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Activity of tyrosinase immobilized on hydroxyaluminum–montmorillonite complexes

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Abstract

Montmorillonite was coated with varying levels of hydroxyaluminum, and the influence of degree of coatings on the adsorption, immobilization and activity of tyrosinase (Cu-containing polyphenoloxidase) was investigated in the absence of buffers. Tyrosinase was considered immobilized on the clay surfaces when after repeated washings with deionized distilled water no protein was detected in the supernatant. The immobilization of tyrosinase on Ca–montmorillonite (Ca–Mte) was not evident at pH 6.5, whereas tyrosinase was immobilized on hydroxyaluminum–montmorillonite ($Al(OH)_x$–Mte) complexes. The amount of tyrosinase immobilized on the clay surfaces increased with increasing level of hydroxyaluminum coatings from 1 to 5 mmol Al/g clay. The specific activity of the immobilized tyrosinase ranged from 19.3 to 62.1% of the free enzyme, indicating some perturbation in the conformation of the protein molecules bound to the coated clay surfaces and the hindrance of some enzyme active sites after binding. Both free and immobilized tyrosinase had an optimum pH of 6.0 and an optimum temperature of 30°C. Immobilized tyrosinase was less sensitive to the change in temperature, indicating the role of hydroxyaluminum coatings in protecting tyrosinase molecules against denaturation. After 30 d of storage at 4 and 25°C, the free enzyme retained 63% and 33% of its initial activity, whereas the immobilized enzyme retained 91% and 49%, respectively, of its initial activity. This indicated the higher stability of tyrosinase after immobilization on the Al-coated clay. Infrared analysis of the tyrosinase–$Al(OH)_x$–Mte complex after reaction with catechol indicated the presence of humic-like polymers adsorbed on the clay surfaces.

Keywords: Enzyme activity; Adsorption; Immobilization; Hydroxyaluminum–montmorillonite; Tyrosinase; Catechol oxidation

1. Introduction

The immobilization of enzymes on soil components such as clay minerals simulates their state in soil environments and permits elucidation of their mechanisms of action. Some enzymes have been immobilized on 'clean' clay minerals, namely, montmorillonite (Mte) and kaolinite [1–4]. Recently, it was shown that after immobilization of aspartase on Ca–Mte in the absence of bridging organic components and buffers, the immobilized enzyme and the clay matrix have a synergistic effect on the catalysis of aspartic acid deamination [5]. In natural environments, clay minerals are usually coated with

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aluminum or iron hydroxides [6]. It has been shown that a fungal laccase and a plant peroxidase, immobilized through a bridging component (glutaraldehyde) on glass bead, montmorillonite, kaolinite and soil, retained a high level of activity, with almost unchanged kinetic properties and a possible reusability [7]. The interaction of some enzymes with hydroxy–Al– or hydroxy–Fe–montmorillonite complexes has been studied [8–11]. More recently, Naidja et al. [12] observed the influence of hydroxy–Al coating on the adsorption of tyrosinase molecules in the interlayers of Al-coated Ca–montmorillonite. Further they reported that the presence of phosphate buffer substantially reduced the adsorption of tyrosinase molecules on $Al(OH)_x$–Mte complexes. The higher the level of hydroxyaluminum coatings, the higher was the suppressing effect of phosphate buffer on the amount of tyrosinase adsorbed, indicating the strong competition of phosphate ions with the tyrosinase molecules for adsorption sites on $Al(OH)_x$–Mte complexes.

The objective of this study was to examine the activity of tyrosinase immobilized on $Al(OH)_x$–Mte complexes as it pertains to the oxidative polymerization of catechol. The effects of pH, temperature and aging on the activity of immobilized tyrosinase were investigated. After reaction with catechol, the enzyme–clay surface was examined by Fourier transform infrared (FTIR) spectrometry. To better understand the mechanism of immobilization of tyrosinase on Al-coated clay, both the immobilization through a bridging organic component such as glutaraldehyde and the use of buffers were avoided.

2. Experimental

2.1. Catechol and tyrosinase

Catechol (1,2-dihydroxybenzene) and mushroom tyrosinase (EC 1.14.18.1) were obtained from Sigma (St. Louis, MO, USA). The desiccated enzyme was stored at −5°C until used.

2.2. Ca–montmorillonite (Ca–Mte)

The Ca–Mte sample (SWy-1 Crook County, Wyoming, USA) was obtained from the Source Clay Repository of the Clay Mineral Society. The purification treatments were previously described by Naidja and Huang [13].

