Review

Biooxidation of indole and characteristics of the responsible enzymes

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Indole, an electron-rich N-aromatic heterocyclic organic compound, functions as a popular component of fragrances, indicator of some diseases and signal molecule in plant, animal and microorganism, respectively. It also serves as the precursor, core building block and functional group of many important biochemical molecules and compounds, such as plant hormones, alkaloids, indigoids, certain proteins and enzymes. Most of these important molecules and compounds if not all, are originated, fully or partly, from biooxidation of indole. This review outlined the progress in the study of biooxidation of indole and responsible enzymes in microorganism and in plant in past two decades, summarized the pathways of indole biooxidation with an emphasis on those leading to formation of indigo and indirubin in plant and discussed the perspectives of the research in indole biooxidation with a focus on the application of indole and its derivatives in agrochemicals, pharmaceuticals and environmental pollution remedy.

Key words: Biooxidation, indole, indole 2, 3-dioxygenase, indole dioxygenase, indole hydroxylase, indole monooxygenase, indole oxidase, indole oxygenase.

INTRODUCTION

Indole is an electron-rich N-aromatic heterocyclic organic compound. Its formula was first proposed by Adolf von Baeyer in 1869 (Baeyer and Emmerling, 1869) and is consisted of a six-membered benzene ring and a five-membered nitrogen-containing pyrrole ring (Figure 1). It is a solid at room temperature, having a flowery smell at very low concentration but an intense fecal odor at higher concentration. Unlike most amines, indole is not basic (Otani et al., 1962).

Indole, in particular in the form of indole nucleus (indole ring as a core building block and key functional group in a compound) has been found present in grand body of naturally occurring compounds, such as important alkaloids, plant hormone, flower scents, tryptophan, dye-stuffs, human feces, and coal tar etc. (Houlihan, 1972; Sundberg, 1996; Sharma et al., 2010). It plays an important role in the secondary metabolism and the metabolism regulation of the living-beings and is usually exploited as pharmaceutical drugs, agrochemicals etc. In microbe, indole as well as its derivatives may function as important signal molecule (Stamm et al., 2005; Lee and Lee, 2010). For example, in *Escherichia coli*, it can enhance switching frequency of the flagellar motor (Montrone et al., 1996), activate transcription of genes such as *astD*, *tnaB* and *gabT* etc. (Wang et al., 2001), and work for biofilm formation (Di Martino et al., 2003; Kuczynska-Wisnik et al, 2010). In plant, indole is a popular component of fragrances such as jasmine oil and organ essential oil etc., and the fragrance is one of the key factors for attracting insect pollinators. It is the nucleus of the most important member of auxin family, such as indolyl-3-acetic acid (IAA), which generates the majority of auxin effects in intact plants and in plant cells,

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tissues and/or organs cultured *in vitro*. It is also the core of other indolic auxins like indole-3-butyric acid (IBA) and 4-chloroindole-3-acetic acid (4-Cl-IAA) etc. that play important roles in the growth and behavioral processes in the plant life cycle. In indigo-producing plants, Chinese woad and woad, for example, indole is present as isatan and indican etc. which are the precursors of indigoids. Hydrolysis of the precursors gives rise to formation of indigo, the most important dyestuffs earliest known to human (Sandberg, 1989), and of indirubin, a new anti-cancer molecule. In practice application, indole is used in perfume industry, for example, in the manufacture of synthetic jasmine oil in one hand, and more widely used in pharmaceutical industry in other hand. Indole nucleus is present in many pharmaceutical drugs of different functions, such as in hallucinogen dimethyltryptamine, anti-inflammatory drug indomethacin, the beta blocker pindolol, and triptans as well as in drugs like serotonin, melatonin and niacin etc. (Stoff et al., 1978; Del Soldato et al., 1979; Safarinejad, 2008; Loder, 2010).

