CHAPTER I

INTRODUCTION

Natural polymers have been playing many important roles for man's wellbeing long before synthetic polymers come into existence. Man knows how to use plant fibers for making clothes, ropes and paper since the ancient time long before nylon or polyester were synthesized. The advancement of science and technology help guiding us to the better use of natural polymers. Many modified natural polymers are now being used in wide variety of fields, ranging from medicine to agriculture and to general industries. We have seen the use of carboxypropylmethyl-cellulose as a high viscosity solution for the viscosurgery such as during the operation on eyes [1]. We know about the use of a number of natural polymers in the controlled-released of drug [2]. Modified starch with high water-retention capacity has promising potential for agriculture [3,4]. Proteins from plants and animals have been used in the process of microencapsulation of feeds for larvae of aquatic animals [5] and to microencapsulate drugs in pharmaceutical industry [6]. In the microencapsulation for larval feeds the polypeptides were cross-linked by an agent to form a membrane encapsulate all the feed ingredient within so that valuable components are retained undissolved in water. Considering another polymer, cellulose we witnessed the use of cellulose acetate in many industries, ranging from making glue, to producing thin sheets such as film strips and other products. The

list of the applications of natural polymers and their modified version is getting longer and longer as time goes by.

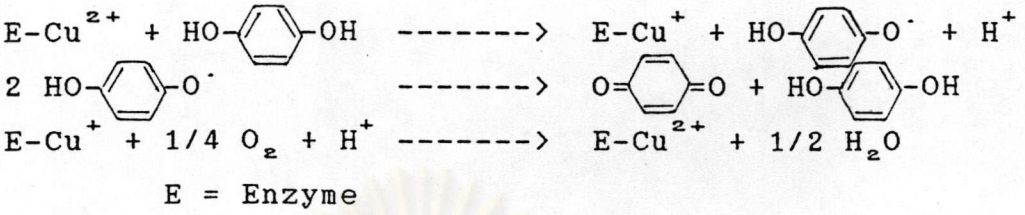
One natural polymer having promising potential for the development of local industries in Thailand is the polymer from the lac tree or lacquer tree. The making of lacquerwares has been practising in the northern part of Thailand since Sukothai period [7]. However the technique has been passing on from generation to generation with little modifications. It is hope that if we study the formation of lacquer from lac tree within the perspective of science we may obtain valuable information that will eventually benefit the industry. It is interesting to note that in the process of oxidation and polymerization of latex into lacquer the reaction require the presence of an enzyme, laccase, as the catalyst. We feel that more is still to be learnt about laccase either isolated from the lac tree and from other sources. Also studying the enzyme we believe that lots will be gained in the future in terms of the application to lacquerware industry and others. This thesis will, therefore, focus on the study of this biocatalyst with a special emphasis on the immobilization of laccase on polymer supports.

1.1 Reactions catalysed by laccase

Laccase (p-diphenol : oxygen oxidoreductase, EC.1.10.3.2) is an enzyme which catalyses the oxidation-reduction of a spectrum of substrates. Those substrates being oxidised by the action of laccase are, for example, syringaldazine, o-dianisidin, diphenol, catechol and catechol derivatives [8,9,10,11,12]. The oxidation of these substrates always accompanied by the reduction of O_2 to

