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Laccase

Properties, Catalytic Mechanism, and Applicability

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ABSTRACT

The present review was dedicated to laccase—the enzyme, belonging to the group of multinuclear copper, containing so called "blue" oxidases. The molecular structure, metals content, substrate specificity, and other physicochemical properties were described in this article. The authors considered the mechanism of enzymatic action and electrocatalytic oxygen reduction catalyzed by laccase in details. The data of laccase application in organic synthesis, biosensors, and immunoenzyme assay were presented.

Index Entries: Laccase; catalytic mechanism; electron-transfer; bioelectrocatalysis; immunoenzyme assay; biosensors; enzymatic synthesis.

INTRODUCTION

Laccase was first obtained from the juice of the Japanese tree *Rhus venicifera*. There are publications about the laccase produced from *R. succedanea* (*1–3*). A notable number of fungi are known to produce this enzyme. The most studied laccases appear to be from *Agaricus bisporus, Podospora anserina, Rhizoctonia practicola, Trametes* or *Polyporus versicolor,*

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Pholiota aegerita, *Pleurotus ostreatus* (*2*), *Coriolus hirsitus* (*3,4*), and *Neurospora crassa* (*2,3,5*).

Most fungi produce both intra- and extracellular enzymes. It was reported (*6,7*) that some compounds, including the substituted phenols, amines, and benzoic acid, can induce the synthesis of laccase by some fungi not producing this enzyme in their absence. The physiological function of fungal laccase is thought to be primarily related to its role in lignin decomposition. There is also a supposition that a major function of laccase is not the oxidation of lignins proper, but its influence on polymerization of their oxidation products (*8*).

Laccase belongs to multinuclear copper-containing oxidases. This enzyme, along with cytochrome C oxidase, ceruloplasmine, and ascorbate oxidase, catalyzes the reduction of molecular oxygen by various organic compounds straightforwardly to water without the step of hydrogen peroxide formation. In the laccase active site, the presence of a few copper atoms of various types, as well as of "simple" substrates, hydrated electron, and oxygen, makes this enzyme highly interesting in the study of the mechanism of enzymatic catalysis and electron transfer in the protein molecule through the catalysis. A wide substrate specificity, the high catalytic constants, and the use of air oxygen as the second substrate provide the ground for laccase applicability in organic synthesis, biosensors, and immunoassay. All these aspects will be discussed in this article.

MOLECULAR STRUCTURE OF LACCASE

Laccase, like all blue oxidases, is a glycoprotein. The carbohydrate moiety of the enzyme (*9*) can constitute 10–45% of the protein molecule by weight (86–91% according to ref. *10*), and includes such carbohydrates as hexoseamine, glucose, mannose, galactose, fucose, and arabinose (*11–13*). The molecular masses of laccases from various sources vary in a wide range; major discrepancies may well be related to the carbohydrate moiety of the molecule. The amino acid chain contains about 500 amino acids (AA). The molecular masses of laccase isoforms produced by the same strain can differ (Table 1).

The investigation of C-terminal amino acids sequence of laccase from *Neurospora crassa* makes possible the establishment of a high degree of homology with the amino acid sequence of ceruloplasmin (*20*) and a less degree of homology with that of plastocyanine and azurine. The laccase molecule usually contains four copper atoms in various surroundings that are admittedly classified into three types. They differ in accessibility to solvents, in spectra, and in the features of their surroundings.

Copper of the first type (copper 1) is available to the action of solvents, including water. It can be removed from the enzyme molecule

Table 1
Molecular Masses of Laccases from Various Sources[a]

Laccase source	Mol mass, kDa	Ref.
Schizophyllum commune Dicaryon	62, 64, 72 (extracellular)	*1*
	36, 38, 42, 45, 48 (intracellular)	*1*
Pleurotus ostreatus	59	*6*
Botrytis cinerea	72–102	*10*
Podospora antiserina	390	*14*
Rigidoporus lignosus	52, 55	*15*
Phellinus noxius	70	*15*
Trametes versicolor	66, 64, 45, 43	*16*
Sycamore	95–100	*9*
Rhus vernicifera	140	*17*
Polyporus versicolor (*Coriolus versicolor*)	62, 64 (two isoforms)	*18*
Coriolus hirsitus	55	*3*
Cerena maxima	67	*3*
Coriolus anisoporus	57.5	*19*

[a]Isoelectric points of laccasses from varous sources are 2.9 (6); 2.6, 2.8 (10); 3.83, 3.32 (15); 4.5 (39). The sedimentation coefficient $S^{20}w$ is 6.0–6.1 S (9).

by various copper complexones, and displaced by mercury with a great loss in the activity (*21*) or else substituted by cobalt (*22*).

Copper of the second type (copper 2) is easy to eliminate (*21*). However, that from laccase in aerobic conditions proved impossible to be disposed of (*23*). In anaerobic conditions, in the presence of a reducing agent and in the absence of diethyldithiocarbamate (DDC) or dimethylglyoxime, EDTA also failed to remove copper atoms (*24*). On the other hand, the copper 2 center was reconstructed both in aerobic and anaerobic conditions (*24*). Copper 2 was shown (*25*) to interact with fluorine ion; the surrounding protein undergoes the conformational changes (*26*).

Copper can be totally disposed of from laccase by the cyanide method (*25*). It proves impossible to re-embed the first and second type of copper. However, it appears possible to embed the third type of copper (copper 3) in apoenzyme. These results are of interest in view of the fact that these sites, in other blue oxidases, are reconstructable. Cyanide may well interact with disulfide bonds, thereby modifying the protein. Conformational changes of the protein are also possible. The cyanide method eliminates copper 1 at pH 7.0 and various types of copper from thiourea in the presence of chloride ions at pH 4.0 (*25*).

Laccase Spectra

Laccase spectrum features the absorption at 280 and 605 nm. Copper 1 imposes an intense blue color of the enzyme (605 nm, and $103M^{-1}/c^{-1}$), features S(cys) --> Cu(II) transfer band, and gives the Electron Paramagnetic Resonance (EPR) spectrum with an exceptionally small hyperfine splitting in the low-field region (*27,28*).

Copper 2 features normal parameters of EPR spectrum, attesting the presence of tetragonal surrounding. It has but an insignificant absorption in the visible region (*29,30*).

Laccase EPR spectra recorded in presence of H_2O^{17} point out the presence of an H_2O molecule in the surroundings of the copper 2 atom. The spin echo method can reveal a ligand nitrogen atom in copper 2 surroundings (*31,32*).