For these indole-derived compounds naturally occurring or artificially synthesized, the indole nucleus forms their structure basis and/or functional group. Among them, some are initiated by oxidation of indole, for example, indigoids. Chemically speaking, the indole is easily oxidized because its electron-rich nature, and a significant body of chemicals can oxidize indole and its derivatives, such as hydrogen peroxide, benzoyl peroxide (Kanaoka et al.,1971), periodate (Dolby and Rodia, 1970), chromium (Meenakshisundaram and Sarathi, 2007a), chlorination, bromination (Kanaoka et al., 1971), sodium performate, chloramines-T, peracetic, persulfuric acids, *N*-bromosuccinimide, peroxo anion and HSO_5^- (Meenakshisundaram and Sarathi, 2007b) etc. However, in living-beings, the oxidation of indole is much more difficult and complicate because of vast biodiversity, cell compartment and different kinds of oxidases involved. Even so, mighty advancements in the research of biooxidation of indole have been made since 1991 when Kamath and Vaidyanathan (1991) comprehensively reviewed the achievements up-to 1990. In this paper, we outlined the progress in the study of oxidation of indole and responsible enzymes in microbe and plant in past two decades and summarized the pathway of indole biooxidation with an emphasis on those leading to formation of indigo and indirubin in plant.
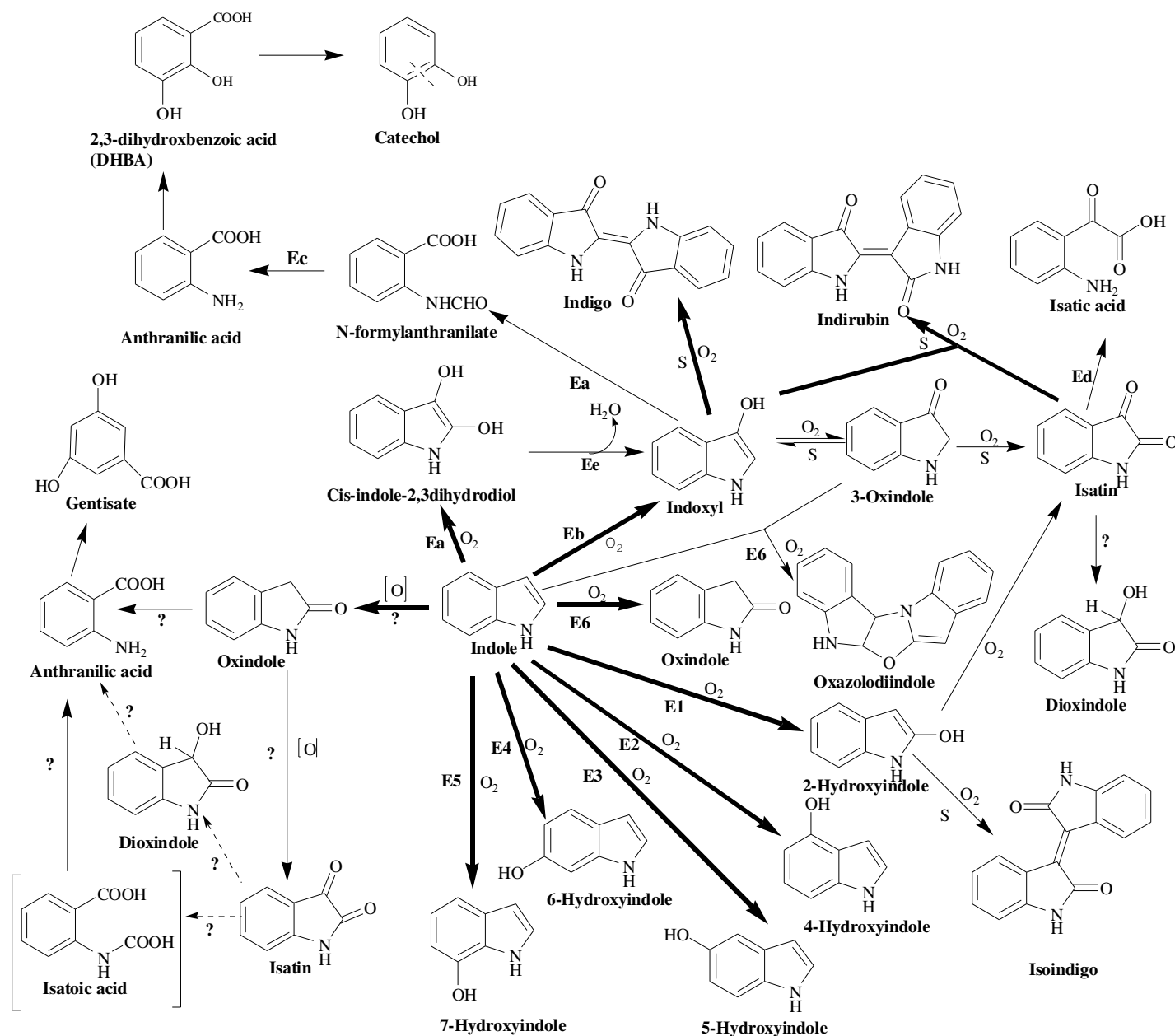
OXIDATION OF INDOLE IN MICROBES