2.3. Hydroxyaluminum–montmorillonite complexes ($Al(OH)_x$–Mte)

$Al(OH)_x$–Mte complexes containing 1.0, 2.5 and 5.0 mmol Al/g clay were prepared by addition of an appropriate aliquot of 1.0 M $AlCl_3$ to 5.0 g of montmorillonite suspension followed by adjustment of pH to 7.0 with dropwise addition of 0.5 M NaOH. The suspensions were stirred for 2 h, dialyzed against deionized distilled water until Cl^- free and then freeze-dried. The nature and the properties of the $Al(OH)_x$–Mte complexes prepared are reported by Naidja et al. [12] and briefly described below. The $Al(OH)_x$ coating polymers prepared had an OH/Al molar ratio of 2.8 and were noncrystalline polycations. The cation exchange capacity (CEC) of Ca–Mte was 103 cmol/kg which decreased to 18 cmol/kg with increasing the level of $Al(OH)_x$ coatings to 5.0 mmol Al/g clay. The external surface area of Ca–Mte, determined by BET method with multipoints N_2 gas adsorption isotherms, was 23.1 m^2/g and increased to 37.1 m^2/g with increasing the level of $Al(OH)_x$ coatings to 5.0 mmol Al/g clay. No micropores were detected, the average diameter and the total volume of mesopores also increased with increasing the levels of coatings and were 3.4 and 4.8 times higher at the level of 5.0 mmol Al/g clay than those of Mte before coatings [12]. These data substantiate the findings of Al coatings of the Mte external surfaces [14]. The total surface area (internal and external) of Ca–Mte, determined by gravimetric method with ethylene glycol monoethyl ether

(EGME) [15], was greatly influenced by the level of $Al(OH)_x$ coatings and it decreased from 707 m^2/g before the coatings to 200 m^2/g at 5.0 mmol Al/g clay. Since the external surface area of Mte is less than 4% of the total surface area, the decrease in the total surface area of Mte from 707 m^2/g before the coatings to 200 m^2/g at the coating level of 5.0 mmol Al/g clay reflects the decrease in the internal surface area which is more than 96% of the total surface area. This indicates that the access of EGME molecules to the interlayers of $Al(OH)_x$–Mte complexes was increasingly limited by the gradual intercalation of $Al(OH)_x$ polymers in Mte, as a stable 'blocking material' occupying the interstitial spaces of the clay [16]. This intercalation was also indicated by the gradual increase of the d_{001} spacings from 9.6 Å before coatings to 15.3 Å at the coating level of 5.0 mmol Al/g clay of the preheated $Al(OH)_x$–Mte complexes at 500°C [12].

2.4. Adsorption and immobilization of tyrosinase on Ca–Mte and $Al(OH)_x$–Mte complexes

Sterilized vessels were used in all experiments which were conducted in aqueous solutions of boiled–deionized–distilled (bdd) water in the presence of toluene (1 ml/l). The suspension of Ca–Mte or $Al(OH)_x$–Mte complexes (4.0 mg/ml) were dispersed by ultrasonification (3 min at 100 W) in bdd water. Tyrosinase was prepared in bdd water at 4°C (1.0 mg tyrosinase/ml). An aliquot of 0.3 ml of the tyrosinase solution was added to the clay suspension which was brought to 2.0 mg clay/ml and shaken until equilibrium was reached (1 h). The suspension was centrifuged at $19\,000 \times g$ and 4°C, using Sorvall RC-5B automatic superspeed refrigerated centrifuge. The amount of tyrosinase protein remaining in the supernatant was determined by the method of Bradford [17], using bovine serum albumin as the protein standard. The amount of tyrosinase protein adsorbed was calculated by subtracting the amount of protein in the supernatant from the initial amount of protein added to the clay. The tyrosinase–clay mineral complexes were washed with bdd water at pH 6.5 until no protein was detected in the washings, using the method of Bradford as mentioned above, and the amount of tyrosinase protein immobilized by $Al(OH)_x$–Mte complexes was calculated by subtracting the amount of protein in the supernatant and in the washings from the initial amount of protein added to the clay. The reported measurements represent the averages of two replicates. The free and immobilized tyrosinase were freshly prepared and kept in an ice bath until used.

2.5. Activity measurements

Sterilized vessels were used for all experiments which were conducted in aqueous solutions of bdd water in the presence of toluene (1 ml/l). The activity of the free or immobilized tyrosinase was determined by measuring oxygen consumption with a membrane-covered Clark polarographic oxygen sensor [3,18] with a temperature sensor (Model 820, Orion, Boston, MA, USA). Catechol was used as a substrate. A temperature-controlled glass reaction vessel with a volume of 82.0 ml was completely filled with a solution of 5.0 mM catechol, which was saturated with oxygen, and the desired amount of free or immobilized tyrosinase (Tables 1 and 3) was injected through a side port. Vigorous agitation was achieved with a Teflon-coated magnetic stir bar. The system was controlled at pH 6.5 by addition of 0.02 M NaOH or 0.02 M HCl, using a Brinkmann 672 titroprocessor (Metrohm, Herisau, Switzerland). The oxygen depleted (consumed by the reaction) during the 2 min reaction period at 25°C was recorded. All measurements represent the averages of two replicates. The enzyme activity was expressed in μmol O_2 consumed min^{-1}. The specific activity was calculated by dividing the activity by unit weight of tyrosinase protein and expressed in μmol O_2 consumed mg^{-1} protein min^{-1}.

