoVo DX and LoVo WT supernatants.](image)
5. DISCUSSION

Application of enzyme based electrodes to different areas of analytical biochemistry and clinical chemistry derives from the combination of an extremely high selectivity with great facility of use and notably reduced costs of operation. These features derive respectively from the presence of a highly specific biocatalytic component (the enzyme) and from the nature of a recorded signal (amperometric or potentiometric), whose detection is performed by relatively inexpensive devices. Many reviews covering the different aspects of bioelectrodes technology and applications have been published in the past few years.

In this work a BSAO based electrochemical method has been developed by means of two different experimental measurement conditions:

1) with free BSAO in solution
2) with immobilized BSAO (BSAO based biosensor)

The results obtained using these two configuration devices can be discussed in terms of the following items: 1) characterization of catalytic activity of enzyme in the presence of three different substrates; 2) analytical determination of polyamines.

According to the experimental results performed with the free BSAO in solution as well as with the immobilized BSAO better electroanalytical features (sensitivity, fast response time and reproducibility) were obtained with using devices that were realized by coupling the BSAO with the hydrogen peroxide electrode instead of those obtained with the oxygen electrode.

The kinetic characterization of the catalytic activity of BSAO was performed with two different devices. The $K_m$ and $V_{max}$ values have been calculated from the obtained experimental data. The characterization with BSAO free in solution are comparable with those obtained with traditional methods. The same experiments performed with the BSAO based biosensor showed different kinetic parameter values respect to those obtained with the BSAO free in solution; this due to the diffusive and kinetic control of the bioelectrochemical devices response and due to modification of the BSAO catalytic activity. As a consequence of this kinetic modification of the BSAO catalytic activity, a better affinity was obtained in respect to the benzylamine, while in the experiments performed with the BSAO free in solution this result was obtained with spermine.

In order to evaluate the influence of the buffer in the BSAO based electrochemical devices performances, two buffer solutions were tested; sodium phosphate and HEPES. The BSAO is more stable in phosphate buffer (0.1 M, pH 7.0), but in the presence of HEPES, the affinity of BSAO towards its substrates increases. By decreasing the ionic strength can also be increased the affinity.

The evaluation of the possible therapeutical use of the BSAO was also studied by means of inhibition experiments in the presence of the classical inhibitors, which covalently bind to the organic cofactor TPQ. The effect of phenylhydrazine and benzylhydrazine on the catalytic activity of BSAO, showed to be more evident with phenylhydrazine than benzylhydrazine, when it was used as inhibitor. Longer incubation time led to an increased response when benzylhydrazine was used as an inhibitor; this most probably because it is a pseudo-substrate.

The development of BSAO based electrochemical devices provide a selective and fast method for the determination of polyamine concentrations. Three different types of methods were used to characterize the possible use of proposed methods for the determination of polyamines in different matrices:
1) measurements \textit{in batch} with free enzyme
2) configuration of “dry” sensor with free enzyme
3) measurements with immobilized enzyme

The first configuration is suitable for the determination of polyamines in matrices, with the best accuracy and rapidity. This method resulted to be ideal on cell culture studies and it could also have an important application \textit{in vivo}. The second configuration presents the possibility to use reduced volumes even though the sensibility is lower. This configuration can be very useful for real matrices. The configuration of biosensor has evidently the advantage of the possibility to use the same configuration for various experiments as the electroanalytical interpretation and, at the same time the enzyme are more stable.

With all these three configurations, it is possible to monitor the reaction process in real time, since polyamines concentration (and consequently BSAO catalytic activity) is continuously monitored, directly and selectively, by the BSAO/H\textsubscript{2}O\textsubscript{2} biodevices, without the need of any sample pre-treatment.
6. CONCLUSIONS AND PERSPECTIVES

The electrochemical methods here proposed present some advantages with respect to all those traditional techniques employed for the \textit{in vitro} determination of BSAO catalytic activity. First of all, by means of this method it is theoretically possible to quantitatively determine the enzymatic activity of BSAO, the operating conditions being only limitations of the BSAO based electrode. Furthermore, the methods here presented are fast, extremely selective, and, as indicated by the data reported, sensitive. Still, the method is extremely simple and does not require the use of any cumbersome experimental apparatus. Nonetheless, the method gives the possibility of monitoring the reaction process in real time by the amperometric biosensor.

The method here described can also represent the first step toward a complete \textit{in vitro} study of the catalytic properties of different molecular forms of BSAO, and towards the kinetic characterization of its inhibitors. These devices are also able to determine the inhibition effects deriving from the interaction between several substances and BSAO. Particularly the use of BSAO biosensor can be recommended for the routine determination of amines in food matrices due to its combination of good analytical features (response time, lowest detection limit and range of linearity), reduced costs of operations and maintenance, prolonged lifetime of operation of enzymatic layer (in the case of BSAO biosensor) and facility of use.

The experimental characteristics of the BSAO based biosensor employed in the present work can be opened by promoting its \textit{in vivo} applications. The biosensor employed in the present work could also successfully cover these kinds of analytical applications. Another future application can be presented by the employment of the proposed devices in the determination of biogenic amines in human cancer cells by assembling a microelectrode.
Bibliography