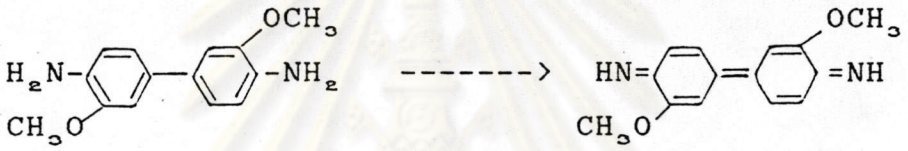
H_2O, hence the term oxidoreductase. Some of the reactions catalysed by laccase are shown in Figure 1.1. Laccase was shown to contain 4 atoms of copper per molecule of the enzyme. The atoms of copper play an essential role in the oxido-reduction process. Yoshida [12] proposed that in the process of oxidation of hydroquinone by *Rhus vernicifera* laccase, three steps could be visualized (Figure 1.1). Firstly, there is a transfer of an electron from hydroquinone to the laccase-Cu^{2+} (E-Cu^{2+}) resulted in the formation of the semiquinone radical and laccase-Cu^{1+} (E-Cu^{1+}). Secondly, the semiquinone radical dismutates into p-benzoquinone and hydroquinone. Thirdly, there is the reduction of O_2 into H_2O accompanied by the conversion of laccase-Cu^{1+} (E-Cu^{1+}) back to laccase-Cu^{2+} (E-Cu^{2+}). the enzyme is then ready to go through yet another cycle of oxido-reduction, converting more hydroquinone to quinone with a concomitant consumption of O_2. Reaction with o-dianisidin, catechol, and syringaldazine showed only the conversion of the substrates to their respective oxidised product. The assay of laccase activity, therefore, can be done by either following the conversion of hydroquinone to quinone or measuring the consumption of O_2. Pending on the availability of equipments a few workers[13,14,15] used the measurement of O_2 consumption as the method of assaying laccase. Once a substrate that produces coloured product is, available it becomes a convenient method to follow the formation of the product spectrophotometrically. Harkin & Obst [16] suggested that syringaldazine is an effective reagent for detecting laccase since the product of the oxidation is red-pink and absorbs light maximally at 530 nm. We adopted this method

Reaction of Hydroquinone



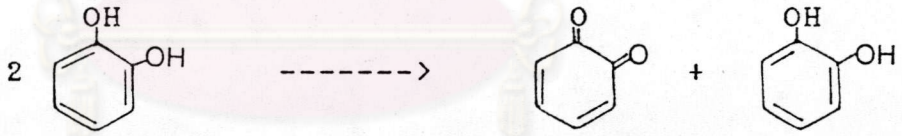
E = Enzyme

Reaction of O-dianisidin

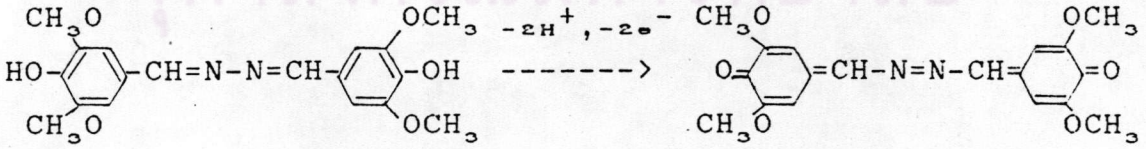


(Orange-brown)

Reaction of Catechol



Reaction of Syringaldazine



(Red-pink)

FIGURE 1.1: Example of reactions catalysed by laccase.

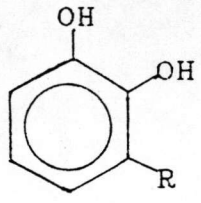
in the assay of laccase throughout this study.

1.2 Sources and roles of laccase in nature

Reports on the existence of laccase up till now have been confined to two groups of living organisms, namely, the higher plants and fungi. Laccase is sometimes referred to as the "blue oxidase" due to its blue colour in solution [17]. Even though laccase from plants and fungi do have many common features the enzyme seems to serve different functions depends on the needs of the organisms. We shall now consider laccase of plants and fungi in more details.

1.2.1 laccase from plants

The study of laccase started first with lacquer trees (for reviews see Mayer [8] and Mayer & Hartel [9]). The lacquer tree, first studies, was the Japanese lacquer tree, *Rhus vernicifera*. Yoshida [12] suggested that the presence of laccase could contribute to the drying of lacquer. The sap of lacquer trees is a latex containing about 20-25 % of water and a mixture of 3-substituted pyrocatechol derivatives as a major component [18]. The structure of pyrocatechol derivatives are shown below.