Copper 3 is undetectable by EPR method because of a strong antiferromagnetic interaction of Cu–Cu dyad. This type of copper is a binary complex with absorption at 330 nm disappearing on the reduction of the active site (*27*).

The spectra can be modified by adding urea, azide, or by use of pH < 4.5 (*31*). Possibly, urea, azide, and low pH values preclude the formation, at low temperatures, of an additional hydrogen bond between the protein and copper 1. The spectra of copper 2 in these conditions do not alter appreciably. Copper 1 and 2 provide a superfine cleavage. The spectrum parameters of two ions vary on the temperature shift from that of liquid nitrogen to ambient. The resonance Raman spectrum of copper 1 also alters in this temperature range. Possibly, the shift in temperature induces the conformational changes in the laccase molecule, but their nature is unclear (*31*).

STRUCTURE OF LACCASE ACTIVE SITE

Oxidases, transferring four electrons to oxygen to form water without formation of hydrogen paroxide, are usually complex enzymes (*28*). The laccase is a relatively simple representative of this family. One of the ligands of copper 1 seems to be cysteine or methionine (depending on laccase source) (*28*). The study of laccase derivatives, containing one mercury atom and three copper atoms, based on the analysis of mercury EPR signal and fluorine binding, showed that copper 2 is bound to three nitrogen atoms. The fourth copper 2 ligand was shown to be a water molecule (*28*).

Chemical and spectral studies on binding anions to the native laccase and the copper 2-depleted laccase from *Rhus vernicifera* (*28*) showed the high affinities of copper 2- and 3-type sites for N_3^-, O_2^{2-}, and F^-. Copper 2 was proven to play an important role in structural nonspecific stabilization of anionic binding in the copper 3 active site.

In addition, it is possible that the atoms of copper 2 and 3 are bound via nitrogen. An electron possibly gets into laccase (at least fungal laccase) vis copper 1 and transfers to copper 2. Removal of copper 2 hinders the electron transfer between copper 2 and 3 atoms. Copper 2 and 3 are possibly cooperative and responsible for oxygen reduction (*21*). Our results obtained by the impulse radiolysis method provided evidence that the primary acceptors of electrons in the other copper-containing oxidase, ceruloplasmin, are cysteine and hystidine, which transfer the electron to copper 1.

From the computer comparison of the primary structures of proteins containing copper of various types, possible schemes were offered for location of amino acid ligands of copper in the polypeptide chain of laccase from *Neurospora crassa* (*33–36*). The binding of copper 1 ion ("blue site") involves the residues His-428, Cys-500, His 505, and possibly Leu-510 (*35*). The copper 2 ion is coordinated with the amino acid residues His-431 and His-435. The ligands of copper ions in the binuclear site are probably three amino acid residues out of the following: His-428 to His-435, His-499, His-501 (one-half of the site), and three out of four residues: His-95, His-97, His-140, and His-142 (the other half of the site). Thus, the binuclear site in laccase may well be formed by amino acid residues located in polypeptide chain areas forming "blue" and "nonblue" sites. Supposedly, the sites of various natures were formed in the immediate vicinity of each other. This is a fundamental event, since it optimizes, in multinuclear oxidases, the processes of intramolecular electron transfer from the oxidized substrate through copper atoms of various types to the protein-bound second substrate, oxygen, with no liberation of any high-reactivity oxygen intermediates into the medium.

REDOX PROPERTIES OF LACCASE ACTIVE SITE

Redox potentials of copper in the prosthetic groups of laccases from *Rhus vernicifera* and *Coriolus versicolor* were explored in refs. (*37*) and (*38*). The disclosed notable discrepancies between the redox potentials of copper 1 and 3 of these enzymes make the similarity of the mechanisms of catalysis by laccases from various sources worth discussion. The redox potentials of copper 1 and 3, obtained in (*4*), related to the absorption of chromophorms at 330 nm in laccase from *Rhus vernicifera*, depended on hexacyanoferrate, used as electron mediator on redox titration. In the excess of this compound, the potentials of copper 1 and 3 were 434 and 483 mV, respectively, for the enzyme from *Rhus vernicifera* (sodium phosphate, pH 7.5, the ionic strength 0.2). The redox potentials of copper of the same types obtained for laccase from *Coriolus versicolor* at pH 5.5 are 785 and 782 mV.

The effect of fluoride, a strong inhibitor of laccase, does not actually affect the redox potential of copper 1, but notably influences the poten-

tials of copper 2 and 3 (*4*). The redox potential of copper 2 of *Coriolus versicolor* laccase in the presence of fluoride ion decreases by more than 210 mV. This fact is explicable by a strong interaction between fluoride ion and copper 2 (*4*).

Catalytic Activity of Laccase

Laccase can catalyze the oxidation of various compounds, including *o,p*-diphenols, aminophenols, polyphenols, polyamines, lignin, some inorganic ions (*8,39*) and aryl diamines (*40*). It also catalyzes demethylation of lignin and methoxyphenol acids (*41,47*). The second substrate of the enzymatic reaction is molecular oxygen (*39*). All known laccases can also catalyze, with equally high efficiencies, both the oxidation of ascorbic acid and phenol substrate (*42*). Unlike the reactions catalyzed by other oxidoreductases, oxygen is reduced directly to water by a four-electron mediator-less mechanism (*43*). The values of the Michaelis constants and k_{cat} for laccase-catalyzed reactions are listed in Table 2.

By the second substrate, oxygen, the K_m values fall around $10^{-5}M$ for laccases isolated from various sources, whereas V_m makes up 50–300 $M*s^{-1}$ (*42,43*).

A number of works (*44,45*) reported a strong pH dependence of both K_m *and* U_m. The Michaelis constant, both by oxygen and by electron donor, was pH-independent, whereas the catalytic constants, both by oxygen and electron donor, are pH-dependent equally: in the acidic region, the k_{cat}^{02} and k_{cat}^{DH} values do not alter, whereas in the neutral region, they decrease (*45*).

The studied pH dependencies of the catalytic constant show that the enzyme active site contains at least two acidic-alkaline groups with close values of p*Ka*(~ 5.5) (*45*).

