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Immobilization of penicillin G acylase in layered double hydroxides pillared by glutamate ions

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Abstract

Immobilized penicillin G acylase (PGA) has been assembled in the interlayer galleries of a layered double hydroxide (LDH) by a three-step process. Glutamate-pillared LDH was first prepared by a novel process involving dissolution of an LDH-carbonate precursor in aqueous glutamic acid, followed by precipitation of the product by addition of base. This method avoids the problem of competitive intercalation by nitrate ions observed with conventional methods involving precipitation of LDHs from solutions of metal nitrate salts. The glutamate ions are strongly held in the interlayer region of the LDH by virtue of the electrostatic interaction between their carboxylate groups and the cationic layers, and act as carriers for the introduction and retention of free amino groups in the interlayer galleries. Subsequent reaction of the amino groups of the immobilized glutamate ions with a glutaraldehyde linker followed by addition of PGA affords the immobilized enzyme (IME). The intermediate and final materials, which can be termed bioinorganic nanocomposites, have been characterized by FT-IR spectra, powder X-ray diffraction (XRD) and thermal analysis. The activity, thermal stability, pH stability and operational stability of the IME have been assayed. After 10 recycles, carried out in succession in a discontinuous reactor, the IME displayed 90% activity retention, with the expressed activity remaining above 430 U/g.

Keywords: Immobilized enzyme; Penicillin acylase; Glutamate; Intercalation; Layered double hydroxide; Hydrotalcite

1. Introduction

Penicillin G acylase (PGA) (EC 3.5.1.11) catalyses the deacylation of penicillin G to afford 6-aminopenicillanic acid (6-APA), an important intermediate in the manufacture of semisynthetic penicillins such as amoxycillin and ampicillin [1]. PGA has previously been immobilized on a variety of supports [2], but efforts to immobilize PGA on new type of carriers are still in progress with the goal of improving the catalytic efficiency of the enzyme and reducing the cost of the support. The insoluble carriers used to date are almost always polymeric resins, natural polymeric derivatives or organic gels and fibers with limited capacity for reuse, creating problems of disposal of organic materials. Inorganic carriers have the advantage of being reusable and are generally resistant to microbial attack and not affected by changes in pH or swollen by organic solvents.

The class of materials known as layered double hydroxides (LDH) or hydrotalcite-like materials have the general formula $[M_{1-x}{}^{II}M_x{}^{III}(OH)_2]^{x+}(Y^{n-})_{x/n}$.

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zH_2O, where the identities of the divalent M^{II} and trivalent M^{III} cations, the interlayer anion Y^{n-} and the stoichiometric coefficient x can be varied widely [3]. They have been much studied in the past for their ion-exchange behavior [4] and are currently attracting renewed interest as host matrices for the preparation of nanocomposite solids via the intercalation of organic species [5]. The interlayer anions can be assembled either as a monolayer or bilayer in the galleries, depending upon their charge, size and packing properties.

Proteins and enzymes including cytochrome *c* and horseradish peroxidase have previously been intercalated in layered solids such as α-zirconium phosphate [6] and shown to retain their structural integrity and activity but no studies of the intercalation of enzymes in LDHs have been reported. LDH materials are usually synthesized by coprecipitation in one of two the ways [7]. Precipitation at variable pH involves slow addition of a mixture of metal salts to a solution of sodium hydroxide and sodium carbonate. Precipitation at constant pH is carried out by simultaneous dropwise addition of mixed salt and base solutions to a reaction vessel at such a rate that the pH remains constant. Once addition is complete in either case, the resulting suspension is then aged at a given temperature. We have developed a new process for the synthesis of carbonate-pillared LDHs with a narrow range of particle sizes [8]. This involves a very rapid mixing and nucleation process, followed by a separate aging process. Mixing and nucleation of a large volume of reactant solutions is complete in a short time and the resulting mixture of crystallite nuclei with a narrow range of particle size is then aged in a separate process resulting in well-formed crystallites with a similarly narrow range of diameters. This process has been successfully scaled up to the pilot plant scale, allowing production of the support using very economical starting materials. We were therefore interested to explore for the first time the potential ability of LDHs to act as supports for immobilization of enzymes such as PGA by intercalation. In some cases, it has been found that use of glutaraldehyde as a linker between the support and the enzyme enhances the stability of the immobilized enzyme. For example, PGA has been supported on Amberlite XAD7 resin modified by transamidation with 1,2-diaminoethane [1]. The primary amine groups thus introduced are suitable for activation with glutaraldehyde through Schiff's base formation, which is followed by covalent coupling of the residual aldehyde groups with the free amino groups of the enzyme. In order to assemble a similar system in the interlayer galleries of LDH, it is first necessary to intercalate a carrier for the primary amine groups. Whilton et al. [9] have recently reported that amino acid anions such as glutamate may be intercalated into LDHs and these materials were chosen as precursor in our procedure. The glutamate-pillared LDHs were prepared by a different method from that employed by Whilton et al. [9] in order to overcome the problem they reported of competitive intercalation by nitrate ions. Reaction of the intercalated amino moieties with glutaraldehyde followed by reaction with PGA should allow the assembly of the IME in the interlayer galleries.