The systems were examined for microbial

Table 1
Adsorption and immobilization of tyrosinase protein on Ca–montmorillonite (Ca–Mte) and hydroxyaluminum–montmorillonite complexes ($Al(OH)_x$–Mte) at different levels of Al coatings

Adsorbent [a]	Tyrosinase protein adsorbed (μg) [b]	Tyrosinase protein immobilized (μg) [b]
Ca–Mte	41.4	ND [c]
$Al(OH)_x$–Mte_1	22.8	16.1
$Al(OH)_x$–$Mte_{2.5}$	29.5	22.3
$Al(OH)_x$–Mte_5	82.0	74.6

[a] 0.143 mg of tyrosinase protein was added to 4.0 mg of clay at pH 6.5 and 25°C. The levels of Al coatings were 1.0, 2.5 and 5.0 mmol Al/g clay for $Al(OH)_x$–Mte_1, $Al(OH)_x$–$Mte_{2.5}$, $Al(OH)_x$–Mte_5, respectively.
[b] Mean of 2 replicates (1.2% ≤ standard error ≤ 3.8%).
[c] Not detectable.

growth before and after reaction with catechol, using the method of Koch [19]. No microbial growth was detected in either the free or the immobilized enzyme systems before and after reaction with catechol.

2.6. Absorbance of the reaction products

Absorbance of the reaction products formed from catechol in the presence of the free tyrosinase was measured after a 2 min reaction period at 470 nm [20], using a Beckman DU 650 microprocessor controlled spectrophotometer (Fullerton, CA, USA). After reaction of catechol with a $Al(OH)_x$–Mte complex or a tyrosinase–$Al(OH)_x$–Mte complex, the suspension was centrifuged at $19000 \times g$ (15 min at 4°C) and the absorbance of the supernatant was measured at 470 nm as described above.

2.7. Effects of pH, temperature and aging on the activity

The pH of the reaction system, which ranged from 4.0 to 8.0, was controlled during the 2 min reaction period at 25°C as described above. To investigate the effect of temperature on the activity of tyrosinase, a constant temperature water bath was used to maintain temperature from 15 to 60°C during the 2 min reaction period at pH 6.5. To test the effect of aging on the activity of tyrosinase, the free and immobilized tyrosinase were stored at constant temperatures of 4 and 25°C for up to 30 d at pH of 6.5. The activity of the free and immobilized tyrosinase was measured as described above. In these experiments, the amounts of the free and immobilized tyrosinase studied in the systems were the same (75 μg).

During aging, the free and immobilized tyrosinase were examined for microbial growth after 10, 21 and 30 d, using the method of Koch [19]. No microbial growth was detected in either the free and the immobilized enzyme systems before and after reaction with catechol.

2.8. FTIR analysis

Fourier transform infrared (FTIR) spectra of tyrosinase–$Al(OH)_x$–Mte complex formed at 5.0 mmol Al/g clay, before and after reaction with catechol at pH 6.0 and 25°C, were recorded on a KBr disk, which contained 1% of sample by weight, using a Biorad 3240 SPS microprocessor controlled spectrophotometer (Cambridge, MA, USA). The spectra were referenced against the spectrum of pure KBr and expressed in absorbance units. The spectrum of the tyrosinase–$Al(OH)_x$–Mte complex before reaction with catechol was subtracted from that of the complex after the reaction to obtain the difference FTIR spectrum of the reaction products adsorbed.

3. Results and discussion

3.1. Adsorption and immobilization of tyrosinase on $Al(OH)_x$–Mte complexes

The level of hydroxyaluminum coatings on Ca–Mte greatly influenced both the adsorption and the immobilization of tyrosinase (Table 1). At the levels of Al coatings of 1.0 and 2.5 mmol Al/g clay, the amounts of tyrosinase adsorbed

on the coated clay were 55.1% and 71.2%, respectively, of that adsorbed on Ca–Mte, indicating that the intercalation of $Al(OH)_x$ polymers in the Ca–Mte limited the access of the protein molecules to the internal surface adsorption sites [12]. At a coating level of 5.0 mmol Al/g clay, the amount of tyrosinase adsorbed on Al coated clay was approximately double that adsorbed on Ca–Mte. At this level of Al coatings, in addition to the intercalation of $Al(OH)_x$ polymers into Ca–Mte, the external surfaces were also coated [12] as discussed in the experimental section. Since no further increase of the d_{001} spacing of $Al(OH)_x$–Mte complex at 5.0 mmol Al/g clay after adsorption of tyrosinase was observed [12], tyrosinase was apparently largely adsorbed on the Al-coated external planar and edge surfaces of $Al(OH)_x$–Mte complex. The intercalation of $Al(OH)_x$ polymers in Mte has been reported [6,16,21–24]; the coatings of hydroxy–Al ions on the external planar and edge surfaces of 2:1 layer silicates have been demonstrated [25].

