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An Immobilization of Extracellular Laccase to Humus-Iron Complex*1

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ABSTRACT

There are some evidence that active enzymatic proteins, *e.g.* fungal laccase, exist in the naturally occured soil humus. This study was performed to investigate the covalent binding of fungal laccase to the humic acid-iron complex, and to measure laccase activity of immobilized ones. Seven methods were adopted to form the covalent binding of fungal laccase with soil humic acids complexed with iron. Using these seven methods it was possible to change the dimension of spacer arm between laccase and support, and also to regulate the mode of covalent binding of this enzyme. The spacer arm was regulated from 2C to 11C. There was not observed any straight relationship between the spacer arm longitude and the laccase activity after immobilization, but the binding mode more effective than the former. Three out of the seven methods gave the high activity of immobilized laccase, and which active products of laccase immobilization was stable up to 10 days after the process. It is indicated that natural soil condition might be prevented the laccase activation by the toxic influence of some phenolic humic compounds. It was shown, for the first time, the possibilities to obtain the high activity of fungal laccase by binding to humic acids, and especially in complex with iron.

Keywords : Humic acid-iron complex, laccase, *Cerrena unicolor*, immobilization, humic acid, fungal laccase

1. INTRODUCTION

The humus in soil is a very complex product formed from decayed plant materials as roots, leaves, stems as well as including materials of animal origin (Haider *et al.*, 1975). It is a commonplace to say that humus is necessary in soil to ensure life reproduction. The chemical formula of humus is still unknown. What we know so far is that humus can be divided into humic acids and fulvic acids, with the differences between these two components depending solely on the physicochemical properties of these soil components.

All components of humus primarily consist of polymerized phenolic compounds, aminoacids (Trojanowski and Lobarzewski, 1961), peptides, and mono- and oligo-saccharides (Haider *et al.*,

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1975). Especially the phenolic compounds are very dangerous to enzymatic proteins (laccase) in nature and produced by microorganisms as well as fungi. Although there is some evidence that many active enzymes are present in the complex mixtures of soil, there are problems with extracting laccase from them (Leonowicz and Bollag, 1987). The naturally occurring humus in soil probably posseses the possibility to bind and stabilize the enzymatic proteins. It is possible that some fractions of humic acids are able to immobilize the active enzymes, *e.g.* fungal laccase, which activity was observed in the naturally occurred soils. But the extraction of laccase from the soil humus was very difficult (Leonowicz and Bollag, 1987).

As the fungal laccase is widely recognized as extracellular enzyme in wood rotting fungi, therefore, it is especially possible that this enzyme containing Cu could not only be adsorbed by the humus in soil, but also be immobilized to some fractions of humic acids. Therefore it would be worthwhile to obtain in model condition the active complex formation of fungal laccase and humic acid-iron fraction in laboratory condition. Until now, fungal laccase has been immobilized only on the usually used controlled porous glass (CPG) (Leonowicz *et al.*, 1988; Rogalski *et al.*, 1995; Rogalski *et al.*, 1991; Rogalski *et al.*, 1989) or on sandy loam or silt loam (Leonowicz and Bollag, 1987; Sarkar *et al.*, 1989; Lobarzewski and Leonowicz, 1989).

This study was performed to investigate the covalent binding of fungal laccase to the humic acid-iron complex, and to measure laccase activity of immobilized ones. Seven methods were adopted to immobilize the fungal laccase by covalent binding to soil humic acids complexed with iron. It was hoping that the results go some way towards imitating the natural conditions under which the active fungal laccase proteins exist in soil.

2. MATERIALS and METHODS

2.1 Humic substances and humic acid-iron complexes

A humic acid (HA) was extracted from a lignite (Leonardite) of North Dakota (USA) with 0.5 N NaOH by a standard procedure (Piccolo, 1988). The protonated HA obtained from the extraction, was further purified by a double precipitation and 0.5% HCl-HF (v/v) treatment as previously described (Piccolo, 1988), dialyzed against water, and freeze-dried. Elemental analysis and other chemical and physical-chemical characteristics for this HA extract were reported earlier (Piccolo *et al.*, 1992).