The pH optimum of the activity of laccase from *C. hirsutus* is at pH 3.5–4.5 (*39,44*), from *Schinus molle* at pH 6.2, from *Schizophyllum commune* Dycarion at pH 5.4–6.0 (*1*), from *Rhizoctonia praticola* at pH 6.0–7.0 (*7*), and from *C. versicolor* at pH 4.0–5.0 (*46*).

Some compounds, such as halides, acetate, and so forth, were shown to have a capacity to inhibit laccase (*46 to 48*). The enzyme activity is also influenced by the ionic strength. In some works (*46,47*), on the activity measurements, the ionic strength was made constant by adding KNO_3 into the medium.

Of interest are the data on oxidation of polymeric compounds in the presence of laccase. The oxidation of some substrates, for instance, thioglycosidized lignin, is accompanied by changes in UV spectrum and in distribution of dimensions of substrate particles (*8*). Two reactions occur: polymer degradation to form low-mol-wt products and lignin condensation to form high-mol-wt fractions. The analysis of results on distribution of peaks formed on gel filtration of products are indicative of condensation–depolymerization equilibrium. The structural study of degradation prod-

Table 2
The Catalytic Activities of Laccases with Various Substrates

Compounds	Laccase sources	K_m, mkM	k_{cat}, s^{-1}	Ref.
Pyrocatechol	*C. hirsutus*	390	492	*39,43*
	C. maxima	123	404	*39,43*
Hydroquinone	*C. hirsutus*	180	257	*39,43*
	C. maxima	—	—	*39,43*
	Fusarium graminearum	—	900	*42*
	Fusarium graminearum	—	800	*42*
K. ferrocyanide	*C. hirsutus*	97	457	*39,43*
	C. maxima	—	—	*39,43*
	Fusarium graminearum	—	2000	*42*
2,6 dimethoxyphenol	*Botrutys cinerea*	190	—	*10*
Quinol	*Botrutys cinerea*	133,533 (for isoforms)	—	10
	Schinus molle	1250	—	*42*
Guaiacol	*C. versicolor*	300	200	*42*
	Fusarium graminearum	—	660	*42*
Vanillic alcohol	*C. versicolor*	400	182	*42*
Vanillin	*C. versicolor*	2000	90	*42*
Vanilic acid	*C. versicolor*	1000	160	*42*
Homovanillic acid	*C. versicolor*	350	160	*42*
Eugenol	*C. versicolor*	600	150	*42*
Dihydroeugenol	*C. versicolor*	260	160	*42*
Guaiacyl-propanol3	*C. versicolor*	—	—	*42*
Verate aldehyde	*C. versicolor*	—	—	*42*
Ascorbic acid	*C. versicolor*	2000	110	*42*
	Fusarium graminearum	—	2300	*42*
Dioxphenylalanine	*C. versicolor*	1000	70	*42*
	Fasarium graminearum	—	1200	*42*
o-Dianisinine	*C. versicolor*	100	130	*42*
	Fusarium graminearum	—	100	*42*
3,3′,5,5′-tetra-methylbenzidine	*Fusarium graminearum*	—	1000	*42*

ucts of compounds, modeling phenolic β-1-lignin substructure, led to an inference about three types of laccase reaction: C α-Cβ cleavage, alkyl-aryl cleavage, and C α oxidation. Phenolic hydroxyls are necessary in this case for oxidation of β 1-substituted substrates (*8*).

CATALYTIC MECHANISM OF LACCASE

A mode of laccase action based on the study of circular dichroism spectra was reported (*55*). On its elucidation, the following experimental data were taken into account:

1. All four copper atoms are involved in the catalysis;
2. Electrons can get into the protein molecule by various ways and in various sequences;
3. The interaction with molecular oxygen can occur at all reduction steps of the reaction.

A relative contribution of this or that pathway of the offered scheme depends on the reducer/oxygen ratio. The mode of laccase action was thoroughly studied by many researchers (*3,11–13,17,37,38,49–53*). The initial step of catalysis by laccase was shown (*49*) to involve a step of one-electron transfer to a specific copper-containing site to form a free radical from an organic substrate. From mass-spectroscopy data, it was established that one of the two water molecules formed on oxygen reduction passes into the solution very quickly (*4,29*). The other water molecule remains fairly tightly bound to the copper 2 ion. The following mechanism of catalysis by laccase was offered. The initial step of oxygen reduction to water consists of the transfer of two electrons from the reduced dyad of copper 3 ions to form O_2^{2-}. This is followed by one-electron transfer from copper 1 to oxygen intermediate to form water molecules and O^- radical. A shift in redox state of the enzyme can stabilize O^- radical. The fourth electron passes from copper 2 ion to O^- radical to form the second water molecule.

The development of this scheme enabled the authors (*29*) to offer close mechanisms for laccase from *Rhus vernicifera* and *Polyporus versicolor*. The basic points of the catalysis mechanism for the enzyme from *Rhus vernicifera* are the following: laccase occurs in two forms; electrons are taken from electron donors one by one; copper 1 plays a central role in the transfer of at least one electron to copper 3 ions. In further studies, the mechanism was specified (*50*). Pecht et al. (*54*) studied the electron transport pathways in laccase from *Rhus vernicifera*. By the impulse radiolysis method, they showed that at least the first electron is gained by copper 1 ion. Other works also show that all electrons pass through copper 1 ions and that the latter is the "entry" for all electrons. These data call in question the mechanism offered in ref. *55*, according to which there can be a few such "entries."

In stationary condition, fluoride ion rapidly inhibits dimethylphenylene diamine oxidation (*25*). Fluoride ion lowers the stationary concentration of oxidized copper 1 and 2, increasing the concentration of intermediate oxygen radicals. The scheme of enzymatic reaction in fluoride presence was studied in detail (*25*). This work showed that in the process of O_2 reduction to H_2O, all three types of copper are involved. Fluorine ion is capable of interacting with copper 2. In stationary conditions, it rapidly inhibits the oxidation of such substrate as dimethylphenylenediamine. Both reducer-dependent and reducer-independent steps of the reaction are inhibited. The authors (*25*) consider that fluoride lowers the stationary concentrations of oxidized copper 1 and 2, elevating the concen-

tration of intermediate oxygen radicals. Andreasson and Reinhammar (*56*) showed that fluoride ion does not affect the reduction of copper 1. It may well be that the gaining of electrons by copper 3 ions occurs via copper 2 ion to which fluoride ion is specifically bound.