After washing with water, no tyrosinase bound to Ca–Mte was detected, indicating that the protein molecules were weakly held to the Ca–Mte surfaces, and thus, easily desorbed by water. Naidja and Huang [13] showed a similar rapid desorption by water of an amino acid adsorbed on Ca–Mte. In contrast, the amount of tyrosinase immobilized on $Al(OH)_x$–Mte complexes increased with increasing the level of coating (Table 1). At 5.0 mmol Al/g clay 91% of the adsorbed protein molecules were retained after washing, and thus, immobilized. The result showed that the protein molecules were strongly held to the Al coated clay surfaces. The immobilization of tyrosinase by the hydroxy–Al coated clay apparently proceeded through ligand exchange whereby the carboxylate groups of tyrosinase displace the hydroxyl or water groups from the hydroxy–Al polymers adsorbed on Mte. This interpretation is in accord with the observation that there is a strong competition between tyrosinase molecules and phosphate ligands for adsorption on the Al coated clay surface [12]. It is known that specific adsorption of phosphate on Al hydroxides proceeds through ligand exchange of phosphate for hydroxyl and water groups from the coordination sphere of Al ions [26,27]. Further, it is generally accepted that the adsorption of organic ligands by Al hydroxides and hydroxy–Al–clay complexes involves ligand exchange reactions [27–30]. Some of the carboxylate groups which are not specifically adsorbed could interact with the positive surface of the Al coated clay by electrostatic attraction as suggested by Sepelyak et al. [28] for the adsorption of the pepsin protein molecules on gibbsite and boehmite. Although formation of hydrogen bonding between the protein molecule moiety (carboxyl and/or amino groups) and the surface hydroxyl and water groups of hydroxy–Al polymers is implicated, these weak bonds are easily broken by washing [31].

3.2. Activity of immobilized tyrosinase

It has been shown that hydroxyaluminum-intercalated clay (pillared clay) may have a catalytic activity in the transformation of some organic compounds [21]. Therefore, to establish the role of the $Al(OH)_x$–Mte complexes in the catalytic process of the oxidation of catechol, the oxygen consumption was determined in the $Al(OH)_x$–Mte complexes systems before immobilization of tyrosinase. During the 2 min reaction period the amount of oxygen consumed by the $Al(OH)_x$–Mte complexes in the presence of catechol was only slightly higher than that consumed in the absence of catechol (Table 2). Since the system was oxygenated before addition of the $Al(OH)_x$–Mte complexes, the oxygen consumption indicated that some oxygen molecules were adsorbed on the $Al(OH)_x$–Mte surfaces. The oxidatively polymerized products of catechol have a characteristic absorbance at 470 nm [20], and the absorbance data indicated that no humic-like polymers were formed from catechol in the presence of $Al(OH)_x$–Mte complexes (Table 3). This confirms the hypothesis

Table 2
Oxygen consumption by catechol during a 2 min reaction period in the presence of Ca–Mte and $Al(OH)_x$–Mte complexes at different levels of Al coatings

Reaction system [a]	O_2 consumed (μmol) [b]
H_2O–Ca–Mte	2.8
H_2O–$Al(OH)_x$–Mte_1	2.8
H_2O–$Al(OH)_x$–$Mte_{2.5}$	3.0
H_2O–$Al(OH)_x$–Mte_5	3.3
Catechol (blank)	1.7
Catechol–Ca–Mte	3.3
Catechol–$Al(OH)_x$–Mte_1	3.1
Catechol–$Al(OH)_x$–$Mte_{2.5}$	3.5
Catechol–$Al(OH)_x$–Mte_5	3.8

[a] The levels of Al coatings were 1.0, 2.5 and 5.0 mmol Al/g clay for $Al(OH)_x$–Mte_1, $Al(OH)_x$–$Mte_{2.5}$, $Al(OH)_x$–Mte_5, respectively. The concentration of catechol was 5.0 mM and the total volume of the solution was 82.0 ml.
[b] Mean of 2 replicates (standard error $\leq$ 5.2%).

that the oxygen consumed in the presence of $Al(OH)_x$–Mte complexes was virtually adsorbed on the $Al(OH)_x$–Mte surfaces which did not appear to exhibit a catalytic activity. Therefore, the oxidation of catechol by the tyrosinase–$Al(OH)_x$–Mte complexes was evidently caused by tyrosinase immobilized on $Al(OH)_x$–Mte complexes.

Table 3
Absorbance of the reaction products formed from catechol at pH 6.5 and 25°C in the presence of free or immobilized tyrosinase on $Al(OH)_x$–Mte complexes

Complex [a]	Absorbance at 470 nm [b]
$Al(OH)_x$–Mte_1	0.000 colorless
$Al(OH)_x$–$Mte_{2.5}$	0.003 colorless
$Al(OH)_x$–Mte_5	0.002 colorless
Tyrosinase–$Al(OH)_x$–Mte_1	0.072
Tyrosinase–$Al(OH)_x$–$Mte_{2.5}$	0.035
Tyrosinase–$Al(OH)_x$–Mte_5	0.164
Free tyrosinase	0.245

[a] The amounts of tyrosinase protein immobilized on $Al(OH)_x$–Mte complexes were reported in Table 1. The amount of free tyrosinase protein was 75 μg (the same as that of the tyrosinase protein immobilized on $Al(OH)_x$–Mte_5 complex).
[b] Catechol (5.0 mM in a total volume of 82.0 ml) was used as blank. For $Al(OH)_x$–Mte complexes and the tyrosinase immobilized on $Al(OH)_x$–Mte complexes, after a 2 min reaction period with catechol, the suspension was centrifuged at 19,000 g (15 min at 4°C) and the absorbance of the supernatant was measured at 470 nm.