2. Experimental

2.1. Materials

Penicillin G potassium salt and penicillin G acylase were supplied by the Hua Bei Medicine Group.

2.2. Method

An Mg/Al-LDH (Mg/Al = 2.21) containing carbonate as the interlayer anion was synthesized by a modified coprecipitation method as described elsewhere [8]. Typically a solution containing $Mg(NO_3)_2 \cdot 6H_2O$ (61.6 g, 0.24 mol) and $Al(NO_3)_3 \cdot 9H_2O$ (45.2 g, 0.12 mol) in 150 ml distilled water and a solution containing NaOH (23.2 g, 0.58 mol) and Na_2CO_3 (25.4 g, 0.24 mol) in 150 ml distilled water were rapidly (over a period of 2 min) added to a colloid mill [10]. The resultant gel was then aged for 5 h under reflux with stirring. The white solid was subsequently isolated by filtration under suction, washed with distilled water until the pH of the washings was below 8 and then dried at 70 °C for 24 h. Elemental analysis of the LDH gave 20.7% Mg and 10.6% Al. Glutamate-pillared LDH was prepared by adding L-glutamic acid (7.5 g, 0.05 mol) to a suspension of the above LDH (4.0 g, 0.008 mol) in 100 ml distilled water. The suspension slowly dissolved with effervescence and a clear solution was obtained. This solution was added dropwise

to an alkaline solution (NaOH (2.0 g, 0.05 mol) dissolved in 100 ml decarbonated water) maintaining the pH above 9, followed by refluxing for 6 h. The resulting solid was recovered by filtration, washed, and dried at 75 °C for 24 h. Elemental analysis gave 6.98% C, 3.72% H, 1.42% N, 11.8% Mg and 5.68% Al.

An aqueous solution of glutaraldehyde (10 ml, 5% v/v) was added to a suspension of glutamate-pillared LDH (0.5 g in 30 ml deionized water) and stirred magnetically at 55 °C for 1 h. The resulting solid was filtered and washed well with water before being suspended in fresh water (80 ml). A solution of 1.25 ml pH 7.93 phosphate buffer was added to the suspension with stirring. A total of 4.5 ml free PGA (167 U/ml) was added (1 U corresponds the amount of enzyme which cleaves 1 μmol of penicillin G to 6-APA per minute at pH 7.9 and 37 °C). The suspension was shaken for 20 h at 30 °C. The immobilized PGA was recovered by centrifugation, washed and dried at ambient temperature.

Activities of both free and immobilized enzymes expressed in the hydrolysis of penicillin G solution (4%, w/w) in 0.05 N pH 7.93 phosphate buffer were measured in a stirred reactor using the pH-stat method at 37 °C for 12 min [11]. About 1 ml of free enzyme and 0.5 g dry weight of immobilized enzyme were used in the assays.

Powder X-ray diffraction (XRD) data were collected on a Shimadzu XRD-6000 powder diffractometer, using Cu Kα radiation between 3 and 70° using step-scans of 0.02° (2θ).

FT-IR spectra were recorded on a Bruker Vector 22 spectrometer. The sample was finely ground for 1 min, combined with oven dried spectroscopic grade KBr and pressed into a disc. The spectrum of each sample was recorded in triplicate by accumulating 20 scans at 2 cm^{-1} resolution between 400 and 4000 cm^{-1}.

Thermal analyses of powdered samples up to temperatures of 700 °C were carried out at a ramp rate of 10 °C/min using a BOIF instrument (manufactured in Beijing).