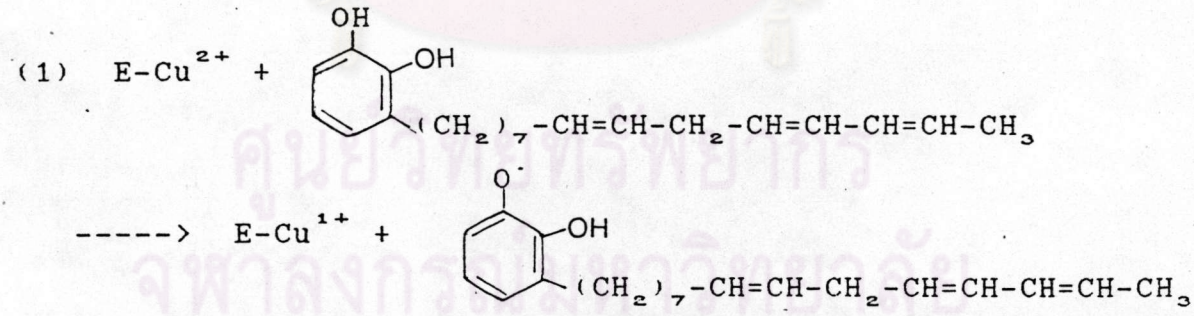


3-substituted pyrocatechol derivatives

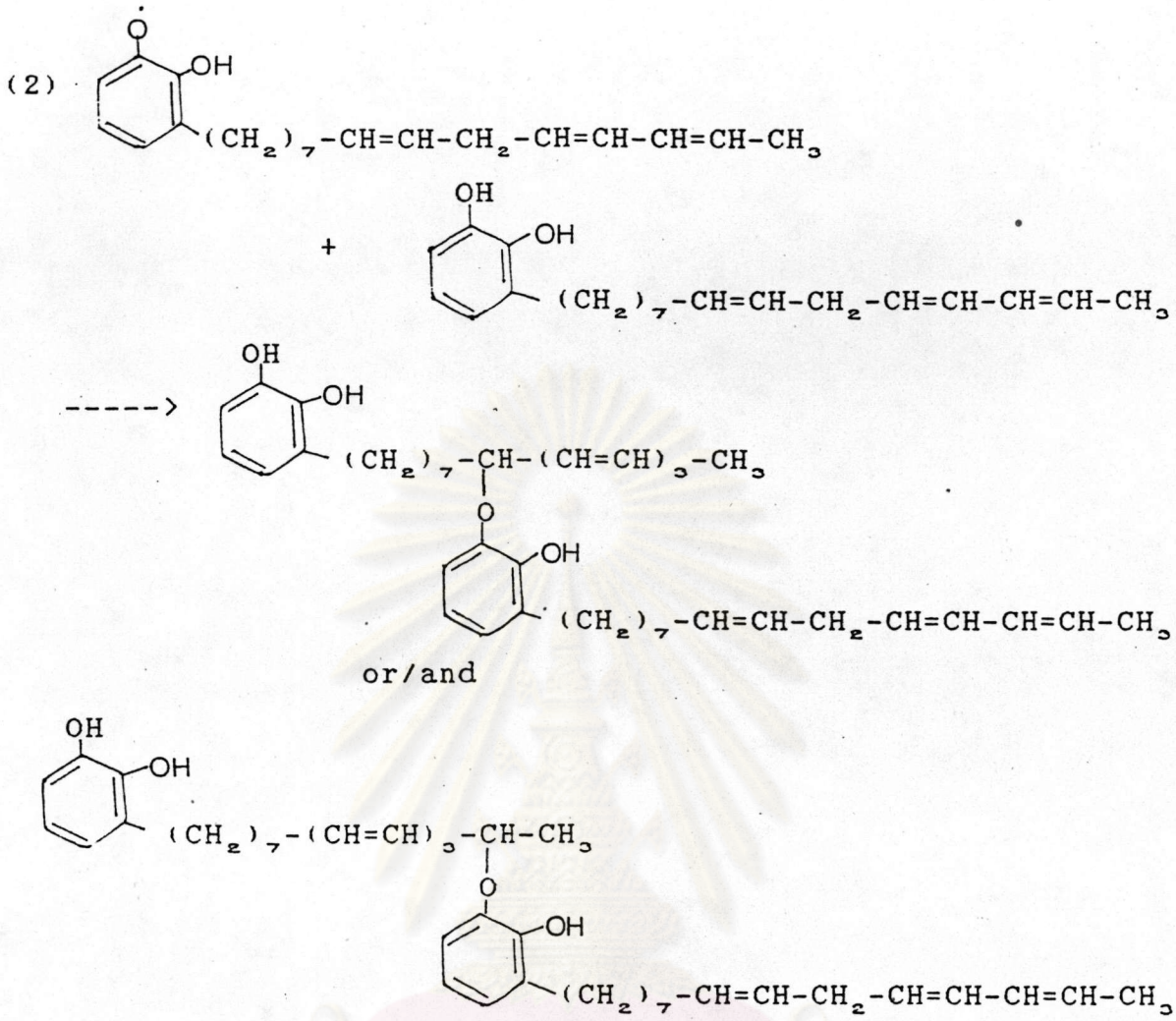
1	R
a	$(CH_2)_{14}-CH_3$
b	$(CH_2)_7CH=CH-(CH_2)_5-CH_3$
c	$(CH_2)_7-CH=CH-CH_2CH=CH(CH_2)_2CH_3$
d	$(CH_2)_7-CH=CH-CH_2-CH=CH-CH=CH-CH_3$