The HA was then suspended in distilled water, redissolved by adding a NaOH solution up to pH 7 and passed through a cation exchange resin Dowex 50X2-400. The HA total acidity was determined by titrating a 50 mL aliquot of the HA eluted solution to pH 9 with a 0.09 N NaOH solution using an Orion 960 Automatic Titrator. The structural features of the HA were assessed by a solution-state quantitative ^{13}C-NMR spectroscopy. Details regarding NMR acquisition procedures are reported elsewhere (Piccolo *et al.*, 1992). The automatic spectrum integration gave the following distribution of C intensities: aliphatic C (0～110 ppm): 29.8%; aromatic C (110～160 ppm): 55.7%; carboxylic C (160～190 ppm): 14.5%. The humic acid-iron (Fe-HA) complex was prepared according to well established procedures (Piccolo and Stevenson, 1982).

2.2 Fungal laccase preparation

Cerrena unicolor was the source of laccase (benzenediol:oxygen oxidoreductase, E.C. 1.10.3.2.). The *Cerrena unicolor* strain was

obtained from our culture collections. The mycelium of *Cerrena unicolor* was cultivated on the Lindeberg and Holm (1952) liquid medium in the submerged condition for 10 days at 28°C. The filtrate was desalted in the Sephadex G-25 (9×25 cm) and the laccase containing fractions were lyophilized. The lyophilized laccase was used after dissolving in the acetate buffer for the immobilization procedure.

2.3 Laccase assays

The laccase activity of lyophilized and immobilized enzymes was determined spectrophotometrically using syringaldazine as a substrate (Leonowicz and Grzywnowicz 1981).

2.4 Protein determination

Protein determination was performed by Schacterle and Pollack method(1973).

2.5 Laccase immobilization methods

The preparation of laccase was dissoved in 0.05 M acetate buffer (the specific activity 172.4 nkat/mg of protein) and used for immobilization on the humic acid-iron complex by following seven methods:

I) Five g of the humic acid-iron complex was activated with 20 mL of 2% aminopropyltriethoxysilane (APTES) (Pierce Chemicals Co, USA) in acetone for 24 hours. After removing acetone solvent at 45°C, the dry product was activated further by difunctional 5% glutardialdehyde for 12 hours at 22°C. Not reacted glutardialdehyde was removed by filtration. The product was washed with 0.05 M acetate buffer of pH 5.0. According to these procedures the humic acid-iron complex support posseses free aldehyde groups which are able to bind the free NH_2 groups of laccase enzyme. This immobilization reaction was done by mixing 30 mg of laccase enzyme per 1 g of activated support (Lappi *et al.*, 1976).

II) Five g of the humic acid-iron complex was mixed with 10 mL mixture of 10% acrylamide (Merck, Germany), 10% bis-acrylamide (Fluka, Switzerland), 2 microliters N, N',N',N'-tetramethylethylenediamine (TEMED) (Fluka, Switzerland) and 0.01% $(NH_4)S_2O_8$, and left for 24 hours. The reaction product is the polyacrylamide adhesively coated support which has active groups to be reacted to NH_2 groups of the enzyme. After filtering and drying at 30°C, the support was activated further by glutardialdehyde (Merck, Germany) as described in method I, and then the laccase was immobilized to this support by its free NH_2 groups (Lappi *et al.*, 1976).

III) Five g of the humic acid-iron complex was mixed with 20 mL of 2.5% carbodiimide (1-ethyl-3-[3-Dimethylaminopropyl] carbodiimide hydrochloride) (Pierce, USA) in water, and left for 12 hours for its immobilization. After this step the support has the free amino groups on its surface which could be bound to the free COOH groups of laccase. The excess carbodiimide was removed from the support by filtration. Then laccase was immobilized to the activated support as described in method I (Lappi *et al.*, 1976).

IV) Five g of the humic acid-iron complex was activated by two ways: a) using APTES and then carbodiimide as described in method I (Lappi *et al.*, 1976). In this step the support obtained free NH_2 groups. b) After the above activation the carbohydrate rings present in the support were opened by 20 mL of 6% of $NaIO_4$ for 12 hours reaction. The excess of $NaIO_4$ was filtered off, and the supports were washed twice with water (Zaborsky *et al.*, 1974). The wet activated supports were used for laccase

immobilization by its free NH_2 to aldehyde groups formed after $NaIO_4$ treatment. The other laccase immobilization would be possible to carbodiimide active centers by its free COOH groups after these activation method .

V) Five g of the humic acid-iron complex was activated one time using APTES, and then with glutardialdehydes as described in metho I. Also the saccharide groups of humic acids were activated by $NaIO_4$ as described in method IV. After these activation the humic acids were able to bound laccase by its free NH_2 to aldehyde groups of glutardialdehyde or to the aldehyde groups present in the saccharide components of humic acids.