In microbes, oxidation of indole can be catalyzed by enzymes encoded by both chromosome gene(s) and plasmid one(s). Therefore, the pathways operating to oxidize indole differed from each other and generated various ultimate products, depending on species, varieties, biotypes and/or their harbored plasmids. Up-to-date, at least 8 pathways are well definite and some

proposed (Figure 1).

The first pathway is that the indole is dioxygenated to form *cis*-indole-2,3 dihydrodiol, and the resulting dihydrodiol, then, is dehydrated to give rise to indoxyl, which is proposed catalyzed successively by the dioxygenase and indole-2,3 dihydrodiol dehydrogenase (Fujioka and Wada, 1968; Ensley et al., 1983). The indole can be also directly monooxygenated to form indoxyl under the catalysis of monooxygenase (Keil et al., 1987). The 3rd pathway is that the indole is converted into oxindole under the direction of certain oxygenase-type P450s. Also in aerobic condition, the indole can be separately oxidized at the C2, C4, C5, C6 or C7 position of its ring to yield 2-hydroxyl-, 4-hydroxyl-, 5-hydroxyl-, 6-hydroxyl- or 7-hydroxyl indole, respectively (Figure 1). Among the enzymes earlier identified responsible for these reactions are naphthalene dioxygenase (Ensley et al, 1983), toluene ortho-monooxygenase, toluene 2-monooxygenase (Rui et al., 2005), and toluene 4-monooxygenase/multicomponent phenol hydroxylase (McClay et al., 2005) etc., respectively. The multi-component phenol hydroxylase (belonging to bacterial multicomponent monooxygenases), 2-hydroxybiphenl 3-monooxygenase variant (HbpAind) and toluene dioxygenase can also produce various indole oxidation derivatives other than 3-hydroxyindole, such as 4-hydroxyindole, 5-hydroxyindole and 7-hydroxyindole etc. (Meyer et al., 2002; Kim et al., 2003, 2005). In anaerobic condition, conversion of indole to oxindole has been also observed (left side in Figure 2) in bacteria (Berry et al., 1987; Madsen et al., 1988; Madsen and Bollag, 1989; Gu and Berry, 1991), but the enzyme responsible for this kind of conversion remains unclear.

Although the pathways of indole oxidation in bacteria are diverse (Figure 1), they can be classed chemically into two categories: Ketonization of indole and hydroxylation of indole. Ketonization of indole, in particular at the C2 position, usually leads to indole ring cleavage and in consequence to formation of anthranilic acid and alike. Whereas the outcome of indole hydroxylation greatly depends upon the position and number of hydroxyl formed. Hydroxylation at both C2 and C3 or sole at C3 of indole ring results in formation of an active but unstable indoxyl. The indoxyl, in aerobic condition, may "spontaneously" dimerize to form indigo and indigoids (Sebek and Jager, 1962; Ensley et al., 1983; Han et al., 2008) in so called "indigo-producing bacteria", such as *Pseudomonas indoloxidans*, *P. putida, P. mendocina, P. sp.*HOB1 and *Sphingomonas macrogoltabida* etc. (Ensley et al., 1983; Yen et al., 1991; Moreno-Ruiz et al., 2003; Pathak and Madamwar, 2010). However, in "non-indigo-producing bacteria", 2,3-dihydroxylation of the indole may also results in cleavage of indole ring between C2 and C3, forming diverse ultimate products other than indigo or indigoids (Up-right in Figure 1). Single hydroxylation of indole at C2 produces 2-hydroxyindole which may "spontaneously" condenses too, but form isoindigo instead of

Figure 1. Oxidation of indole in microorganisms. Ea: Dioxygenase, Eb: Monooxygenase, Ec: N-formylanthranilate deformylase, Ed: Isatin hydrolase, Ee: Indole-2,3 dihydrodiol dehydrogenase, S: Spontaneous reaction, ?: Unclear reaction, E1: Toluene ortho-monooxygenase (wild type) (TOM), E2: Toluene-2-monooxygenase (mutant) (T2MO), E3: Toluene-4-monooxygenase (mutant) (T4MO), E4: Human cytochrome P450, E5: Toluene-2-monooxygenase (wild type) (T2MO) / Multicomponent phenol hydroxylase (mPHs), E6: Cytochrome P450, - - - - ▸: Hypothetic reaction, ⎯⎯⎯▸ : Indefinite reaction, ⎯⎯⎯▸ : Defined reaction.

