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Peroxidase Conjugate of Cellulose Nanocrystals for the Removal of Chlorinated Phenolic Compounds in Aqueous Solution

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Abstract: The study was conducted to immobilize peroxidase (E.C. 1.11.1.7) on to the rodlike cellulose nanocrystals after activation with cyanogen bromide treatment. The resulted bioactive conjugates were used to remove chlorinated phenolic compounds in aqueous solution. Gas phase Fourier transfer infrared spectroscopy was used for the detection and quantification of ammonia released from the immobilization reactions *in situ*. Results revealed that cyanogen bromide treatment of cellulose nanocrystals generated cyclic imidocarbonate group and cellulose carbamate. Covalent bonding between the activated nanoparticles and peroxidase generated ammonia as one of byproducts and the ammonia generation at an elevated temperature was more significant. Immobilization of enzyme at room temperature resulted in the bioactive conjugates with enzyme activity of 594 unit g^{-1}. Increase of immobilization temperature to 50°C led to thermal deactivation of enzyme although immobilization probably proceeded fast. Comparing to its soluble counterpart, the immobilized peroxidase demonstrated high removal of chlorinated phenolic compounds. This capability might be attributed to the stabilization effect of immobilization toward enzyme deactivation and the precipitate formation of oxidized phenol products inducing by the amino group from carbamate on the bioactive conjugates.

Key words: Peroxidase conjugate, enzyme immobilization, cellulose nanocrystals, CN-Br activation, chlorinated phenolic compounds

INTRODUCTION

Peroxidases can induce polymerization of phenols through generation of radicals by electron-transfer reactions (Liu *et al*., 2001). Because they are relatively cheap, the enzymes have been intensively studied for bioremediation, such as the removal of aromatic amines from textile waste water (Biswas *et al*., 2007) and the removal of phenols from effluents (Cooper and Nicell, 1996). It was found that the phenol removal efficiency was high if coagulant was combined with peroxidase in the applications (Tatsumi *et al*., 1994). The added coagulant would bind the oxidized phenol products to form precipitates and it thus prevented the peroxidase from being deactivated. Similarly, Arseguel and Baboulène (1994) found that the combined utilization of talc with peroxidase could decrease the deactivation of enzyme.

General experiences have also indicated that the immobilization of enzymes on the supports by multipoint attachment can stabilize the enzymes (Pedroche *et al*., 2007) and this can certainly enhance the capability of peroxidase in the removal of toxic phenol compounds in aqueous solution. Many works have been done on the immobilization of peroxidases on solid carriers, which include cinnamic carbohydrate esters (Rojas-Melgarejo *et al*., 2004), porous glass (Grzywnowicz *et al*., 1992; Lai and Lin, 2005), glass beads (Gómez *et al*., 2006), cellulose derivatives membranes (Murtinho *et al*., 1998) and magnetite (Tatsumi *et al*., 1996). Recent attentions have been directed to the selection of proper materials as support. Non-porous solid particles in small size may generate less diffusion limitation than the porous analogues do in the solution. As a result, reactions catalyzed by non-porous enzyme conjugates are more close to the homogenous ones (Jia *et al*., 2002). Also, particle size can play an important role in loading capacity of enzyme. Small size will have high surface area and thus may have high enzyme loading capacity. In light of the support particle size, new emergent nanoparticles can become top candidate for enzyme immobilization.

Acidic treatment of wood fibers can generate rodlike cellulose nanocrystals (CNCs), which are non-porous

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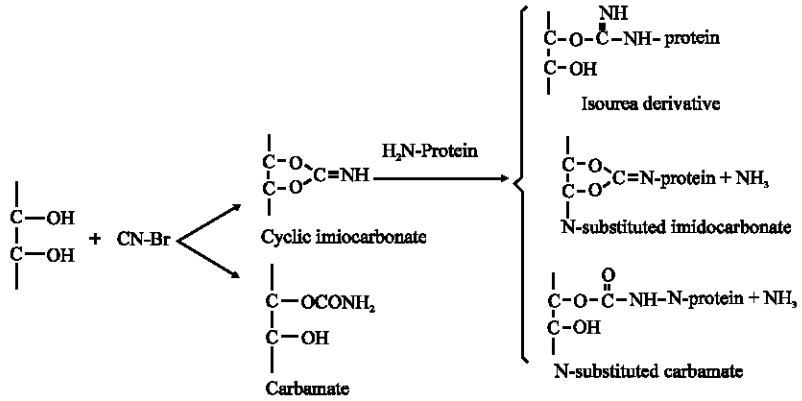