Laccase catalysed the oxidation of these catechols. Subsequent polymerization of these catechols [11] resulted in a black, highly viscous solution used in the making of lacquerwares.

The process of polymerization is thought to occur as shown below



(2)



or/and

The action of laccase resulted in the formation of pyrocatechol derivative free radicals. The free radicals can, in tern, attack the double bond of R-group resulted in the formation of a dimer. Subsequent attack by another free radical resulted in the formation of a trimer, and so on. At the end a polymer was obtained. Laccase contributed to the drying of lacquer through the above process.

Studies of lacquer trees from other regions of asia, namely, *Rhus succedanea* from Vietnam, *Melanorrhoea usitata* and *Melanorrhoea laccifera*

from Thailand, Burma, Laos and Cambodia revealed similar compositions [19,20] as shown in Table 1 and Table 2, respectively.

Some variations in the nature of pyrocatechol derivatives and the laccase content should be noted, however. The lacquer tree of Thailand, the *Melanorrhoea usitata*, contains gummic substance having the major component as thitsiol, while the *Rhus vernicifera* and *Rhus succedanea* contain urushiol and laccol, respectively (Table 1). The content of gummic substances is the highest in *Melanorrhoea usitata* together with its long C_{17} carbon chain attached to the pyrocatechol derivative make the Thai (or Burma) finished lacquer strongest and most durable of all (Table 1). The content of laccase (Table 2), on the other hand, is the lowest in *M. usitata* making the drying process longer than others [21,22]. Attempts had been made to mix the sap from the Japanese lacquer tree with the sap from the Thai lacquer tree and found that the mixture required shorter time for drying. Presumably the high content of laccase from the Japanese sap help enhancing the drying process.

Our preliminary survey indicated that laccase has been playing an indirect role for the production of lacquerwares in our country more than 1,000 years ago since the Sukothai period. The lacquerwares (Figure 1.2) are real national and cultural treasures. The lacquerware industry is now becoming and important industry in the northern part of Thailand. The demand for Thai Lacquerwares in overseas markets is so high that the industry cannot produce enough to meet the order recieve from overseas distributors. (Veeranan Neeldanuwong,

personal communication [21]). The Center for Industrial Promotion of the Northern Region (Ministry of Industry) has a section looking into ways to improve this situation. We hope that our research concerning laccase will one day pay a role in promoting the progress of this industry.

TABLE 1.1: Compositions of catechol derivatives in sap from different types of lacquer trees. [20]

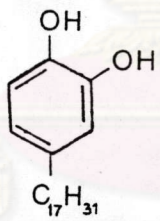
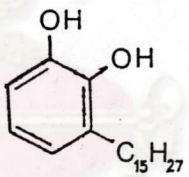
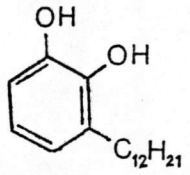
Substance	*Melanorrhoea usitata*	*Rhus vernicifera*	*Rhus succedanea*
	Thitsiol	Urushiol	Laccol
Formular	$C_{23}H_{36}O_2$	$C_{21}H_{32}O_2$	$C_{18}H_{28}O_2$
Molecular weight	344	316	274
Structural formular of Diphenol benzene			

TABLE 1.2: Overall compositions of sap from different types of lacquer trees showing the compositions of gummic substance, water and laccase plus other unidentified components. [20]

Lacquer tree	Composition (Percent %)		
	Gummic substance	Water	Laccase & Others
Melanorrhoea usitata	87	8.1	4.9
Rhus vernicifera	70	19.5	10.5
Rhus succedanea (1)	33.07	43.57	22.88
(2)	52.0	29.0	19.0

FIGURE 1.2: Example of lacquerwares produced at the Center of Industrial Promotion of the Northern Region of Thailand. The products are in great demand overseas and have brought substantial income to the country.