indigo. The 2-hydroxyindole may also be further oxidized to produce isatin which can heterodimerize "sponta- neously" with indoxyl to form indirubin (right side in Figure 1). In a non-gelatinous strain of *Chromobacterium violaceus* ATCC-533, Sebek and Jager (1962) observed that the indole both from L-tryptophan degradation and artificial supplement might directly condense to violacien in the presence of air, which was named "Violacein pathway". This pathway was later strongly challenged by

numbers of workers around world. For example, after feeding violacien-producing *E. coli* with a mixture of [2-^{13}C]- and [indole-3-^{13}C]-tryptophan, of [3-^{13}C]- and [indole-3-^{13}C]-tryptophan *in vivo* or after reconstitution of violacien biosynthesis *in vitro* with E. coli-expressed and purified 5 proteins VioA-E which were originally contiguously encoded in *C. violaceus*, Momen and Hoshino (2000), Antonio and Crecynski-Pasa (2004) and Balibar and Walsh (2006) showed that the violacein was
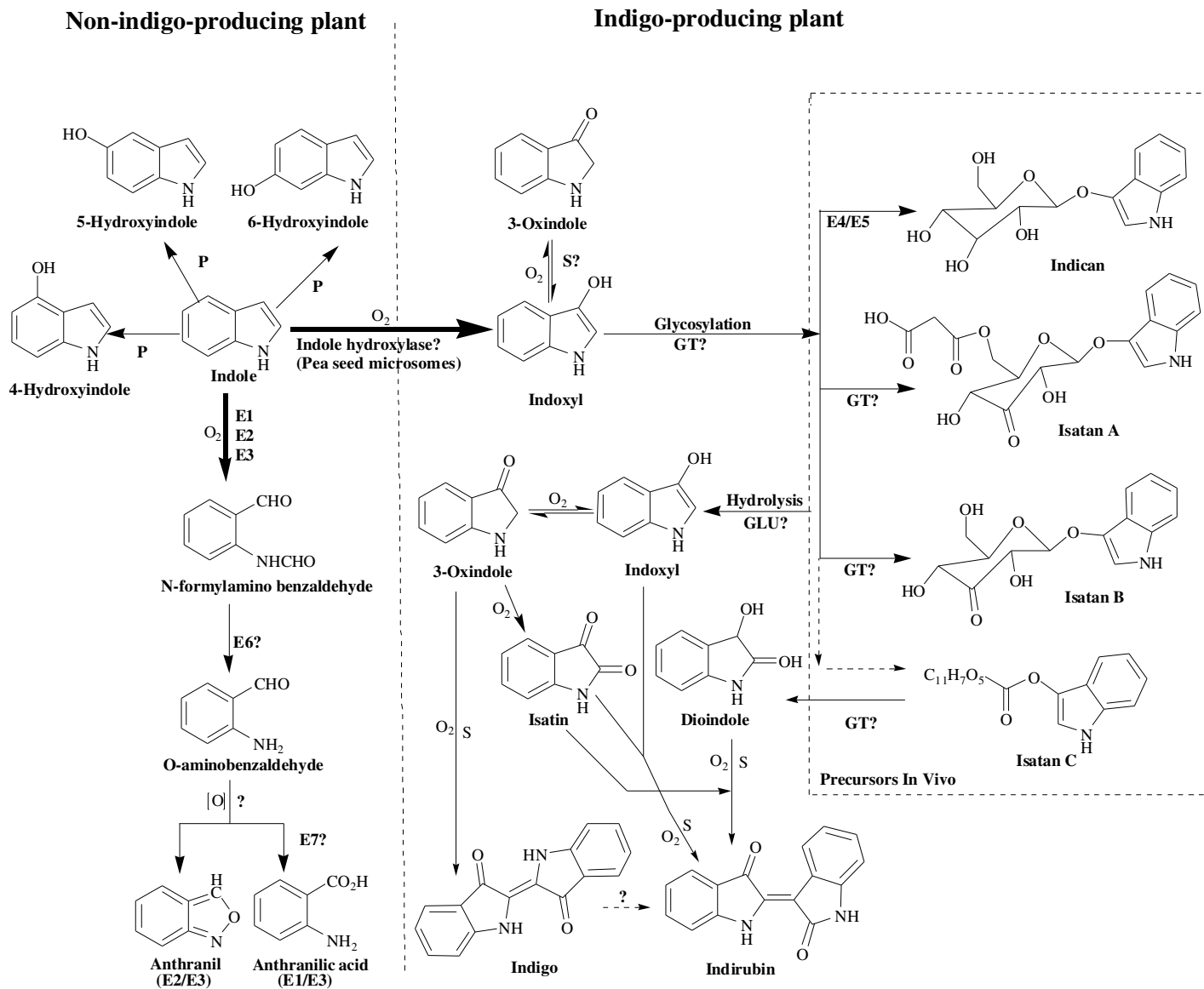
Figure 2. Oxidation of indole in higher plants. E1: Indole oxygenase, E2: Indole oxidase, E3: Indole 2, 3-dioxygenase, E4: Indican synthase, E5: Indoxyl-UDPG-glucosyltransferase, E6: Formylase, E7: Aldehyde oxidase, S: Spontaneous reaction, P: Plant tissues and organs, GT?: Glucosyltransferase, unidentified, GLU?: Glucosidase, unidentified.