Scheme 1

particles and have typical dimensions of 50-60 nm long by 5-10 nm wide (Beck-Candanedo *et al*., 2005). After the cellulose surface is activated, CNCs can serve as support for peroxidase immobilization. Many procedures have been developed for the activation of cellulose materials (Shishkina *et al*., 2001) for the binding with bioactive materials. Among those, CN-Br (cyanogen bromide) treatment under alkaline condition was widely used even back in the 1970's. Although the linkages between support and protein may cleavages under some circumstances (Shishkina *et al*., 2001), the low cost and simplicity of the method still make the procedure attractive. The mechanism for the covalent linkage between the support and enzymes is due to the coupling reactions between the active groups generated from CN-Br treatment and amino group in protein, as depicted by the following scheme (Axèn and Ernback, 1971; Kennedy *et al*., 1980).

Although the postulated reaction scheme is generally accepted, detail reaction mechanism is still not very clear. While early investigators claimed that ammonia was released from the covalent bonding reactions (Axèn *et al*., 1967; Bartling *et al*., 1972), some others believed that ammonia was actually not produced since most of nitrogen from the starting materials was accountable in the products (Kågedal and Åkerström, 1971). Apparently, confirmation of ammonia generation in the covalent bonding reactions can clarify the mechanism of enzyme immobilization on CN-Br activated supports.

The use of chlorine-contained compounds for fiber bleaching in the pulp and paper industries generates a wide variety of CPCs (chlorinated phenolic compounds), including chlorinated phenols, chlorinated guaiacols, chlorinated vanillin and chlorinated catechols (Oliveira *et al*., 2002). Because of their resistance toward biodegradation under natural environment, the CPCs may eventually find their ways getting into the food chains. Some of those compounds are found to be carcinogenicity and over twelve of them have been listed as controlled toxic substances by US Environmental Protection Agency (McKague and Taylor, 2001). Although great efforts have been taken to reduce the level of those hazardous compounds in the effluent, study with immobilization of cheap peroxidase for the removal of CPCs from pulping effluent is still of great interesting

In this study, we wish to report the immobilization of peroxidase on CN-Br activated CNCs through covalent bonding. A gas phase FTIR unit was used to monitor the released ammonia *in situ*. The generated peroxidase conjugates were used to treated aqueous solution containing CPCs, the removal efficiencies of the hazardous compounds were compared with those from the treatment with soluble peroxidase counterpart.

MATERIALS AND METHODS

This study was carried out in Georgia Institute of Technology, USA and GuangXi University, China in the period of 2006 to 2007.

Chemicals and enzyme: Cyanogen bromide was purchased from VWR International. 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) was purchased from Sigma-Aldrich and used as it was. 3,4,5-trichlorophenol, 4,5-dichloroguaiacol, 6-chlorovanillin, 3,4-dichlorocatechol, 4,5,6-trichloroguaiacol, 3,4,5-trichlorocatechol were all purchased from Helix Biotech Corporation. Peroxidase (E.C. 1.11.1.7) was a crude enzyme produced by submerged fermentation of a genetically modified *Aspergillus* sp. and it was provided as a gift by Novozyme North America.