The process of preparing Yang Rak (lacquer) ready to be used in the making of lacquerwares is quite tedious and requires patience. If we want to make the lacquer ourselves we have to identify the lac trees first and obtained sap from them. Lac tree commonly grown in the north is the *Melanorrhoea usitata* as shown in Figure 1.3. To obtained a quantity of sap one has to make a deep cut through a relatively thick bark of the trunk. As a rough estimate, if one wants about 100 ml. of the sap from one cut one may have to wait for a week or more.

The process of making the black lacquer (Figure 1.4) from sap has changed very little our the time. The initial step involves the stirring of sap in good contact with air (to get O_2) at 30-40 °C for several hours. In this process evaporation of water occures and the oxidation and polymerization takes place leading to lacquer with approximately 3 % (w/w) water. The lacquer will have an appropriate viscosity for use in the production of lacquerwares. The production of a typical lacquerware involves a number of steps as illustrated in Figure 1.5.

Apart from laccase from the above mentioned lacquer trees another plant the sycamore tree (*Acer pseudoplatanus L*) was reported also to contained the blue laccase. Cells of this species were grown in culture and found to excrete large amount of laccase into the culture medium [23,24,25] . The enzyme was also purified by this group of workers after culturing a large quantity of cells in 20 litre batch cultures [14]. It is also interesting in the biosynthetic point of view to note that sycamore cells, cultured under copper-deprived conditions, excreted the laccase apoprotein (laccase without copper) at the same rate as the sycamore cells;

FIGURE 1.3: Lacquer tree, *Melanorrhoe usitata*, grown in Chiangmai. Top is the features of leaves and flowers (that bloom only once a year in December). Bottom is the trunk showing thick bark. Note the previous injury on the trunk that resulted in the secretion of sap when upon contacted with O_2 was oxidised to black lacquer.

FIGURE 1.4: Appearance of the Yang Rak after the action of laccase. The lacquer in this case ready to be used.

FIGURE 1.5: Steps involved in the production of a lacquer-ware using the lacquer from *M. usitata*.

cultured under copper-supplied conditions, excreted the active copper containing laccase. When the culture medium was initially supplied with limiting amounts of copper, the active laccase was first excreted until all copper atoms were used up. Then the laccase apoprotein was excreted, thereafter [24].

1.2.2 Laccase from fungi

The study of fungal laccase has centered mostly around wood-destroying white-rot fungi and mushrooms [8,9]. It was suggested that laccase in this case play a role in lignin degradation and/or detoxification of lignin degradation products [26,27]. It is worth noting that fungi secreted laccase to function outside the cells [28,29] similar to other microorganisms that secreted an array of extracellular enzymes, such as proteases, lipases, amylases, lactases etc. to function outside their cells [30]. The ultimate aim is, of course, to catalyse various reactions for the benefit of their growths. Man has learnt how to exploit these extracellular enzymes for a number of years. Many of them have been produced in industrial scales and have been used in wide variety of industries. It is anticipated that laccase will also find its prominent position in the future.

1.3 Properties of laccase

One prominent feauture of laccase is that it is a glycoprotein. The amount of sugar reported in lacquer trees ranged from 32-45 % . The percentage of sugar in laccase isolated from fungi is lower, however, being 11 % in *Neusospora* and 14 % in *Polyporus*, respectively [31]. The higher the amount of carbohydrate in a protein the more stable that protein will be.

It is not surprising, therefore, to observe that latex from lacquer trees can be kept for a long period of time before the drying process is started and laccase still function well [32].