The study of laccase-oxygen interaction showed the following:

1. Oxygen molecule can interact with some copper site (2 or 3 type) in a rapid reaction only after copper 1 has gained one electron;
2. The limiting step seems to be the intramolecular electron transport or some related conformational change; and
3. Fluoride ion can hinder laccase reoxidation.

From the published data (*30,57-61*), the scheme including one-electron steps was offered for oxygen reduction catalyzed by *Rhus vernicifera* laccase. This supposition is at variance with a prevalent opinion that the process should start with two-electron transfer to prevent the formation of superoxide radical (*32*). However, this argument is based on thermodynamics of free particles in solution and underestimates the importance of oxygen-laccase binding (*61*).

The high energy for breaking the O—O bond is a major reason for a comparatively slow reduction of H_2O_2 by inorganic reagents. The authors of the above works supposed that the lowering of this energetic barrier could be reached by an "asymmetric" coordination of O_2^{2-} with two different types of copper present in various oxidation states and polarizing the O—O bond, thereby leading to its breakdown at a low energy.

The anaerobic reduction of *Rhus vernicifera* laccase by hydroquinone was reported (*62*). Copper 1 and 3 ions were shown to be reduced simultaneously with commensurable rate constants of the second order: $3.25*10^2$ and $4.57*10^2 M^{-1}S^{-1}$, respectively. Laccase was found (*63*) to contain copper ions in reduced and oxidized states. According to the model, offered by the authors, the enzyme can be in the active and inactive forms. Copper 1 and 3 in active states have an intramolecular relationship. However, in the inactive state, this interaction is absent. The active molecule interacts with the O_2 molecule only when all copper ions are in the monovalent state. The inactive molecule does not interact with oxygen. The laccase molecule in the active and inactive states interacts with such oxidizers as ferricyanide ion and H_2O_2. The inactive laccase molecule can be oxidized by the active enzyme molecule as a result of a slow intramolecular electron transfer. Substrates are oxidized by copper ions in laccase by one-electron mechanism.

A molecular model was offered (*64*) to demonstrate the processes occurring in the enzyme catalytic site. Steps 1-5 were postulated from the data of the electrochemical assay. Steps 6-9 can be considered a result of kinetic assay of homogenous oxidation of electron donor substrate by laccase. Comparison of kinetic results and information on the composition

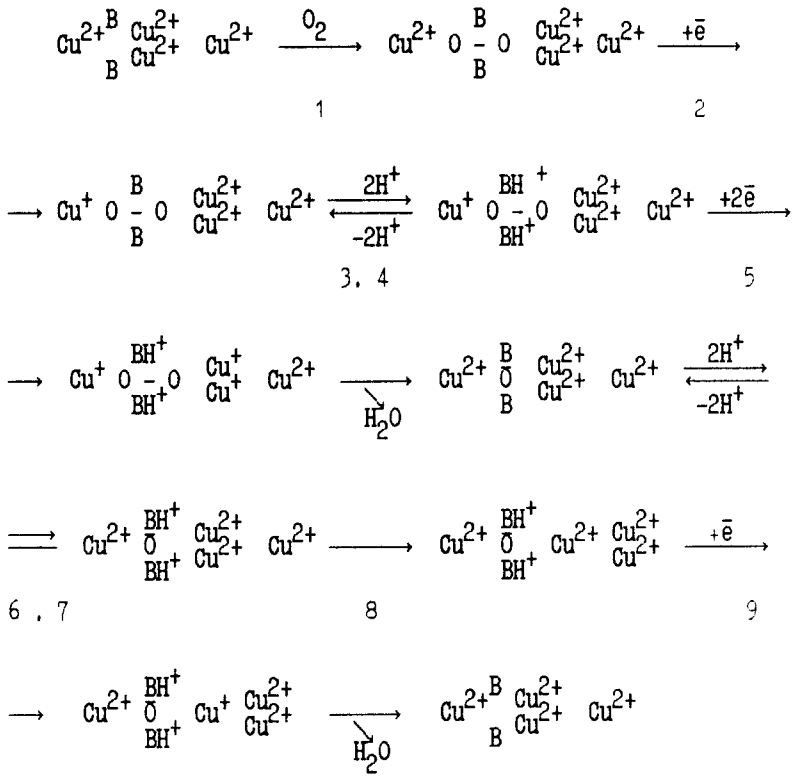

Fig. 1. Molecular model of the processes occurring in laccase active site in catalysis.

and structure of laccase active site can provide the following pattern of the molecular mechanism of oxygen reduction by this enzyme (Fig. 1).

There are a few works on the steady-state kinetics of laccase activity. The mode of *Rhus vernicifera* laccase action was shown to belong to the group of ping-pong mechanisms (*51*).

MECHANISM OF ELECTROCATALYTIC OXYGEN REDUCTION CATALYZED BY LACCASE

Laccase is one of few enzymes that was persuasively shown to effect the enzymatic and electrochemical reactions in absence of a low-molecular-weight carrier of electrons (*65*). The electrode is used in place of one of the substrates of the enzymatic reaction being a donor of electrons. The summary reaction is the electroreduction of oxygen to water (Fig. 2). It is to be noted that at present, one of the most efficient catalysts of this reaction is metals of the platinum group. The equilibrium oxygen potential (1.23 V) was successfully realized only on the electrodes of specially treated platinum in the most rigid conditions (*66*).

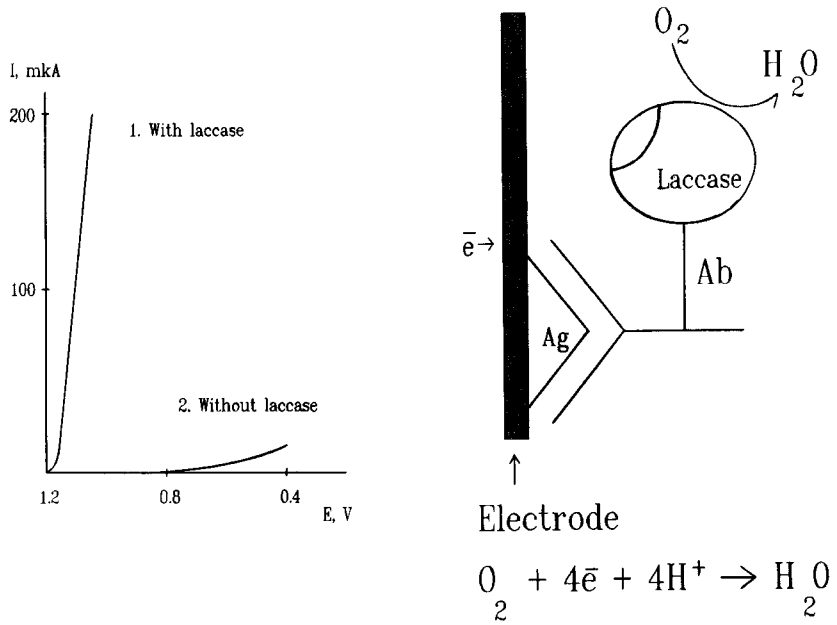

Fig. 2. The scheme of mediator-less electroreduction of oxygen on the electrode with immobilized laccase.