Cellulose nanocrystals: CNCs were prepared from No. 4 Whatman filter paper following a procedure described in literature (Beck-Candanedo *et al*., 2005). The generated CNCs were in a stable colloidal form with a pH value around 3.5. This CNCs suspension was stored in refrigerator at 5°C until used. The size of CNCs was then examined by using transmission electronic microscopy (JEOL JSM 100C). Before preparing samples for TEM analysis, the concentration of original CNCs stock suspension (around 0.5% consistency, w/w) was first diluted by 100 folds. 10 μL of this diluted solution was added into 1 mL ethanol (VWR international, 99.5%) and shaken vigorously. Three microliter of this ethanol-CNCs solution was dried over a film grid (Electronic Microscopy Science) for CNCs image collection. A small portion (5 mL) of CNCs suspension was neutralized to pH 7.0 and dried with a Centrivap Concentrator equipped with Centrivap Cold trap (Labconco®). The dried CNCs were characterized by using FTIR (Nicole 5000) in the form of KBr pellet containing 0.8% sample.

Activation of CNCs: Activation was conducted by the reaction of CNCs with CN-Br in a modified procedure described by Axèn *et al*. (1967). pH of the reaction medium was manually maintained above 11.5 by the addition of concentrated sodium hydroxide solution. The final activated CNCs product was stored in refrigerator until used. Twenty six milligram of the product was dried and was analyzed with FTIR as stated in section Cellulose nanocrystals.

Coupling of activated CNCs with glycine or peroxidase: 1 g activated CNCs product (52.8 mg CNCs solid) was weighed into 50 mL round bottom flask with standard 24/40 joint. Fifty milliliter 0.5 M sodium bicarbonate was used to dissolve 50 mg glycine or 5 μL peroxidase (activity: 20.11 unit μL^{-1}) and the resulted solution was added into the flask containing activated CNCs. pH of the suspension was 8.5. The flask was connected to the gas phase FTIR through the standard joint after 12 h reaction (room temperature reaction) or immediately after the flask was soaked in the water bath at 50°C. For the preparation of peroxidase conjugate, 10 g activated CNCs product (528 mg CNCs solid) was weighed into a 250 mL flask and added with 150 mL 0.5 M sodium bicarbonate. After 50 μL peroxidase enzyme (activity: 20.11 unit μL^{-1}) was added, the flask was sealed and set at room temperature for 12 h with periodically shaking. Similar procedure was used to prepare conjugate at 50°C. After the immobilization, the content in the flask was transferred to centrifuge tubes and was washed with 0.5 M sodium bicarbonate, 1 M NaCl and deionized water respectively. The final water washing supernatant did not have positive response to an assay medium containing 0.05 mM ABTS and 0.05 mM hydrogen peroxide. The washed CNCs-peroxidase conjugates were stable over three months in a refrigerator. Dried CNCs conjugate was analyzed with FTIR as stated in section Cellulose nanocrystals above.

Detection of released ammonia with gas phase FTIR: A procedure employing extractive FTIR technique was used to detect and quantify the released ammonia from the coupling reaction *in situ*. The methodology has been used for gas phase sample analysis (Reyes *et al*., 2006) and has been adopted as standard procedure by US Environmental Protection Agency. A portable gas phase FTIR unit from Midac Corporation was used in this study. The sample cell of the FTIR unit had 10 m path length (2 L cell volume) and its temperature was precisely controlled at 150°C. The instrument was equipped with a low-noise detector that was cooled with liquor nitrogen. The sample cell was connected to the reaction flask through a stainless steel tube with 1/4" diameter. The tube was wrapped with heating tapes and maintained at 130°C to prevent any condensation of analyte during the measurement. A water bath was fixed on a jack and it could be raised to heat the reaction flask. A stream of compressed air at 1 L min^{-1}. flow rate was introduced to the reaction flask. The air purged the ammonia out of the flask and carried the analytes to the FTIR sample cell. A computer interface controlled by AutoQuant Pro© software was employed for data acquisition and treatment. Method for data treatment was established by using noise-free standard ammonia FTIR spectra supplied by Midac Corporation. Three regions in the spectrum, 1043 to 1056 cm^{-1}, 989 to 994 cm^{-1} and 923 to 937 cm^{-1}, were used for the quantification. The use of the selected regions for the quantification is to minimize the overlap of absorption from other components in the sample stream. The resolution of recording spectrum was set at 0.5 cm^{-1}.