Table 4
Activity of free tyrosinase and tyrosinase immobilized on $Al(OH)_x$–Mte complexes at different levels of Al coatings at pH 6.5 and 25.0°C

Complex [a]	Specific activity (μmol O_2 mg^{-1} protein min^{-1}) [b]	% residual specific activity [c]
Free tyrosinase	127.4	100.0
Tyrosinase–$Al(OH)_x$–Mte_1	30.1	23.6
Tyrosinase–$Al(OH)_x$–$Mte_{2.5}$	24.6	19.3
Tyrosinase–$Al(OH)_x$–Mte_5	79.1	62.1

[a] The amounts of tyrosinase protein immobilized on $Al(OH)_x$–Mte complexes were reported in Table 1. The amount of free tyrosinase protein was 75 μg (the same as that of the tyrosinase protein immobilized on $Al(OH)_x$–Mte_5 complex).
[b] For the calculation of the specific activity, the contribution of the $Al(OH)_x$–Mte complexes to the oxygen consumed in the presence of catechol was subtracted.
[c] The % residual specific activity was calculated by comparison with the specific activity of the free tyrosinase.

The mechanism of the catalytic activity of free tyrosinase in the oxidation of catechol and the paramount role of the binuclear Cu active site of the metalloenzyme have been reported earlier [32–34]. Hence, the decrease in the specific activity of tyrosinase upon immobilization (Table 4) indicated that some of the enzyme Cu active sites were hindered by the $Al(OH)_x$–Mte complex surfaces as discussed below.

Compared with the level of coatings of 1.0 mmol Al/g clay, the decrease in the absorbance at the level of coatings of 2.5 mmol Al/g clay (Table 3) might be attributed either to the retention of some reaction products by the enzyme–clay complex as discussed below or to the agglomeration of the $Al(OH)_x$ polymers causing a steric hindrance to some of the active sites. Similarly, the residual activity of immobilized tyrosinase (expressed as percentage of the specific activity of free enzyme) on $Al(OH)_x$–Mte complexes was 23.6 and 19.3% at 1.0 and 2.5 mmol Al/g clay, respectively at pH 6.5 (Table 4), whereas at a higher level of coating, 5.0 mmol Al/g clay, the residual specific activity of immobilized tyrosinase was 62.1% (Table 4). Rao et al. [35] showed that the residual specific activity of immobilized acid phosphatase on

$Al(OH)_x$–Mte complexes increased from 20 to 50% of that of the free enzyme with increasing the level of Al coatings from 1.0 to 6.0 mmol Al/g clay at pH 5.0 in the presence of acetate buffer. Also, the specific activity of invertase was reduced after immobilization on $Al(OH)_x$–Mte complexes [8]. The decrease in the specific activity after enzyme immobilization was attributed to the inhibition of the enzyme molecules by the clay mineral surfaces [8]. This inhibition probably resulted from the hindrance of the active sites of the protein molecules, but the mechanism still remains unclear. We propose a schematic diagram (Fig. 1) showing the effect of Al coatings on the immobilization of tyrosinase by $Al(OH)_x$–Mte complexes and how some of the immobilized protein molecules would be oriented towards positions that may not affect their catalytic activity.

As discussed above, in addition to the intercalation of hydroxy–Al polymers which resulted in a gradual increase of the d-spacing of Mte with increasing the level of Al coatings from 1.0 to 5.0 mmol Al/g clay, the coatings of the Mte external surfaces also increased with increasing the Al level as indicated by the gradual increase in the external surface area of the coated clay [12]. The fact that tyrosinase was immobilized on the $Al(OH)_x$–Mte complexes and not on Ca–Mte surfaces indicated that the protein molecules were bound to the $Al(OH)_x$ polymers. Upon immobilization of tyrosinase on the Al coated external surfaces of clay, the orientation of some of the protein molecules appeared to be in such a way that both the substrate and the oxygen molecules were accessible to the binuclear Cu active site. At the lower level of Al coatings (1.0 mmol Al/g clay), the further increase of the d-spacing, even at 500°C, of the $Al(OH)_x$–Mte complex from 10.9 to 11.9 Å after adsorption of tyrosinase indicates that some of the protein molecules were intercalated and apparently coexisted with interlayered $Al(OH)_x$–polymers [12]. The cointercalation of non-protein high-molecular-weight organic substances and $Al(OH)_x$ polymers in