Considering the nature of protein itself it was reported that laccase from lacquer trees existed as a single polypeptide chain having a molecular weight of about 120,000 in the case of *Rhus verniciferus* [11] and 97,000 in the case of *Acer pseudoplatanus* cell [14]. Laccase in fungi can exist in more than one form. Von Hunolstein et al (1986) [33] reported that the white-rot fungi, *Trametes versicolor*, synthesized two forms of laccase designated as A and B. The amount of A was 9 times greater than the amount of B. De Vries at al (1986) [28] reported that the basidomycete, *Schizophyllum commune* 's laccase consisted of 3 forms as separated by DEAE-Sephacel chromatography. The molecular weights of fungal laccases vary from one organism to another. For example the M.W. of the *polypolus* 's laccase is around 60,000-65,000 while that of *Aspergillus sp.* is about 117,000.

Perhaps one of the most interesting features of laccase is that at high protein concentration the solution appears blue by the naked eyes. The enzyme is, therefore, sometimes referred to as "blue oxidase". The blue colour is due to the complex between the cofactor, the copper atoms, and the enzyme molecule. Laccase from all sources described was shown to contain 4 gram-atoms of copper per molecule of enzyme [34]. Many copper oxidases and proteins exist in nature as shown in Table 3. It is interesting to note that the content of copper varies from 1 to 8 atoms per molecule of protein. Some blue protein such as

azurin is useful as a marker in experiments deal with isoelectric focusing of proteins on polyacrylamide gel. The blue colour makes it possible to follow the migration of the protein.

The interaction of copper to the polypeptide chain in laccase render a unique property to the enzyme. All purified laccases when scanned for their maximum absorption in the visible range using a spectrophotometer showed a characteristic peak at around 600 nm. The role of Cu in the transfer of electron has aroused great interests among chemists, especially the inorganic chemists and the physical chemists. The use of Electron Paramagnetic Resonance (EPR) technique together with other techniques resulted in the assignment of all 4 Cu atoms as type I, type II and type III. One Cu atom each as type I and type II while another two Cu atoms are type III [9,17,34]. The nature of interactions is complex and is not a major concern to us at this stage.

1.4 Immobilization of laccase

To our knowledge only one article exists reporting the immobilization of laccase. Wollenberger et al (1986) [35] experimented on the coimmobilization of laccase with glucose oxidase in a gelatin membrane placed over a modified oxygen electrode [36]. The electrode with immobilized enzymes is used in the determination of glucose concentration. The immobilization of glucose oxidase also is well known and has been used in real applications for a number of years [37,38]. The system, however, suffers from interfering reductive substances such as ascorbate which is generally-present in biological fluids. The coimmobilization of laccase helped to eliminate

Protein and source	Molecular weight	Copper content (gram-atoms/mole)	Activity or function
Azurin (*Pseudomonas, Bordetella*)	16,000	1	"Blue" electron carriers: non-autooxidizable
Stellacyanin (lacquer tree)	20,000	1	
Plastocyanin (chloroplasts)	21,000	2	
Tyrosinase (*Neurospora*)	33,000	1	Oxygenases
Dopamine-β-hydroxylase (adrenal glands)	290,000	4-7	
Laccase (lacquer tree, fungi)	64,000 110,000	4	"Blue" oxidases: catalyze the reduction of O_2 to H_2O
Ascorbate oxidase (cucumber, squash)	130,000	8	
Ceruloplasmin (animal serum)[a]	160,000	8	
Benzylamine oxidase (pig plasma)	190,000	2	"Non-blue" oxidases: catalyze the reduction of O_2 to H_2O_2
Diamine oxidase (pig kidney)	190,000	2	
Galactose oxidase (fungi)	43,000	1	
Uricase (liver)	120,000	1	
Cytochrome *c* oxidase (mitochondria)[b]	~100,000 (monomer)	1	Terminal oxidase

[a]Has weak oxidase activity but unknown physiological function.
[b]Contains 1 mole of heme iron per 100,000 as well as copper.

TABLE 1.3: List of some copper-containing proteins. Sources of these proteins vary from bacteria to fungi to plants and to animals. Some are blue in colour some are non-blue [34].

interfering reductants.

The immobilization of laccase and glucose oxidase as outlined above is generally known as the entrapment method. In fact other methods for the immobilization of enzymes exist as illustrated in Figure 1.6. We shall discuss these methods briefly.