Experiment with aqueous ammonia solution showed that over 99% of ammonia could be purged out of the flask, as indicated by the titration of residual aqueous solution after the purging. Temperature plays an important role in determining the time for complete ammonia purge. At 25°C, it took over 90 min, whereas at 50°C it took only 10 min. The recorded ammonia in gas phase quickly reached a peak maximum and then reduced to the base line slowly. The total area under the peak (as total ppmv) was used to calibrate against the known amount of ammonia in the solution. Thus, after the purges of a series of ammonia solutions, a calibration curve can be established. Figure 1 is a calibration curve with ammonia ranging from 9 to 484 μg in 25 mL water and

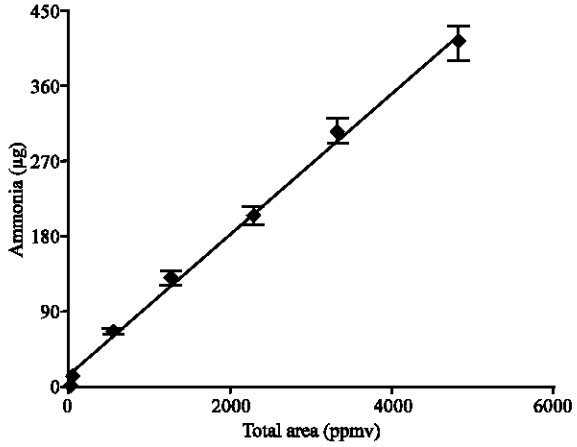

Fig. 1: Calibration plot of ammonia vs. total peak area

its R square value is 0.9943. A series runs with known ammonia indicated that the difference between measured result and used ammonia was bellow 5%. It thus indicates that the method can be used to determine ammonia content accurately.

Bioactivity determination: Enzyme activity was determined following the published spectrophotometric procedure (Rojas-Melgarejo *et al*., 2004). Peroxidase oxidized ABTS in the presence of hydrogen peroxide to generate ABTS cation radical ($ABTS^{\bullet+}$), which has an absorption maximum at 414 nm (ε31.1 mM^{-1} cm^{-1}). The assay medium consisted of 0.05 mM ABTS and 0.05 mM hydrogen peroxide in pH 5.5 sodium phosphate buffer solution. Enzyme activity was determined from the absorbance increase at 414 nm in a reaction period between the 10th second and the 70th second at 25°C. One enzyme unit equaled to the generation of 1 µm of ABTS cation in 1 min. The activity of the conjugate was expressed as enzyme units per milligram of dry CNCs (unit g^{-1}).

CPCs solution treatment and analysis: The study of using peroxidase conjugates to remove CPCs was conducted at room temperature. Individual compound was prepared into stock solution by dissolving in methanol. Desired volume of each stock solution was measured into a 25 mL vial containing enzyme and 10 mL water. The reaction was initialized by the addition of hydrogen peroxide and continued for 15 min under stirring. The residual CPCs monomers were recovered by solvent extraction following the published procedure (Sharma *et al*., 1999). In briefly, reacted mixture was first acidified to pH 2.0 and then was extracted 3 times with 15 mL of ethyl ether-acetone mixed solvent (90:10, v/v). The organic extract was dried over sodium sulfate overnight, concentrated and transferred into 10 mL volumetric flask and made up into mark. Because the recovery of chlorinated catechols by solvent extraction was very low (Sharma *et al*., 1999), the compounds were recovered by drying 1 mL reacted aqueous mixture in Centrivap Concentrator as stated in section Cellulose nanocrystals.