formed from L-tryptophan, but not from indole. They proposed that the formation of violacein was mediated by intermediate 5-hydroxytryptophan. Sanchez et al. (2006) expressed VioA-E genes in both *E. coli* and *Streptomyces albus*, and discovered that the violacein came from a decarboxylative fusion of two tryptophans, and one of the tryptophan experienced an unusual 1→2 shift of the indole ring. Jiang et al (2010) reconstructed the violacein biosynthetic pathway by using *VioA-E* from *Duganella sp.* B2 in *E. coli, Citrobacter freundii* and *Enterobacter aerogenes*, and found that all recombinant strains did produce violacein, although with marketable differences in the protein expression profiles relating to violacein biosynthesis and in crude violacein productivity

and composition.

In bacteria, a large number of enzymes have been identified being able to oxidize indole to form indoxyl, and in consequence to form indigo and/or indigoids, and almost all these enzymes belong to aromatic mono-oxygenase and aromatic dioxygenase (See review: Han et al., 2008 and literatures within). Besides, numbers of laboratory-engineering cytochrome P450s could also convert indole into indigo and/or indigoids, such as P450 BM-3 (Li et al., 2008; Hu et al., 2009; Park et al., 2010; Huang et al., 2011) and P450cam (Manna and Mazumdar, 2010). Some microbial peroxidases were also reported being able to rapidly oxidize indole in the presence of H_2O_2. For example, chloroperoxidase from

Caldarimyces fumago converted indole into indoxyl (Burd et al., 2001); the chloroperoxidases isolated from some strains of *Streptomyces lividansand* and *Pseudomonas pyrrocinia* oxidized indole, indolylacetic acid and tryptophan to give rise to indigo, isatin, and anthranilic acid (Burd et al., 2001). In addition, fungal chloroperoxidase catalysis of indole conversion into oxindole was also reported (Corbett and Chipko, 1979). Even immobilized on mesoporous molecular sieves, the chloroperoxidase remained the ability to oxidize indole to indigo and/or indigoids in the presence of glucose oxidase (Jung et al., 2008; Jung and Hartmann, 2008, 2010). It is notable that few enzymes responsible for cleavage of indole ring have been identified up-to-now although the number of non-indigo-producing bacteria may be much larger than that of indigo-producing one.

OXIDATION OF INDOLE IN HIGHER PLANTS

In higher plants, indole is from the shikimic acid pathway, either via tryptophan or indole-3-pyruvate (Xia and Zenk, 1992). Its oxidation takes completely different pathways based on whether the plant can produce indigoids or not (Figure 2).