VI) Five g of humic acid-iron complex was activated two times using APTES and glutar-dialdehyde as described in method I. Also the saccharide groups of humic acids were activated by $NaIO_4$ as described on method IV. After this activation the laccase was immobilized by its free NH_2 to two active centers of the suppport as in method V.

VII) Five g of humic acid-iron complex was activated by carbodiimide as in method III, and also by $NaIO_4$ as in method IV. After this activation the supports posses the possibilities for binding laccase by its free NH_2 groups. The supports were able to link laccase having active aldehyde groups in the saccharide components of support or NH_2 groups from carbodiimide bound to the matrix.

3. RESULTS and DISCUSSION

Enzyme immobilization to the insoluble supports is possible by adsorption, inside the pores and on the surface of support or by covalent bonds. The covalent bonds are more stable than the others. As covalent bonds are formed in very mild alkaline (pH 8.5) condition, it would be possible to be the right environment for this covalent linkage between the enzymes and humic acids in soil. In general the covalent binding between the matrix and enzyme protein depends on the presence on both surfaces of special groups which could link one to the other easily. It means that applying the above procedure it is possible to use NH_2 groups, COOH groups or aldehyde groups in the support and enzyme. The NH_2 or COOH groups in the proteins are present when these proteins contain alkaline or acidic aminoacids. The hydroxyl groups are always present in tyrosine in protein. Free aldehyde groups can be formed by using $NaIO_4$ as an oxidant in humic acids containing saccharide components (Haider *et al.*, 1975). The free aldehyde groups are capable of binding tightly with NH_2 groups of the enzymes, *e.g.* laccase.

Concerned to the active covalent binding between the humic acid-iron complex and laccase protein, it takes only the application of proper method of binding to obtain the active enzyme after the immobilization process. In the immobilization procedure of enzyme protein to the support, it is not only important to have special active group, but also spacer arm. This spacer arm could regulate the distance between the large beads of the support and comparably small dimension of the protein. For these reasons seven procedures were used for the laccase immobilization to the humic acid support. The spacer arm was regulated from 2C to 11C.

After all three activation agents for humic acid-iron were used; silanization, oxidation and spacer arm introduction by carbodiimide. First, silanization refers an introduction of free NH_2 group on the surface of the support. Second, oxidation with $NaIO_4$ is able to selectively cleave between C_2 and C_3 positions of the saccharide rings and produces dialdehyde saccharides. Third, the spacer arm with

carbodiimide allows to bind one side free NH_2 groups to the other free COOH groups. In these procedures there was not observed any straight relationship between the spacer arm longitude and the laccase activity after immobilization (Table 1 and Table 2). The binding mode of laccase to the support may be more effective than the spacer arm longitude (compared to Table 1 and 2).

There are two possibilities of laccase binding by its free NH_2 groups, one to glutardialdehyde or carbodiimide, the others to aldehyde groups in the saccharide components of the support. As shown in Table 2, the immobilized laccase was active, and this activity was more stable. In this case, the binding process is quite beside of our control in each mode of immobilization of proteins. It is not clear which active free groups

Table 1. Results of laccase (*Cerrena unicolor*) immobilization on the humic acid-iron complex.

Procedure	The mode of laccase immobilization and spacer arm longitude	Immobilized protein (mg/g humic acid)	Protein bound (%)	Laccase activity
I	laccase immobilized by NH_2 groups 8C spacer arm	1.24	12.40	0
II	laccase immobilized by NH_2 groups 5C spacer arm	0.22	0.90	0
III	laccase immobilized by COOH groups 2C spacer arm	1.78	12.70	0
IV	laccase immobilized by COOH or NH_2 groups 5C spacer arm	0.37	7.95	0

Table 2. Results of laccase (*Cerrena unicolor*) immobilization on the humic acid-iron complex.