One milliliter solvent extract was dried under a stream of nitrogen at room temperature. The residues from the solvent extract and from aqueous mixture were converted to trimethylsilyl derivatives following by GC/MS analysis with splitless injection. The instrument was a Hewlett-Packard 5890 II GC equipped with Hewlett-Packard 5971A Mass Selective Detector. A 0.25 mm×60 m DB-5 fused silica capillary column was used for the chromatographic separations. The column had a 25 µm coating stationary phase. The GC was programmed as following: initial temperature 100°C; initial time 5 min.; rate 15°C min^{-1}; final temperature 260°C; inject port temperature 250°C. The mass detector was operated by using the following conditions: EI model; 70 eV; filament on delay time 8 min; mass scan range: 45 to 650 mu. Quantification of CPCs based on the area of selected ion peak. GC response factor of individual compound was obtained from the injection of the correspondent authentic compound.

RESULTS AND DISCUSSION

Characterization of CNCs: Figure 2 is a TEM image of CNCs. The length of CNCs is in a range of 25 to 40 nm and the width is around 10 nm. The length of CNCs products is slight shorter as compared to the published data and this may be due to the variation in hydrolysis time (Beck-Candanedo *et al*., 2005). A FTIR spectrum of CNCs is shown in Fig. 3 and it revealed that the nanoparticles posses high crystalline structure. It is well known that the cellulose fibers consist of crystalline region and amorphous region. As it was proposed by Åkerholm *et al*. (2004), the relative portion of the two regions can be estimated by the ratio of peak height around 1372 cm^{-1} to that around 2900 cm^{-1}. The higher of the ratio, the more of crystalline region in the sample has. For most of cellulose materials, this ratio is less than one. In the CNCs sample, the ratio was found to be around 0.96 as compared to 0.78 for the starting filter paper sample. It thus follows that the acidic treatment of filter paper fibers has removed substantial amount of the amorphous regions and the resulted CNCs are the parts of fiber with high proportion of crystalline structure.

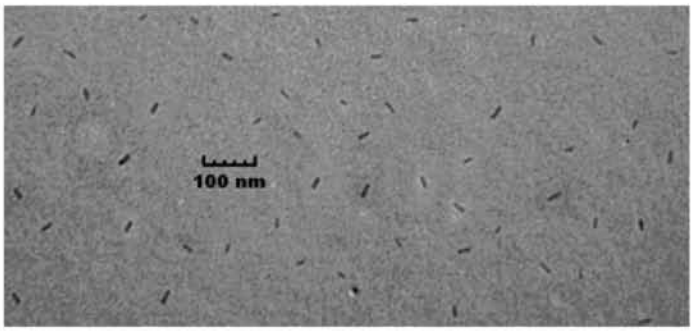

Fig. 2: TEM image of CNCs

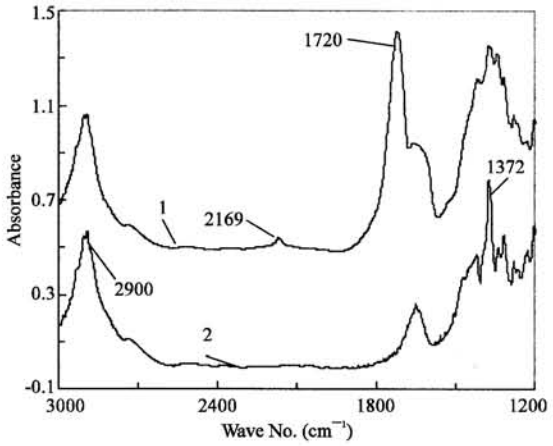

Fig. 3: FTIR spectra of CNCs and cyanogen bromide activated CNCs
1: CN-Br activated CNCs, 2: Original CNCs

Activation of CNCs: The activation of CNC with CN-Br introduced new features to the nanoparticles. As indicates in Fig. 3, FTIR spectrum of the activated CNCs displays a strong absorption band at 1720 cm^{-1} and a small peak at 2169 cm^{-1}.