In non-indigo-producing plant, oxidation of indole usually leads to decycling of the indole ring directly and in consequence to formation of anthranilic acid and/or anthranil as the end products (Nair and Vaidyanathana, 1964; Chauhan et al., 1978; Divakar et al., 1979; Kunapuli and Vaidyanathan, 1982, 1983, 1985, 1991a, b; Pundir et al., 1984; Sarmiento and Garcia, 1995). In this pathway, neither indoxyl nor other hydroxyindoles were detectable though various intermediates were detected and analyzed (Kamath and Vaidyanathan, 1991). *N*-formylaminobenzaldehyde was identified as the direct product of indole decycling in *Tecoma stans* and *Jasminum grandiflorum* and the enzymes responsible for the ring cleavage were partly then full purified and named "indole oxygenases" (Divakar et al., 1979; Kunapuli and Vaidyanathan, 1983, 1991a, b). The *N*-formylaminobenzaldehyde, once forming, was converted to *o*-aminobenzaldehyde which was further oxidized to yield anthranilic acid, the end product (left side in Figure 2). Also in the leaf of *T. stans,* one enzyme partly purified and named "indole oxidase" oxidized indole with the anthranil, but not anthranilic acid, as its end product, although the intermediates identified and proposed were the same as for "indole oxygenases" (Nair and Vaidyanathana, 1964). Unclear remains why the same *o*-aminobenzaldehyde was converted to anthranil in the indole oxidation catalyzed by "indole oxidase" but to anthranilic acid in that catalyzed by "indole oxygenases" in the same *T. stans* leaves. In maize leaves, an enzyme called "indole 2, 3-dioxygenase" was reported to be able to oxidize indole with both anthranilic acid and anthranil as its end products (Pundir et al., 1984). Horseradish

peroxidase could oxidize indole to give rise to 2,2-bis-(3-indoly)-indoxyl and other products in the presence of H_2O_2 (Holmes-Siedle and Sauders, 1957). Besides the enzymes identified and isolated, some detached plant tissues and organs, such as etiolated pumpkin seedlings and green leaves (Horvath, 1977a), pedunculate oak leaf infiltration (Medvedev et al, 1977) and *Tridanscantia* leaves and stem tissue (Horvath et al, 1975; Horvath, 1977a, b) were also reported to oxidize indole to form various hydroxyindoles (4-, 5- and 6-hydroxyindole) other than 3-hydroxyindole. Exception is the pea seed microsomes that were demonstrated to convert indole into indoxyl in the presence of hydroperoxide (Ishimaru and Yamazaki, 1977). This kind of diversity in end-products and/or in intermediates remains to be verified with cell-free system for enzymatic oxidation of indole plus more sophistical analysis techniques such as HPLC, HPLC-MS and NMR etc. to identify the intermediates and the end product(s) of the reaction.

In indigo-producing plant such as *Isatis tinctoria*, *I. indigotica*, *Indigofera tinctoria* and *Polygonum tinctorium* etc. the indole has been postulated oxidized to form indoxyl, and the resulting indoxyl is then glycosylated to give rise to corresponding glycosides or esters such as indican, isatan A, isatan B and/or isatan C which are stored as indigo precursors (Maier et al., 1990; Frey et al., 1997; Marcinek et al., 2000; Minami et al., 2000; Maugard et al., 2001, 2002; Zou and Koh, 2007) (Figure 2).

The precursors, together with their stock organs, are hydrolyzed *in vitro*, in most case in basic condition, to release indoxyl, and the released indoxyl then dimerizes, in the presence of air, to form indigo, indirubin and other indigoids, which is the most classic method to produce indigo and indigoids. However, for long time, no information about direct detection and identification of the indoxyl has been available in the indigo-producing plants, to our knowledge. Recently we detected the indoxyl formation and in consequence the indigo formation by using a cell-free reaction of indole oxidation catalyzed by crude enzymes of *Isatis tinctoria* and *I. indigotica* etc. (Xiao et al, 2007; Liu, 2007; Yuan, 2010). Even so, the enzyme(s) responsible for converting indole into indoxyl in higher plants remains to be identified and isolated.

COMMON CHARACTERISTICS OF THE ENZYME RESPONSIBLE FOR OXIDATION OF INDOLE

Based on their activity mode, the enzymes catalyzing oxidation of indole may be classified into three categories: Monooxygenase, dioxygenase and peroxidase (chloroperoxidase). The monooxygenase is believed to add one molecular oxygen to indole, while the dioxygenase add two, which may be proceeded in the presence or absence of the air. The peroxidase, however, was found to be able to add to indole one mole (Burd et

al., 2001) and two moles (Kobayashi et al., 1989) of oxygen, depending upon the origin of the enzyme, and the oxidation does not take place without presence of H_2O_2. The enzymes responsible for indole oxidation may be also classified into hemechrome-containing oxygennase and flavin-containing one, based on their structure of active center. The heme-containing oxygenase requires in general NADH or NADPH for indole oxidation; some even require still other cofactors, such as ferrodoxin for electron transfer (Han et al., 2008 and references within). While for indole oxidation catalyzed by flavin-containing oxygenase, flavin reductase is sometimes involved.