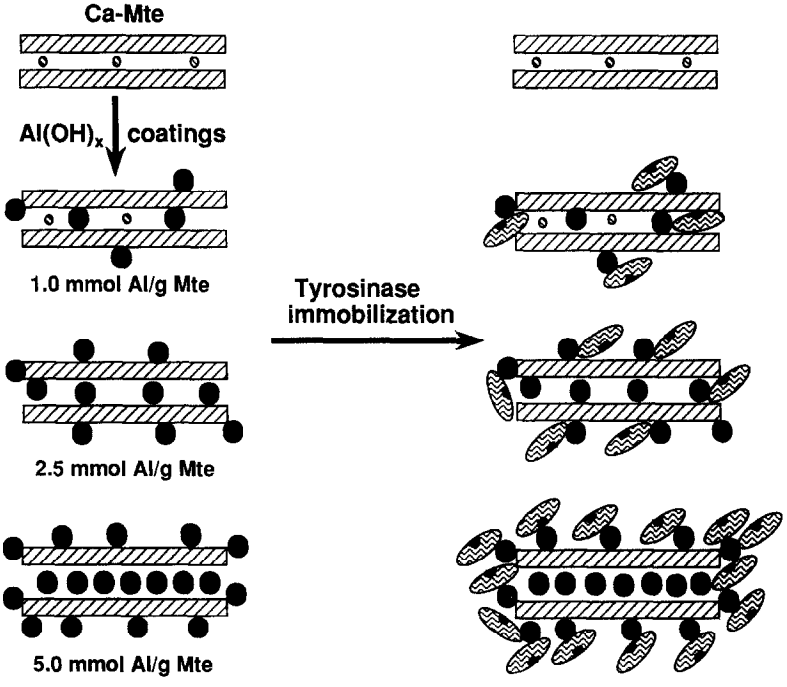

Fig. 1. Schematic diagram showing the immobilization of tyrosinase on $Al(OH)_x$–Mte complexes. (shaded) Ca^{2+}, (black) $Al(OH)_x$ polymers, (wavy shaded with black spots) tyrosinase; the dark region represents the binuclear Cu active site of tyrosinase.

Mte has been reported [29,36]. Besides intercalation some protein molecules appeared to be bound to the external surfaces as depicted in Fig. 1. At the level of 2.5 mmol Al/g clay, more of the external surface of Ca–Mte was covered with $Al(OH)_x$ polymers but the increase of immobilized tyrosinase molecules (1.4 times) was apparently not sufficient (Table 1) to overcome the steric hindrance of hydroxy–Al polymers; some of the tyrosinase molecules might be trapped between the hydroxy–Al polymers, and thus lower the activity of the enzyme (Table 4). When the level of Al coatings was doubled (5.0 mmol Al/g clay), there was 3.3-fold increase in the amount of immobilized tyrosinase. Therefore, more active sites were accessible and the activity of the immobilized tyrosinase was the highest at this level of Al coatings. Based on these results, the effect of pH, temperature and aging on the activity of the immobilized tyrosinase was investigated using the $Al(OH)_x$–Mte complex at the level of coatings of 5.0 mmol Al/g clay.

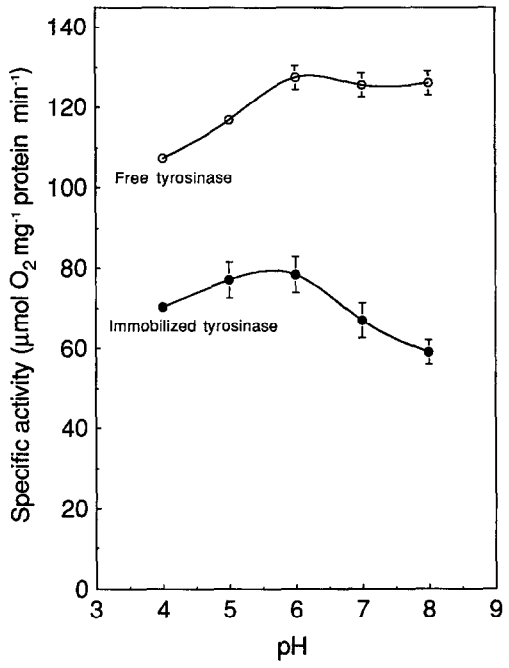

Fig. 2. Effect of pH on the specific activity of free tyrosinase and tyrosinase immobilized on $Al(OH)_x$–Mte complex at a level of coatings of 5.0 mmol Al/g clay. Reactions were performed at 25°C with 5 mM catechol and 75 μg tyrosinase in a total volume of 82 ml.

3.3. Effect of the reaction pH

Both free and immobilized tyrosinase had an optimum pH of 6 (Fig. 2). At levels of pH lower than 6, the activity of the immobilized tyrosinase was less sensitive to the pH change than that of the free tyrosinase, indicating a better resistance of the immobilized protein molecules to the ionization, since the ionic state of the functional groups in or close to the active center has a great effect on its activity [37]. On the other hand, at pH levels greater than 6, the activity of immobilized tyrosinase was much more affected by the pH change than that of the free enzyme. It is known that at the level of Al coatings higher than 2.5 mmol Al/g clay, the point of zero charge (PZC) of Al coated clay approached that of synthetic hydrous oxides [14]. The PZC of $Al(OH)_x$ polymers is 9.7 [38]. The positive surface charge of $Al(OH)_x$–Mte complex at the Al level of 5.0 mmol Al/g clay should decrease with increasing pH and so does the net charge of tyrosinase whose isoelectric point (pI) is 6.1[20]. Therefore, the repulsive electrostatic forces might have caused a split in the enzyme–Al coated clay complex, resulting in a less favorable conformation of the enzyme molecules. Furthermore, hydroxy–Al coated clay was sensitive to the pH change and might have formed Al precipitation products which inhibited the activity of the immobilized enzyme. The activity of immobilized invertase on $Al(OH)_x$–Mte complex was also pH-dependent [8] and sharply decreased from pH 5.5 (optimum) to pH 7.0 compared to the activity of free invertase.