It is postulated that cyclic imidocarbonate functionality and the cellulose carbamate moiety are generated after the activation of cellulose materials with CN-Br (Axèn and Ernback, 1971; Kennedy *et al*., 1980). The absorption at 1720 cm^{-1} was assigned to the stretching vibration of C=N in cyclic imidocarbonate by the earlier investigators (Axèn *et al*., 1967). Indeed, stretching vibration of C=N can cause absorption centering at this wave number. Working with the synthesized compounds, Addor (1963) demonstrated that 2-imino-1,3-dionolane hydrochloride, a model compound of the proposed cyclic imidocarbonate in the activated polysaccharides, gave absorption at 1720 cm^{-1}. But at the same time, carbonyl groups in the proper molecular structure can also cause absorption around this wave number. Pinchas and Ben Ishai (1957) found that carbonyl group in ethyl carbamate, a cellulose carbamate analogue, would give absorption band around 1728 cm^{-1}. More recently, Nada *et al*. (2000) had demonstrated that cellulose carbamate caused an absorption band around 1720 cm^{-1}. It thus follows that the strong band at 1720 cm^{-1} probably arises from both C=N in cyclic iminocarbonate and C=O in cellulose carbamate.

In agreement with the early report from Axèn *et al*. (1967), a small but clear peak arises around 2169 cm^{-1} in the activated CNCs spectrum. This absorption is assigned to the stretching vibration of cyanate structure. It was proposed that the formations of cyclic imidocarbonate and cellulose carbamate during CN-Br treatment proceeded through cyanate intermediate (Axèn and Ernback, 1971). The occurrence of this peak in activated CNCs indicates that the postulated intermediate is probably quite stable. However, the analogous alkyl cyanates are reportedly instable even under room temperature (Pasinszki and Havasi, 2003). It is not clear if the bulky solid CNCs in this study have stabilized the CNCs cyanate moiety.

Coupling of the activated CNCs with glycine and peroxidase: Based on the study with model compounds, it is hypothesized that the nucleophilic attacks of amino groups in protein on cyclic imidocarbonate led to the formation of covalent bonds between the support and protein (Axèn and Ernback, 1971). The cellulose carbamate formed during activation is practically inactive toward immobilization reactions. The main coupling products are substituted isoureas, N-substituted imidocarbonate and N-substituted carbonic acid ester respectively. Formations of the last two products should concomitantly release ammonia as byproduct. In order to detect ammonia, a sophisticated FTIR system has been built as described in the experimental section. A simple amino

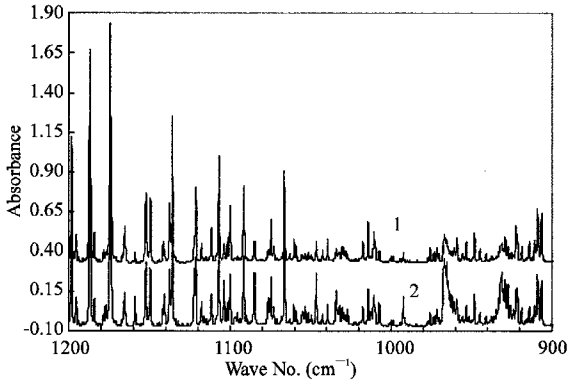

Fig. 4: A comparison of FTIR spectra from purges of glycine-CNCs reacted mixture and standard ammonium hydroxide solution
1: Spectrum from purge of reacted mixture containing 52.8 mg activated CNCs, 50 mg glycine, 15 mL 0.5 M sodium bicarbonate; 2: Spectrum from purge of 25 mL ammonia solution containing 63.6 μg ammonia at 50°C

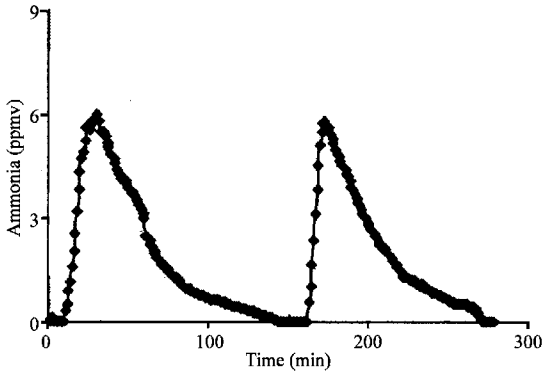

Fig. 5: Ammonia recorded from duplicate experiments of covalent bonding of glycine with activated CNCs
Reaction temperature: 50°C; reaction time: 120 min; purging air flow rate: 1 L min^{-1};
Reaction mixture: 52.8 mg activated CNCs, 50 mg glycine, 15 mL 0.5 M sodium bicarbonate

acid, glycine, was used to react with the activated CNCs at 50°C. Figure 4 shows a comparison of gas phase FTIR spectra from the purge of reaction mixture and the purge of ammonium solution. It indicates that the gas phase spectrum of reaction mixture contains all the features that a gas phase of ammonium solution has. Result thus indicates that ammonia does generate during the coupling reactions.