Electroreduction of oxygen and direct transport of electrons were studied in the system of the oxygen-laccase-carbon electrode (*65,67–70*). The enzyme was on the surface of the electrode in the adsorbed state. Introduction of various sources of laccases into the system shifts the stationary potential of the electrode in the region of positive values and accelerates the electroreduction of oxygen in the range of potentials 1.2–0.6 V; the values of potentials and electric currents at the given potential increased with the amount of the enzyme adsorbed on the electrode. This effect was observed on the electrodes made of various materials (gold, pyrographite, glassy carbon, carbon, and carbon black). In the presence of laccase inhibitors (fluoride or azide anions), the electrocatalytic process rate sharply fell, and the polarization curve actually coincided with the background curve.

It was established (*67*) that the potentiodynamic curves, recorded in anaerobic conditions on carbon black electrodes with the adsorbed *Coriolus versicolor* laccase, showed the reversible cathode and anode maxima of the enzyme reduction oxidation at 0.75 and 0.85 V, respectively. The experiments with apoenzyme, adsorbed on carbon-black electrodes, in anaerobic conditions showed the absence of electric current maxima on potentiodynamic curves, which attests to electrochemical conversions of copper ions and not the protein moiety of the enzyme (*67*), nor did the electrode with immobilized apoenzyme accelerate the electroreduction of oxygen. The nature of primary electron acceptor in the protein molecule

was shown via electroreduction of the enzyme on platinum electrode at E_r = 0.55 V in anaerobic conditions. In the process of electroreduction, spectral shifts of laccase were controled at λ = 610 nm. In these conditions, copper 1 reduction was shown to occur. The process is reversible, which affords laccase reoxidation to be conducted via saturation of the system with oxygen. Thus, for laccase, both in the homogeneous enzymatic catalysis and in the electrochemical system, the primary electron acceptor is copper 1.

The steady and reproducible value of the potential 1.2 V afforded a detailed study of oxygen electroreduction mechanism near the equilibrium potential using the approaches that are conventional in electrochemical kinetics (*65*). The linear dependence between the electric current and overvoltage in oxygen electroreduction in laccase presence remains in the range 1.2–1.18 V.

In the range of potentials 1.18–1.15 V, the slope of the polarization curve increases to the value $\partial E/\partial$ lgi ~0.03 V. In the region of the polarization curve 1.20–1.15 V, the reaction is the first order by oxygen and the pH dependence features the value $\partial E/\partial$ pH ~0.06 V. The calculated values of the stoichiometric number were close to unity. This makes possible the supposition that the decelerated step in oxygen electroreduction is repeated once.

Of great importance for elucidation of the mechanism of oxygen electroreduction are the experiments on the rotating ring-equipped disk electrode. This method affords one to record hydrogen peroxide, formed on the test electrode, by its oxidation in the ring, and thus, to establish if the test reaction runs via hydrogen peroxide production or not, as well as to calculate the rate constants for single steps of the summary process of oxygen reduction. It was shown (*65*) that oxygen electroreduction catalyzed by laccase runs without hydrogen peroxide formation as an intermediate product.

It is to be noted that laccase from *Coriolus versicolor* does not catalyze water decomposition at the potentials higher than 1.2 V. This is explicable by the fact that at higher potentials, laccase is reversibly inactivated as a result of overoxidation of catalytically important groups of the enzyme (*65*).

From experimental data of electrochemical kinetics as well as the data obtained on studying the kinetics of laccase action in solubilized state, the kinetic scheme was offered (*42,71,72*). Its distinctive feature is the presence of a slow step of synchronous transfer of two electrons, which is explicable by the fact that the laccase molecule has a two-electron acceptor, from which two electrons are quickly transferred to oxygen.

APPLICATION OF LACCASE IN ENZYME IMMUNOASSAY (EIA)

At present, EIA is used in various fields of biology, medicine, the food industry, and so forth (*73,74*). One of the most important advantages

of EIA is its high sensitivity, which is usually reached via employment of the conjugates of marker enzymes with ligands of various natures (antigens, antibodies, cofactors, biotin, protein A, and so on). Thus, the selection of the marker enzyme and the procedure of its conjugation with the ligand are the decisive factors for a successful performance of EIA. The range of enzymes used as markers in EIA is fairly wide (*75,76*). One of the most prevalent marker enzyme is, however, horseradiah peroxidase (HP) (*75–77*). Despite the obvious advantages of this enzyme, because of its catalytic and physicochemical properties, it has some drawbacks: a high background staining related to application of the enzymatic reaction of hydrogen peroxide, as the second substrate, and to the formation of an unproductive enzyme–substrate complex with hydrogen peroxide through the catalysis. Peroxidase has some other demerits quite fully described in the literature (*73,77*).

Skorobogatko et al. (*78,79*) offered for use as marker enzyme the laccase isolated from the cultural fluid of the basidial fungus *Coriolus hirsutus*. It was noted above that laccase (1) catalyzes the oxidation of polyphenols and polyamines as well as some inorganic ions by oxygen, and (2) by electron donor, has the substrate specificity fairly close to horseradish peroxidase. So, the values of K_M for laccase and of HP by a general series of chromogenic substrates are close: 0.18–0.39 and 0.2 m*M*, respectively (*76,77,80*). The K_M by oxygen for laccase from various fungi is $10^{-5}M$; because of this, the enzymatic reaction runs oxygen-saturated in the air. In addition, laccase is fairly stable.

The advantages of this enzyme as marker are:

1. The use of air oxygen as the second substrate, and as a consequence, no laccase inactivation through the reaction because of the absence of unproductive enzyme–substrate complexes;
2. A lower, compared to peroxidase, sensitivity for the content of metalic ions of variable valence in the medium; and
3. The employment of the same reagents and equipment in the immunoassay as in EIA based on peroxidase as marker enzyme.