3. Results and discussion

3.1. Characterization of materials

The XRD pattern for the carbonate-LDH showed the characteristic reflections corresponding to a well-crystallized layered phase with an interlayer spacing of 0.76 nm (Fig. 1(a)) with an IR spectrum

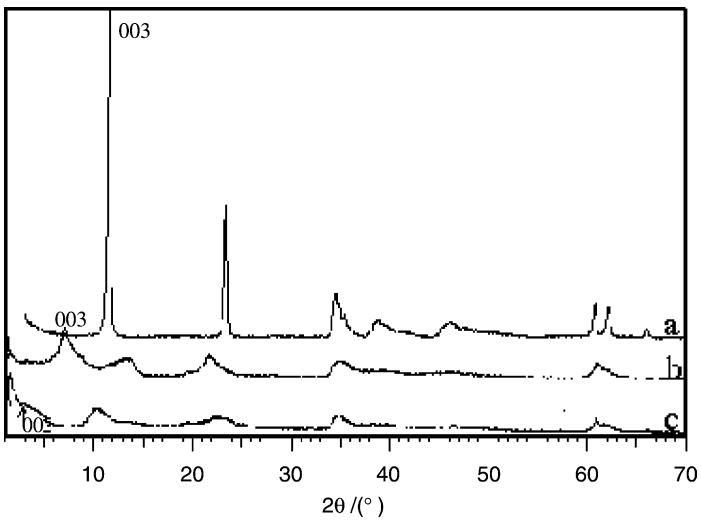

Fig. 1. Powder XRD patterns for (a) Mg/Al-carbonate LDH, (b) glutamate-pillared LDH and (c) penicillin acylase immobilized on (b) by introduction of glutaraldehyde linker. Replacement of carbonate ions by glutamate leads to an increase in interlayer spacing, as shown by the shift in the (0 0 3) reflection to lower angle, and loss in crystallinity. There is a further increase in interlayer spacing and loss of crystallinity after immobilization of the enzyme.

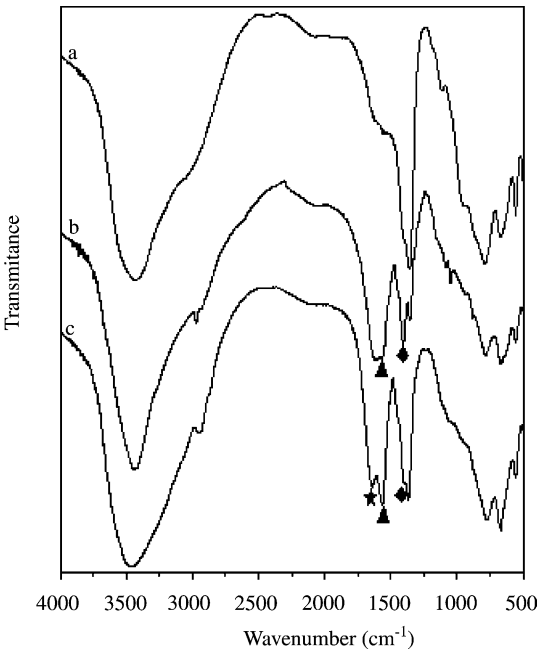

Fig. 2. FT-IR spectra for (a) Mg/Al-carbonate LDH, (b) glutamate-pillared LDH and (c) penicillin acylase immobilized on (b) by introduction of glutaraldehyde linker. The symmetric (▲) and asymmetric (◆) stretches of the glutamate carboxylate groups are clearly seen in both (b) and (c). The peak (★) at 1639 cm^{-1} in (c) can be attributed to the C=N stretch of the Schiff's base linkage.