Figure 5 recorded the ammonia from duplicate runs of coupling reaction at 50°C using 52.8 mg CNCs solids and 50 mg glycine. The amount of ammonia is determined

Table 1: Immobilization of peroxidase on activated CNCs under different temperatures[a]

Activated CNCs, mg (solid)	528	528
Addition of peroxidase, μL (unit)	50.0 (1005.5)	50.0 (1005.5)
Temperature (°C)	25.0[b]	50.0[c]
Enzyme activity remained in supernatant (%)	67.8	43.5
Peroxidase conjugates activity g^{-1}	594.4	298.0

[a]: Immobilization pH 8.5, [b]: Immobilized for 12 h at a sealed flask; [c]: Immobilized for 30 min

be 38.7±1.9 μg and 35.5±1.8 μg, respectively and this will give about 41.3 μM active groups per gram of activated CNCs (41.3 μM g^{-1}). Since glycine is the smallest amino acid, it has little restraint in accessing to the available reaction sites in activated CNCs, the data should be interpreted as the maximum cyclic imidocarbonate units that can form N-substituted imidocarbonate and N-substituted carbomic acid ester products under the specified reaction conditions.

Temperature plays crucial role in the covalent bonding reactions leading to the formation of ammonia. At 25°C room temperature, the released ammonia is not detectable in the first 2 h. Although ammonia is found in the reacted mixture after 12 h, the total detected ammonia is only 11.2 μg, about 30% of that in the reaction at 50°C. When low reaction temperature is used, most of the bonding products are probably isourea derivative, which generates no ammonia as byproduct. At elevated temperature, however, the reaction may favor the formation of N-substituted imidocarbonate and N-substituted carbomic acid ester products and the formation of ammonia becomes significant.

Peroxidase was immobilized on the CN-Br activated CNCs at two different temperature levels: 25°C room temperature and 50°C, respectively. For the immobilization at room temperature, the reaction was terminated after 12 h. The reaction of enzyme with activated CNCs should have completed within this period (Axèn *et al*., 1967). At elevated temperature, enzyme activity is detrimentally affected due to thermal deactivation. Blank test indicated that the lost in enzyme activity is about 85% at 50°C for 30 min. Further increase in temperature and time can cause significant decrease in enzyme activity. As a result, the immobilization of enzyme at 50°C only continued for 30 min.

Table 1 shows the immobilization results. The covalent bonding of activated CNCs with enzyme at room temperature resulted in conjugates with much high bioactivity, twice of that from 50°C immobilization. The low temperature immobilization also ended up with 56% high residual bioactivity in the supernatant. This result clearly demonstrates the thermal deactivation of the enzyme. However, in the separated experiment, it was indicated that the released ammonia from the elevated

Table 2: Removal of chlorinated phenolic compounds by peroxidase*

CPCs	Initial concentration (μM)	CPCs remained after treatment (%)			
		Control[a]	Conjugate 1[b]	Conjugate 2[c]	Peroxidase[d]
3,4,5-trichlorophenol(255)[e]	77.1	92.6±0.7	2.6±0.6	2.4±0.3	12.9±0.5
4,5-dichloroguaiacol(234)	213.5	93.5±0.7	1.9±0.7	1.6±0.5	11.4±0.3
6-chlorovanillin(228)	80.4	98.8±0.5	3.7±0.6	2.7±0.2	22.0±0.2
3,4-dichlorocatechol(322)	217.2	92.6±0.5	1.5±0.4	1.9±0.5	13.7±0.2
4,5,6-trichloroguaiacol(270)	197.6	96.8±0.3	4.9±0.6	4.2±0.3	15.6±0.3
3,4,5-trichlorocatechol(358)	151.5	91.3±0.1	4.6±0.2	4.1±0.2	12.4±0.3