Procedure	The mode of laccase immobilization and spacer arm longitude	Immobilized protein (mg/g)	Immobilized protein (%)	Time (days)	Laccase activity (nkat/l/ g wet support)	Laccase activity changes (%)
V	activation + 1 time silanization + $NaIO_4$ + glutaraldehyde + laccase immobilized by NH_2 groups 8C spacer arm	0.70	12.5	0	1846.1	100
				2	1481.1	80
				4	128.2	41
				8	58.4	19
				10	0.0	0
VI	humic acid-iron complex activation + 2 times silanization + $NaIO_4$ + glutaraldehyde + laccase immobilized by NH_2 groups 8C spacer arm	1.83	22.4	0	9743.2	100
				2	7948.4	80
				4	6153.6	63
				6	2307.6	24
				10	1384.6	14
				16	0.0	0
VII	humic acid-iron complex activation + carbodiimide + $NaIO_4$ + laccase immobilized by NH_2 groups 2C spacer arm	1.24	32.0	0	5128.0	100
				2	5640.8	110
				4	5640.8	110
				6	2820.4	50
				10	0.0	0

of the protein and supports are used for covalent binding. Generally the immobilization process stabilizes the activity of enzymatic proteins. But in the case of method I to IV, preparations (Table 1) of immobilized laccase loss their activities at once. Also, laccase alone, when mixed with humic acid-iron, decreases its activity over the time. In these experiments laccase losses its activity after a few hours. The same results were obtained in four out of seven experiments (Table 1). The humic material may have a poisonous effect on the soluble, very labile structure of proteins.

In further experiments presented in Table 2, the sequence of activation steps of humic acid-iron complex was changed. In method 5 (Table 2) the humic acid-iron complex was silanized once, while in method 6 it was silanized twice (Table 2). By twice silanization the higher stability of laccase activity was obtained with more protein being bound following the immobilization. After silanization of humic acids (either once or twice) we attempted to modify some components of this material using $NaIO_4$ as an oxidative agent. This oxidation gave the positive results in terms of laccase immobilization and high laccase activities. This result would be derived due to partly blocking some groups which are poisoning to the enzyme protein.

In addition, the use of carbodiimide and $NaIO_4$ oxidation (method VII), resulted in interesting a stable rate of activit
y in a laccase-humic acid-iron complex. The rate of laccase protein immobilization was up to 32%, and the enzymatic activity remained stable for up to 6 days. The method VI showed 22.4% of immobilized protein and 10 days stabilized laccase activity. The only 10 days lasting active preparations of immobilized laccase were not surprising effect. As indicated before, the support (humic acid-iron complex) for laccase immobilization might be very dangerous for labile laccase protein. That is why we decided to take some special procedure to decrease the extent of the harmful effect. Therefore it is necessary to find out the adequate sequence of humic acid activation. The positive results of laccase immobilization as in Table 2 gave us some indication as to the mode of laccase binding with humic acids in soil. The oxidation with $NaIO_4$ is able to selectively cleave between C_2 and C_3 positions of the saccharide rings and produces dialdehyde saccharides. It would be believed that the activation of the support may have had the oxidative action on the components of humic acid, thus decreasing the toxic effect of humic acids on laccase.

The earlier results of fungal laccase immobilization (Leonowicz *et al.* 1988; Lobarzewski and Leonowicz 1989; Rogalski *et al.* 1989 and 1995; Sarkar *et al.* 1989) cannot be compared to present results. It is because this experiment was performed by using humic acids isolated from soil, which are then chelated with iron. This complex, as we indicated above, is a highly complex polymer containing saccharides, aminoacids (aliphatic carbon, 29%), phenolic compounds (aromatic carbon, 55%) and iron (8%) (Piccolo *et al.* 1995). The humic acid-iron complex is highly dissociated, thus originating in many positive and negative charges. These charges could have a considerable impact on the laccase activity. This effect was observed easily in the first experiment after mixing soluble laccase with the humic acid-iron complex and also after the immobilization of laccase as seen in Table 1. During all these experiments it was observed clearly that the sequence of the support activation process in laccase immobilization was the most important factor in obtaining an active immobilization product.

4. CONCLUSION

The humus, as an aromatic complex, is a natural substrate for the laccase enzyme, so inhibition by its excess can be expected, and an iron inhibits the activity of laccase. It is well known that laccase acts in soil, where the enzyme could be probably joined to humic acid-iron fractions in nature. In this study three activation agents for humic acid-iron were used; silanization, oxidation and spacer arm introduction by carbodiimide. The sequence of the support activation process in laccase immobilization was the most important factor in obtaining an active immobilization product. Based on results of this experiment, the laccase activity was shown in the immobilized laccase on humic acid-iron complex for the first time. In addition, the possibility of such laccase-humus active complexes could be presented in the natural condition in the soil.

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