It is notable that both heme-containing and flavin-containing oxygenases consist of monooxygenase, dioxygenase and peroxidase. For example, flavin mono-oxygenases and 2-hydroxybiphenyl 3-monooxygenase as well as chloroperoxidases from *Streptomyces lividansand* and *P. pyrrocinia* belong to the flavin-containing oxygenase, while styrene monooxygenase, cytochrome P450 BM-3 mutant, naphthalene dioxygenase, toluene dioxygenase, tetralin dioxygenase from bacteria, cytochrome P450 2A6 and cytochrome monooxygenase from human and chloroperoxidase from *C. fumago* are the heme-containing oxygenase. In higher plant, the indole 2,3-dioxgenase of maize and indole oxygenase of *T. stans* and *J. grandflorum* are members of flavin-containing oxygenase, while *T. stans* indole oxidase pertains to heme-containing one.

It's also notable that some enzymes responsible for oxidation of indole have the substrate-specificity and product-exclusiveness, while others have wider substrate and end-product spectrum. For example, styrene mono-oxygenase from *P. flurorescene* ST oxidizes indole to form sole 3-hydroxyindole (O'Connor et al., 1997), whereas naphthalene dioxygenase from some strains of *P. putida* can catalyze oxidation of indole and indole derivatives to yield more than 15 dyestuffs (Kim et al., 2003). Furuya and Kino (2010) have proved that CYP199A2 did not exhibit any activity towards indole and indole-3-carboxylic acid, but did oxidize indole-2-carboxylic acid, indole-5-carboxylic acid and indole-6-carboxylic acid.

PERSPECTIVE

Indole and its derivatives widely exist in animal, microorganism and plant, and so do the enzymes catalyzing the metabolism and in particular oxidation of indole and its derivatives. In the past two decades, a great progress was made in the indole oxidation, the pathways of indole oxidation and the enzymes responsible for indole oxidation, and particularly in microbe. Now we have got to know that the biooxidation of indole is an enzymatic proceeding, and the enzyme catalyzing this reaction includes heme- and flavin-containing monooxygenase,

dioxygenase, chloro-peroxidase and those with the same or similar enzymatic activity. We also have got to know that enzymatic oxidation of indole follows two main routes: oxidizing cleavage of indole ring and hydroxylation of indole without decyclizing its ring. The cleavage leads to complete degradation of the indole, which may be helpful to remedy or to eliminate environment contamination caused by indole and its derivatives, whereas the hydroxylation, especially at the C3 position, may convert indole to intermediate(s) such as indoxyl and/or end-products that may not only be re-utilizable for the body of living-being itself but also be utilizable for pharmaceutical, agrochemical, dyes-making and food industries etc. We are aware that there are still a lot unknown and a lot remaining to be elucidated and/or clarified in the indole oxidation, the pathway of indole oxidation and the enzymes responsible for indole oxidation, and in particular in higher plants.

First of all, we are going to investigate what is (are) the key characteristics that confer on an enzyme the ability to cleave indole ring or to hydroxylate the indole directly without breaking its ring, because both indole ring-cleaving enzymes and indole-hydroxylating enzymes cover not only heme-containing but also flavin-containing monooxygenases, dioxygenases and even chloro-peroxidases, and in particular in microbe. In order to speed up the investigation, it would be better to distinguish easily and clearly the enzyme cleaving indole ring from those hydroxylating the indole from their names, which is not the case at present. For instance, by their name, it is very hard to distinguish the function of "styrene monooxygenase"of *P. putida* S12 and CA-3 which oxidizes indole to yield sole indoxyl from that of "indole oxidase" of *T. stans*, "indole oxygenase" of *T. stans* and *J. grandiflorum* and "indole 2,3-dioxygenase" of maize which decyclize the indole (Figure 2). We propose to use the name "indole hydroxylase" for enzymes that hydroxylate indole (including indole derivatives) without decyclizing the indole ring, as once used by Otani et al (1962), and the name "indole oxidase" (including "indole oxygenase", "indole monooxygenase" and "indole dioxygenase") for those that cleave the indole ring by oxidation, at least in higher plant. The specific position of hydroxlation may be added to the name, such as "indole-3-hydroxylase" used by Oshima et al. (1965) for a *P. indoloxidans* enzyme that converted indole to 3-hydroxyindole (indoxyl).