Methods for the immobilization of enzymes are generally divided into 3 types. The first one called "carrier-binding" involves the attachment of soluble enzymes to the water-insoluble carriers. The attachment can be achieved through (i) physical adsorption, such as the adsorption of enzymes to activated carbon, concanavalin A, alumina, silica gel, or kaolinite (ii) ionic binding, such as the ionic binding of enzymes to various anion exchangers or cation exchangers, and (iii) covalent binding, generally uses linking agents such as glutaraldehyde, epichlorohydrin, and hexamethylene diisocyanate to form covalent bonds with carriers and enzymes. In this respect one side of the linker molecule form a covalent bond with the carrier and another side of the molecule form another covalent bond with the enzyme. This method, if successful, results in a very stable immobilized system.

The second method of immobilization is the "cross-linking" method. The method relies on the fact that individual enzyme molecule is soluble in aqueous solution. But the enzyme can become insoluble if cross-linked to one another (Figure 1.6 b). The cross-linking agents are, for example, glutaraldehyde, toluene diisocyanate, and hexamethylene diisocyanate. The cross-linking agents are generally bifunctional molecules that form covalent bonds with the functional groups on the enzyme molecule.

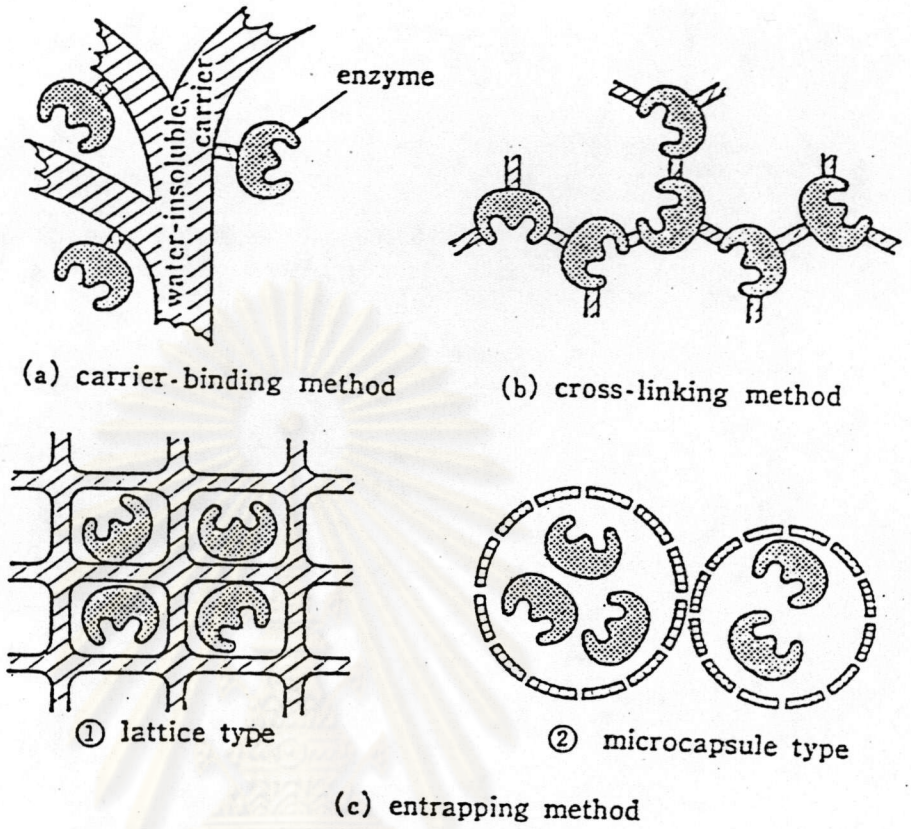


FIGURE 1.6: Three methods for the immobilization of enzymes, namely, (a) carrier binding, (b) cross-linking, and (c) entrapment [39].

The third method for immobilization is termed the "entrapping method". As the name implies the molecules of enzyme are entrapped either within the polymer matrix (Figure 1.6 c1) or within microcapsule (Figure 1.6 c2). In both cases the polymer matrix and the membrane of the microcapsule must have sufficient pore sizes to allow substrates to reach the entrapped enzyme and also for products to leave.

We will try to use some of those techniques in the immobilization of laccase.

1.5 Aims of the investigation

(i) Attempt to purify laccase from locally available materials.

(ii) Try the immobilization of laccase on various polymer supports and test the stability of the immobilized laccase.