The periodate method was used (*78*) to obtain the conjugates of laccase with various antibodies with 75–80% of the initial enzymatic activity retained during the synthesis of conjugates. Evidently, the oxidation of laccase carbohydrate moiety leads to no changes in the active site and, hence, to no loss in the enzyme activity.

The authors (*78*) presume that this affords the application of the periodate method for synthesis of immunolaccase conjugates with a high percentage of retained activity. The conjugate does not alter the immunological and enzymatic activity for a year on storage in 50% glycerol at −18°C. The authors demonstrated an application of immunolaccase conjugates in various modifications of the immunoassay ("sandwich," competitive, indirect EIA) to quantify insulin, and IgG in human and mice

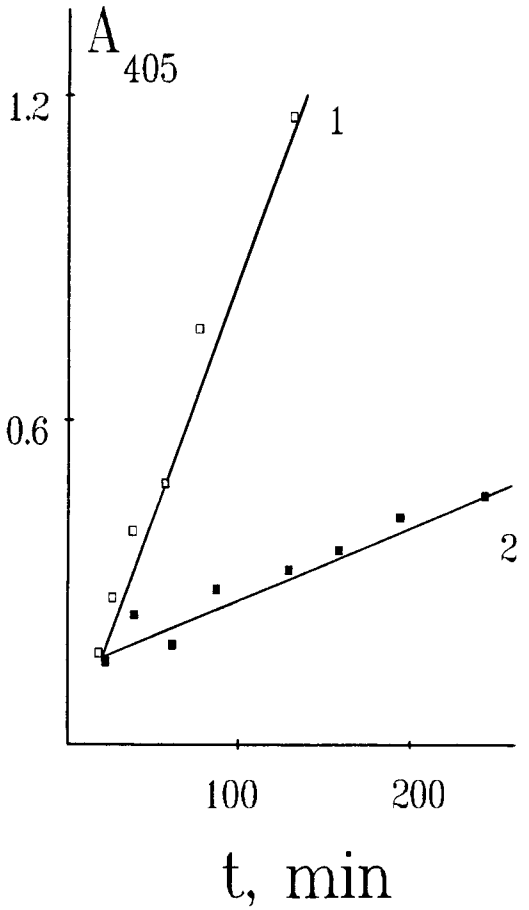

Fig. 3. Comparison of immunolaccase (1) and immunoperoxidase (2) conjugates.

serum. So, the "sandwich" method can detect 2.5*10−12*M* IgG of mice, which is indicative of a high sensitivity of immunolaccase conjugate.

Comparison of the properties of immunolaccase and immunoperoxidase conjugates showed that the absolute sensitivity of antibody–laccase conjugates was three times that for composition-analogous conjugates with peroxidase (Fig. 3). In addition, the authors showed a simpler modification of the immunoassay using laccase in place of peroxidase as a result of applying air oxygen as the second substrate of the enzymatic reaction.

Laccase was offered (*81*) to be used as enzymatic label for EIA based on homovanilinic acid (HVA); the product of oxidation of the latter by oxygen in the presence of laccase fluoresces at λ exc.= 295 nm and λ irr.= 495 nm. This approach sensitizes EIA, in particular, owing to the decrease in the background HVA oxidation by oxygen in the enzyme absence.

LACCASE-BASED BIOSENSORS

At present, to solve a wide range of tasks related to testing biologically active compounds, it is necessary to elaborate high-sensitivity assays that are simple, available, and automated. The devices meeting the above requirements have been given the general term of "biosensors." The functioning of a biosensor supposes a combination of biochemical and physicochemical interactions. The interaction of a biologically sensitive material with a test substance alters the system parameters transferred by a converter to relevant physical changers. A biochemical basis of sensors can be enzymes, antibodies, receptors, and cells. Physical converters can be optical, amperometric, field-effect transistors, piezoeffect, thermistor, and so on. The most promising principles of signal recording seem to be the first four. Each has some advantages on application with a definite biological object and in particular assay conditions. The changes in concentration of this or that compound by use of biochemical systems are finally converted into electric signals (*82*).

Laccase can be applied in biosensors of a few types. Their principles are described below.

ENZYMIC ELECTRODE TO ASSAY TEA CATECHOLS USING LACCASE

The enzymic electrode was elaborated to assay a number of phenolic compounds in tea on the basis of oxygen electrode and immobilized laccase (*83*). Oxidation of catechols by oxygen in presence of immobilized laccase results in altering the oxygen concentration recorded by the oxygen electrode.

A high-activity and stable immobilized laccase was obtained by use of a fibrous DEAE-cellulose pretreated with the Woodwordt reagent. Two types of the sensor were used: butch and flow-injection. The sensor affords the detection of tea leaf catechols at various steps of tea production and on the control of the final product. Efficiency: 20 assays/h; the assay time is 2–3 min; the measurement limits 0–1.5 mg/mL tannin. The immobilized enzyme affords not less than 500 assays and retains its activity for over 2 mo at room temperature. In principle, this type of laccase-based sensor can be also used to assay lignins and some phenols in waste waters, as well as phenolic compounds in wine.

POTENTIOMETRIC IMMUNOSENSOR BASED ON LACCASE

The capacity of laccase to catalyze the reactions of mediator-less electroreduction of oxygen and the possibility of conducting the laccase-assisted immunoassays underlied the elaboration of a new type of sensor

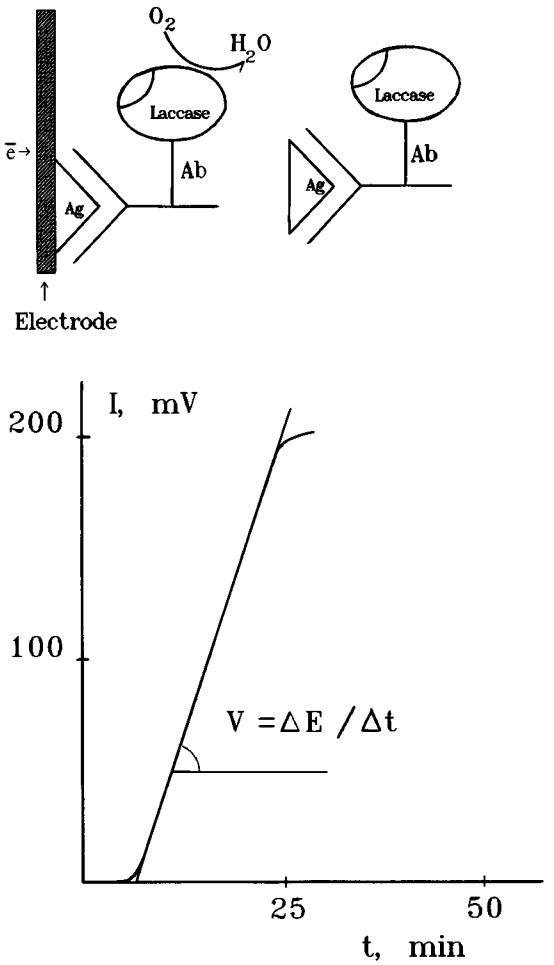

Fig. 4. The scheme of functioning of a potentiometric immunosensor with immobilized laccase.