Secondly, we are going to identify, isolate and characterize more "indole hydroxylases" and "indole oxidase", especially in higher plants. Hydroxylation of indole, especially at the C3 position in plant, and particularly in so called "indigo-producing plants, is a very important, if not a major, pathway of indole oxidation, but the responsible enzyme(s) remains completely unknown, although they have been approved existent *in vivo* and in cell-free system (Ishimaru and Yamazaki, 1977; Xiao et al., 2007; Liu, 2007; Yuan, 2010). In addition, elucidation

of the enzymatic and biochemical mechanism to hydroxylate at a specific position of the indole ring will be surely a big challenge, because of co-existence of the enzymes that hydoxylate the indole at one specific position and those at two or even more positions (Figures 1 and 2).

Thirdly, we are going to identify, isolate and characterize enzymes that are responsible for oxidation of indole to oxindole, and in particular at C2 position which often leads to decyclization of indole ring and in consequence to formation of anthranilic acid and alike (Claus and Kutzner, 1983). Indole ring cleavage is especially interesting for elimination pollution of indole and its derivatives and for remedy of soil and water etc contaminated. At the same time, we need elucidate and clarify the precise proceeding of conversion from indole to oxindole. In bacteria, 2-oxindole was postulated resulted from "direct" ketonization of indole, whereas 3-oxindole from two steps of successive indole oxidation, that is, oxidation of indole to form 3-hydroxyindole (indoxyl) and then oxidation of the indoxyl to yield 3-oxindole (Figure 1). In plus, in the two steps of successive oxidation, whether the second step is enzymatic reaction or spontaneous one remains to be investigated, since in the cell-free system, the indoxyl is usually spontaneously condensed to indigo and indigoids in the presence of air. Besides, in non-indigo-producing plant *T. stans*, both indole oxidase and indole dioxygenase cleaved indole ring to yield *N*-formylaminobenzaldehyde (or 2-formylaminobenzaldehyde) as direct intermediate, but the former had the anthranil as its end-product (Nair and Vaidyanathana, 1964) whereas for the later, its end-product was anthranilic acid (Divakar et al., 1979; Kunapuli and Vaidyanathan, 1983, 1991a, b).

These observations seem implying that both enzymes, indole oxidase and indole oxygenase are responsible not only for cleavage of the indole ring to form *N*-formylaminobenzaldehyde, but also for conversion of the *N*-formylaminobenzaldehyde to the end-product(s). Kamath and Vaijyanathan (1991) hypothesized that after indole oxygenase (*T. stans*) cleavage of indole ring, certain formylase(s) and then aldehyde oxidase(s) were successively involved for transformation of the resulting *N*-formylaminobenzaldehyde to anthranilic acid, the end-product. However, they did not explain why the resulting *N*-formylaminobenzaldehyde from indole oxidation by indole oxidase (*T. stans*) was converted into anthranil other than anthranilic acid. The answer may be come from the cell-free system tests with isolated indole oxidase and in parallel with indole oxygenase from *T. stans* as unique catalyzing enzyme and indole as only substrate, under the help of more advanced analyzing methods and instruments. Here, as in indigo-producing plants and microbes, rapid and precise detection of very unstable indoxyl will be critical and challenging.

Finally, we are going to identify and isolate genes encoding for the enzymes responsible for indole oxidation and involved in the entire pathway of indole oxidation in plants, and in particular in higher plants, just as what has been done in microbe. With these genes, the entire process of indole biooxidation would be reconstituted *in vitro* just as reconstituted violacein synthesis from L-tryptophan by enzymes encoded by contiguously genes *vioA-E* (Balibar and Walsh, 2006; Sanchez et al., 2006; Jiang et al., 2010), which will give a "certificate" to the proposed and or speculated pathways of biooxidation of indole outlined in Figure 2.

In conclusion, better understanding of the indole biooxidation, especially indole hydroxylation will benefit production of bio-indigo and bio-indirubin, which contributes not only to textile, food and pharmaceutical industries, but also to environment protection.

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