3.4. Effect of the reaction temperature

The influence of the reaction temperature on the activity of the free or immobilized tyrosinase is shown in Fig. 3. Both free and immobilized tyrosinase showed an optimal temperature of 30°C. With increasing the temperature of the

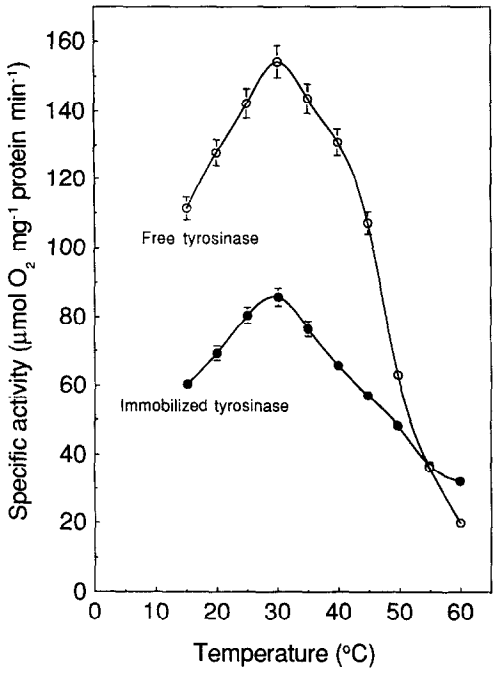

Fig. 3. Effect of temperature on the specific activity of free tyrosinase and tyrosinase immobilized on $Al(OH)_x$–Mte complex at a level of coatings of 5.0 mmol Al/g clay. Reactions were performed at pH 6.5 with 5 mM catechol and 75 μg tyrosinase in a total volume of 82 ml.

reaction from 30 to 60°C, the free tyrosinase only retained 13% of its optimum activity whereas the immobilized tyrosinase retained 37% of its optimum activity. At a temperature higher than 55°C, the activity of immobilized tyrosinase was higher than that of the free tyrosinase. These results indicated that the $Al(OH)_x$–Mte complex protected immobilized tyrosinase molecules against the effects of increased temperature. It has previously been shown that tyrosinase adsorbed on kaolinite was more sensitive to temperature than in free state [4]. This was attributed to the specific surface acidity and/or the thermal accumulation capacity of kaolinite, which may account for a lower thermal resistance of the adsorbed enzyme [4]. In contrast, Leonowicz et al. [39] reported that immobilized laccase on porous glass beads (CPG) was more resistant than free laccase to temperature change.

3.5. Influence of aging on the stability of immobilized tyrosinase

The stability of the immobilized tyrosinase was compared with that of the free enzyme at 4 and 25°C. After 30 d of storage at 4°C, the free tyrosinase retained 63% of its initial activity whereas the immobilized tyrosinase retained 91% of its activity (Fig. 4). When stored for 5 d at 25°C, the activity of the immobilized tyrosinase was preserved at 100%, whereas the activity of the free enzyme drastically decreased.

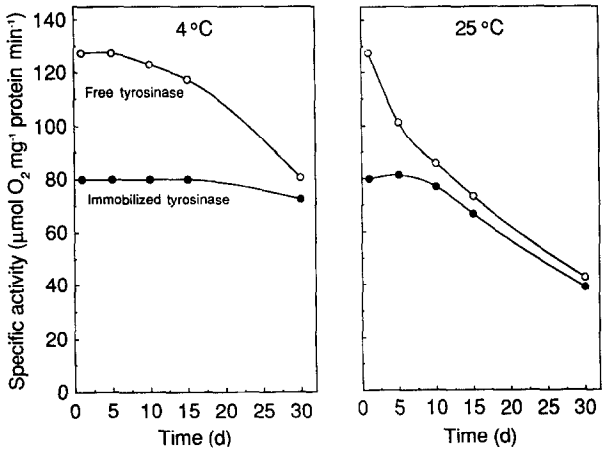

Fig. 4. Influence of aging at 4 or 25°C on the stability of free tyrosinase and tyrosinase immobilized on $Al(OH)_x$–Mte complex at a level of coatings of 5.0 mmol Al/g clay. The amounts of the free and immobilized tyrosinase protein systems were the same (75 μg), and the pH was 6.5.

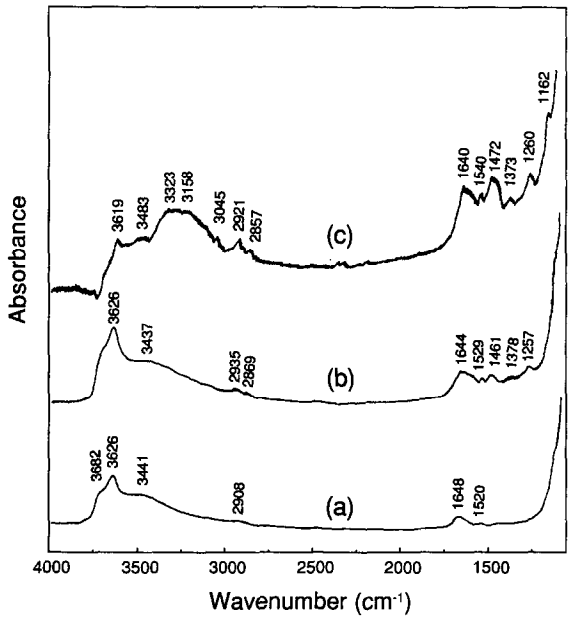

Fig. 5. FTIR spectra of the freshly prepared Tyrosinase–$Al(OH)_x$–Mte complex (5.0 mmol Al/g clay) before and after reaction period of 2 min with catechol at pH 6.0 and 25°C. The sample was washed with bdd water and the residue was air dried. (a) Tyrosinase–$Al(OH)_x$–Mte complex, (b) Tyrosinase–$Al(OH)_x$–Mte complex after reaction with catechol and (c) difference spectrum [(b)−(a)] of the reaction products adsorbed on tyrosinase–$Al(OH)_x$–Mte complex.