(*84,85*). The mode of action of this sensor is shown in Fig. 4. The test antigen is crosslinked to the electrode of carbon material. Addition of laccase–antibodies conjugate solution into the reaction unit leads to binding the conjugate to antigen on the electrode surface. This results in a rapid growth of the potential on the electrode owing to the mediator-less catalysis of oxygen electroreduction by laccase. A preliminary addition of the test antigen into the reaction unit decelerates the growth of electrode potential because of the competition for binding free and immobilized antigens. Figure 5 illustrates a possibility of insulin assay by this method. The assay time is 20 min.

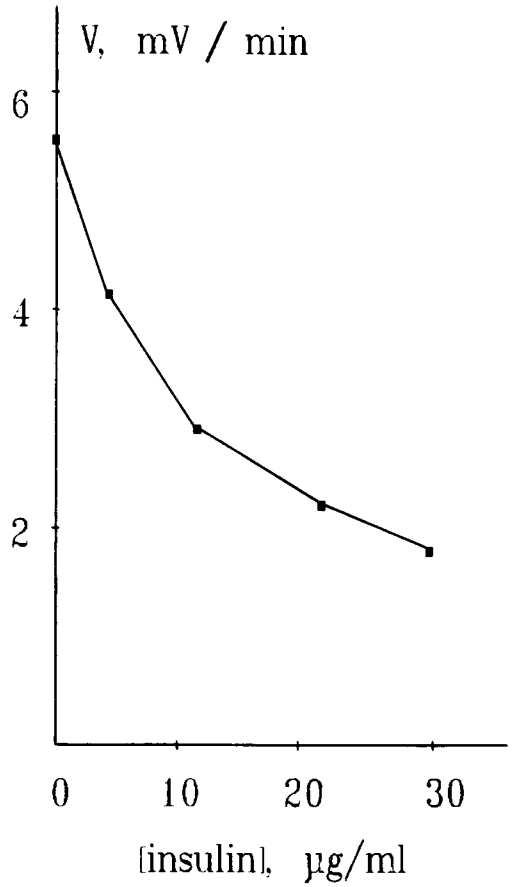

Fig. 5. Calibration curves for assaying insulin by potentiometric immunosensor.

LIGHT-ADDRESSABLE POTENTIOMETRIC SILICON SENSOR (LAPS)

The advantages of potentiometric sensors constructed by the silicon technology are congruent to the advantages of laccase as marker enzyme in immunoassay. It is possible to create the multisensors for a concurrent assay of many samples with the light-addressable signal. The operational principle of the multisensor is based on employment of the field effect in semiconductors to measure the photocapacity of semiconductor-dielectric-electrolyte structures (*86,87*). The photocapacity value of this structure is influenced by pH change in the solution near the surface or by the redox potential change in the solution when the dielectric is coated with the semiconductor. A silicon plate is coated with a thin layer of gold, which acts

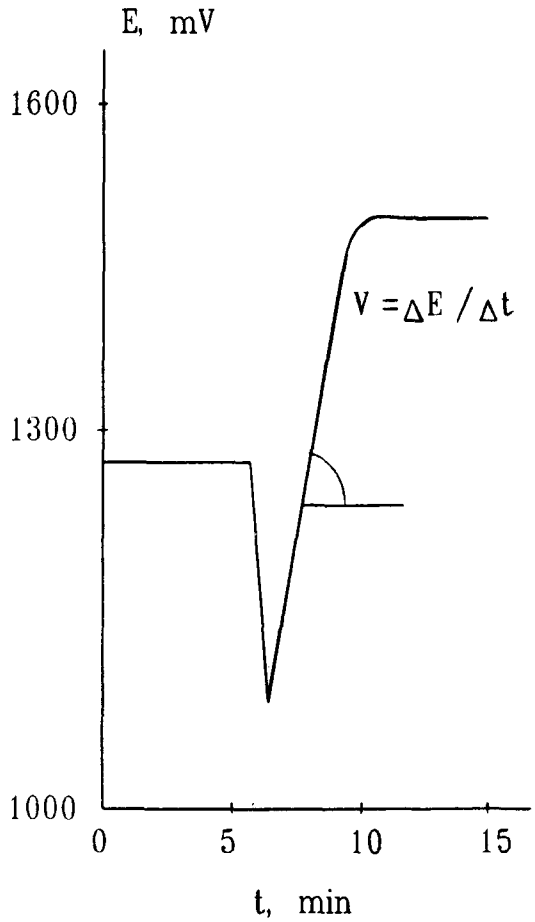

Fig. 6. Potentiometric light-addressable silicon sensor with immobilized laccase.

as a "working" electrode. A nitrocellulose filter with immobilized laccase is pressed to the "working" electrode. The working unit contains a reduced ferrocene solution. Its interaction with oxygen in laccase presence oxidizes ferrocene enzymatically with a shift in the potential of the solution recorded on the electrode (Fig. 6). Similar experiments were conducted without ferrocene when performing the mediator-less electrocatalysis.

APPLICATION OF LACCASE IN ORGANIC SYNTHESIS

The capacity of laccase to catalyze the oxidation of various compounds makes its application promising in organic synthesis. Two works (*88,89*) published the experiments on laccase-catalyzed oxidation of steroids (estradiol, estrone, estriol, and so on) and the related hormones in the mixture of water with organic solvents. The steroid hormones were shown to be oxidized by oxygen in the presence of laccase in the phenol group of the

A ring. The reaction was dependent on temperature, pH, laccase concentration, and the type of organic solvent used. Laccase retained the activity for a few days. The results afford the application of this biphasic system for conversion of large amounts of steroids in small volumes.