*: Reaction was carried out at room temperature in aqueous solution for 15 min with an initial pH of 6.18. Data are average of three runs; [a]: control run was conducted without peroxidase; [b]: peroxidase conjugate prepared from 25°C immobilization; [c]: peroxidase conjugate prepared from 50°C immobilization; [d]: soluble peroxidase; [e]: No. in parenthesis is m/z value of fragment ion used for quantification of the compound

temperature immobilization was 90% higher than that from the room temperature immobilization, even though the immobilization time was only 0.5 h instead of 12 h. Data thus indicates that high temperature immobilization can accelerate the cross linkage reactions leading to the formation of ammonia and ultimately increases the immobilization speed. Consequently, the selection of immobilization temperature should be balanced between the thermal deactivation and immobilization speed.

Removal of CPCs: Aqueous solutions containing CPCs were treated with peroxidase in its immobilized forms and soluble form. The enzyme charge was 0.8 unit mL^{-1}. The reactions were initialized by the addition of hydrogen peroxide. The remained CPCs after the treatments are showed in Table 2.

Data indicates that the remained CPCs after the treatments with the two peroxidase conjugates are lower than those treated with the soluble counterpart. The performances of the two peroxidase conjugates in removal of CPCs are similar and the removal rates are above 95%. Gómez *et al*. (2006) reported that the immobilization of horse radish peroxidase on glass bead had protective effect against inactivation or inhibition. As a result, the use of the immobilized peroxidase could reach high phenol removal rate. Similarly, the immobilization of peroxidase on CNCs may have improved enzyme stability and thus results in high removal of CPCs. Besides the enzyme protection, however, the adsorption of oxidized products to form precipitates is also believed to be helpful in enzymatic removal of phenol (Tatsumi *et al*., 1994). In this study, it is observed that the peroxidase conjugates of CNCs can induce precipitate formation during the treatment. When peroxidase conjugates were used, the reacted mixture was immediately turned to turbidity following by the formation of color precipitates. However, when soluble enzyme was used, the reacted solution only turned to dark brown color without precipitate formation. A similar phenomenon was also reported by Wada *et al*. (1995) when tyrosinase was used to treated phenols and aromatic amines. In their study, chitosan or a coagulant containing amino group must be used to create precipitates. It was proposed that the amino group in the additives could attract the oxidized phenols to form precipitates. For the treatment with peroxidase alone, only high dosage of enzyme (up to 2 unit mL^{-1}) can produce precipitates because extra enzyme serves as the provider of amino group for the adsorption of oxidized products (Tatsumi *et al*., 1994). In this study, peroxidase conjugates of CNCs have amino group on the surface. During the activation of CNCs, cellulose carbamate moiety was generated along with the cyclic imidocarbonate. The carbamate survived the reactions in the coupling with peroxidase and it may provide amino group for the adsorption of oxidized CPCs to form the precipitates.

CONCLUSIONS

The particle size of CNCs from sulfuric acid hydrolysis of filter paper is in the nano-scale. The nanoparticles can be activated through CN-Br treatment to introduce active group of cyclic imidocarbonate. The subsequent reaction of the active moiety with amino acid functionalities in peroxidase can result in the formation of enzyme conjugates through the covalent bond formation, with the concomitant release of ammonia as one of byproducts. At an elevated immobilization temperature, ammonia generation is more significant and the immobilization is probably fast, although the enzyme may suffer thermal deactivation. The generated peroxidase conjugates of CNCs at both room temperature and 50°C are more effective in the removal of chlorinated phenolic compounds than its soluble counterpart. The enhancement is probably due to the protection effect of immobilization toward the enzyme deactivation and the product precipitate formation inducing by amino group in carbamate on peroxidase conjugate of CNCs.

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