After 30 d of storage at 25°C, the free tyrosinase retained 33% of its initial activity, whereas the immobilized enzyme retained 49% of its activity. This clearly indicated that the immobilized tyrosinase on $Al(OH)_x$–Mte complex was more stable and less sensitive to the aging process than the free tyrosinase. Leonowicz et al. [39] showed that whether stored at 4 or 24°C for 27 d immobilized laccase on porous glass beads was more stable than soluble laccase.

3.6. Reaction products adsorbed on the tyrosinase–clay complex

It has previously been shown that the oxidative polymerization of catechol by tyrosinase leads to formation of catechol–melanin, a dark-color humic substance [40,41]. In the present study, after reaction with the catechol, the tyrosinase–$Al(OH)_x$–Mte complex suspension was dark in color as were the supernatant and pellet upon centrifugation. The darkness of the pellet suggested that catechol–melanin was adsorbed on the tyrosinase–$Al(OH)_x$–Mte complex, and it appeared likely that other reaction products were adsorbed there as well. This idea was confirmed by FTIR analysis of the enzyme–Al coated clay complex before and after reaction with catechol (Fig. 5a and b, respectively), and by the difference spectrum (Fig. 5c) which indicated that the adsorbed reaction products included humic-like polymers. The infrared absorption band assignments in the difference spectrum of the reaction products (humic-like polymers) formed from catechol and adsorbed on tyrosinase–$Al(OH)_x$–Mte complex are summarized in Table 5. The main absorption bands

Table 5
Assignment of the infrared bands of the reaction products formed from catechol and adsorbed on tyrosinase–$Al(OH)_x$–Mte complex at a level of Al coatings of 5.0 mmol Al/g clay

(a)	(b)	(c)	Assignment
3682	—	—	Al–OH, $Al(OH)_x$–Mte complex
3626	3626	3619	Al–OH, Ca–Mte
—	—	3483	OH, hydrogen-bonded
3441	3437	—	OH, H_2O
—	—	3323–3158	OH, phenolics
—	—	3045	CH, aromatics
—	2935	—	CH, acyclic
2908	—	2921	CH, acyclic
—	2869	—	CH_2, acyclic
—	—	2857	CH_2, acyclic
1648	1644	—	Amide I, Ty–$Al(OH)_x$–Mte complex
—	—	1640	C=C, C=O, polymeric quinones, COO^-
—	—	1540	C=C, aromatics, quinones
1520	1529	—	Amide II, Ty–$Al(OH)_x$–Mte complex
—	1461	1472	CH, C=C, aromatics
—	1378	1373	CH, OH bending, $C–CH_3$
—	1257	1260	CO, C–OH, phenolics, C=C rings, COOH
—	—	1162	Si–O–C, C–OH, C–C aliphatics, polymeric substances

(a) Tyrosinase–$Al(OH)_x$–Mte complex.
(b) Tyrosinase–$Al(OH)_x$–Mte complex after reaction with catechol at pH 6.5 and 25°C. The sample was washed with bdd water and the residue was air dried.
(c) The reaction products formed from catechol and adsorbed on tyrosinase–$Al(OH)_x$–Mte complex. Difference spectrum of spectra (a) and (b).

are discussed below. The broad band at 3323–3158 cm^{-1} was attributed to the OH stretching vibration of phenolics [42]. The weak and sharp absorption band at 3045 cm^{-1} was produced by the CH aromatic stretching vibration [43]. The existence of the aliphatic (acyclic) nature of CH bonds is clearly indicated by the CH and CH_2 stretching vibrations in the regions 2921 and 2857 cm^{-1} [44,45]. Aromatic rings as well as polymeric quinones may give rise to the 1640 cm^{-1} and 1540 cm^{-1} bands of C=C or C=O double bonds [43,46]. On the other hand, the band at 1640 cm^{-1} may also indicate the presence of carboxylate ions [46]. The band at 1162 cm^{-1} was assigned to C–C bond in aliphatics and polymeric substances [47], or to the Si–O–C group [46], indicating a possible linkage of some humic-like polymer moiety to a Si–O group at the edges of the tyrosinase–$Al(OH)_x$–Mte complex.

4. Conclusions

The immobilization of tyrosinase by $Al(OH)_x$–Mte complexes was influenced by the level of Al coatings which provided a better protection for the enzyme molecules in acidic conditions than in neutral to alkaline conditions. The tyrosinase immobilized on Al coated clay mineral surfaces was less sensitive than free tyrosinase to the change in temperature and retained its activity for a longer period of time. The catalysis of the catechol oxidation by immobilized tyrosinase on the Al coated clay resulted in the formation of dark color humic-like polymers including catechol–melanin adsorbed on the enzyme–Al-coated clay complex.

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