One of the known possible ways to protect the carboxyl group in peptide synthesis is the use of phenylhydrazide. The sphere of its application is, however, limited by the clearing process in rigid conditions according to the scheme:

$$-CO-NH-NH-C_6H_5- \xrightarrow{[0]} -CO-N=N-C_6H_5 \longrightarrow -CO-OH + N_2 + C_6H_6$$

A new enzymatic procedure using laccase was offered (*90*) to eliminate phenylhydrazide protection. The enzymatic clearing runs at neutral or close to neutral pH and at room temperature. The oxidizer is air oxygen. Tryptophan, tyrosine, and methionine, the amino acids most sensitive to oxidation, were shown not to be amenable to modification through enzymatic clearing. The clearing mechanism seemed also to include the step of oxidation to the high-activity, unstable phenyldiimide with its consequent self-decomposition. Laccase was also shown to be applicable for elimination of phenylhydrazide protection blocking the side carboxyls. The model dipeptide substrate Glu($NHNHC_6H_5$)—Gly—OMe was synthesized, in which γ-carboxyl of glutamic acid was shielded by phenylhydrazide. The clearing process catalyzed by laccase runs to completion for 3 h.

Another example of laccase application is the synthesis of vinblastine. Vinblastine is the best out of the present-day known cytostatic successfully applied in therapy of malignant diseases, especially leukemia. A major source of vinblastine at present is the plant *Catharanthus roseus*. The amount of vinblastine in it is very low. However, two structural fragments of its molecule, the alkaloids katarantine and vindoline, are contained in these plants in large amounts and are comparatively inexpensive. In view of this, a most urgent task is to develop the synthesis of vinblastine from katarantine and vindoline.

In recent years, a few purely chemical procedures were offered for vinblastine synthesis from its structural fragments. Some of these procedures give comparatively high yields of vinblastine. However, a notable drawback of the chemical synthesis is its cumbersomeness, high cost, and toxicity of the reagents.

A more promising method is the enzymatic synthesis of vinblastine, in which the covalent conjugate of katarantine with vindoline is produced enzymatically. We have effected the scheme of enzymatic-chemical synthesis of vinblastive from katarantine and vindoline in a yield of about 40% (Fig. 7). The preparations were identified by the thin layer chromatography and HPLC. At present, the works on optimization of synthesis conditions and on increase in the product yield are under way.

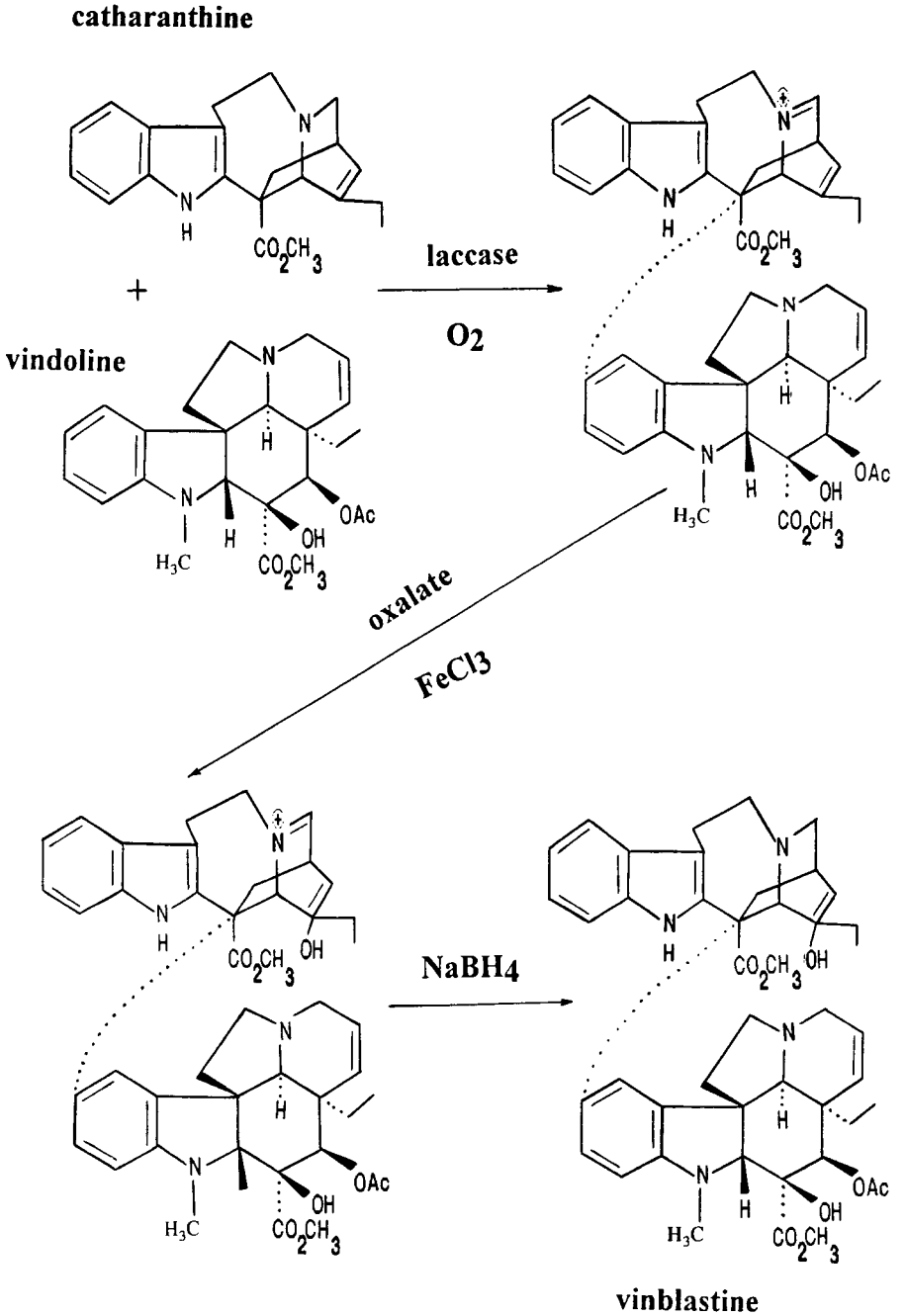

Fig. 7. The scheme of enzymatic chemical synthesis of vinblastine from katarantine and vindoline.

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