(Fig. 2(a)) similar to that reported in the literature [3]. LDHs containing anions other than carbonate are usually synthesized under a nitrogen atmosphere by coprecipitation at variable pH (dropwise addition of mixed salt solution to alkali) or constant pH (simultaneous dropwise addition of mixed salt and alkali solution to the reaction vessel at such a rate that the pH remains constant). Exclusion of atmospheric carbon dioxide by use of a nitrogen atmosphere is necessary because of the high affinity of LDHs for carbonate ions. Even so, contamination by precursor anions can sometimes occur. Thus, when glutamate-pillared LDHs were prepared by Whilton et al. [9] using precipitation at constant pH under nitrogen, the resulting materials contained both glutamate and nitrate ions in the interlayer region, the latter arising from the magnesium and aluminum nitrate precursors. We have developed a new synthetic method to avoid nitrate contamination. A magnesium–aluminium carbonate LDH suspension was dissolved by addition of an excess of glutamic acid and the resulting mixed glutamate salt solution was used to synthesize glutamate-pillared LDHs by precipitation with aqueous NaOH at variable pH. Even though a nitrogen atmosphere was not used, the product was not contaminated with any carbonate ion (as shown by XRD, IR and the absence of any effervescence when the product was reacted with dilute hydrochloric acid). Pillaring of the LDH by glutamate using this new method gave a material with the characteristic diffraction pattern of a poorly crystalline LDH showing a broad (0 0 3) diffraction peak centered at 1.24 nm, similar to the value reported by Whilton et al. [9] and consistent with the intercalation of the glutamate anions in the interlayer region. The interlayer spacing is similar to that expected for an α,ω-dicarboxylic acid with five carbon atoms [12]. Given that the thickness of the brucite-like layer of LDH is 0.48 nm, the gallery height in the glutamate-pillared material is 0.76 nm, compared with 0.28 nm in the carbonate-containing LDH. These data suggest a monolayer arrangement for the intercalated amino acids.

Elemental analysis results for the LDH carbonate precursor and the glutamate-pillared LDH were consistent with the formulae shown in Table 1. The Mg/Al ratio in the glutamate-pillared LDH sample (2.34) was slightly higher than that in the carbonate precursor (2.21) indicating that precipitation of the trivalent cation is slightly incomplete. In contrast, Whilton et al. [9] reported that exchange of nitrate ions in an LDH precursor with glutamate ions led to

Table 1
Analytical data for LDHs materials

Samples	Chemical composition	Mg/Al ratio
LDH	$[Mg_{0.69}Al_{0.31}(OH)_2](CO_3^{2-})_{0.155}\cdot 0.66H_2O$	2.21:1
Glu[a]-LDH	$[Mg_{0.70}Al_{0.30}(OH)_2](C_5H_7NO_4^{2-})_{0.15}\cdot 3.4H_2O$	2.34:1

[a] Glu: glutamate dianion.

a significant decrease in Mg/Al ratio. The elemental analysis (see Table 1) indicated that the glutamate ions were incorporated as dianionic species in the interlayer region. The analysis also suggested that a large amount of water was adsorbed on the surface of glutamate-pillared LDHs.

The FT-IR spectrum of the glutamate-LDH showed absorption bands corresponding to the intercalated organic anions (Fig. 2(b)). Characteristic alkyl C–H stretches were observed in the region 3000–2800 cm^{-1} of the spectrum and the asymmetric and symmetric stretches RCO_2^- at 1405 and 1594 cm^{-1}, respectively, were also evident. No peaks due carbonate ions, which are observed at 1377 cm^{-1} were seen in the spectrum [13]. A broad absorption between 3600 and 3200 cm^{-1} was associated with the stretching modes of hydrogen bonded hydroxyl groups from both the LDH sheets and interlayer water.

Thermogravimetric analysis results for the carbonate-LDH showed an initial distinct reduction in mass between 80 and 200 °C (about 15%, w/w) arising from loss of surface adsorbed and interlayer water. A major mass loss was observed starting at 260 °C and continuing up to 500 °C, resulting from concomitant dehydroxylation of the inorganic layers and decomposition of intercalated carbonate anions (about 27%, w/w) (Fig. 3(a)).

The corresponding DTA trace for the carbonate-LDHs showed two endotherms centered at 242 and 407 °C associated with these processes (Fig. 4(a)). In contrast, the glutamate-LDH underwent a more gradual mass loss between ambient temperature and 250 °C, respectively (about 20%, w/w) (Fig. 3(b)), resulting from loss of surface adsorbed and interlayer water. The onset of a rapid and major mass loss ensued between 250 and 450 °C (about 30% loss, w/w) as dehydroxylation and decomposition of the organics occurred in an exothermic process (Fig. 4(b)).

After reaction of the glutamate-pillared LDH with glutaraldehyde followed by immobilization of PGA, the resulting solid showed a weak (0 0 3) diffraction peak at 4.4 nm in the XRD (Fig. 1(c)), indicating that PGA had been intercalated into the interlayer galleries but with significant stacking disorder. The gallery spacing for the intercalated immobilized PGA was 3.92 nm, which is consistent with the size of the enzyme reported in the literature [14]. The IR spectrum (Fig. 2(c)) for the immobilized enzyme showed peaks corresponding to the asymmetric and symmetric stretches of the carboxylate groups of the glutamate ion and in addition a peak at 1639 cm^{-1} which can be

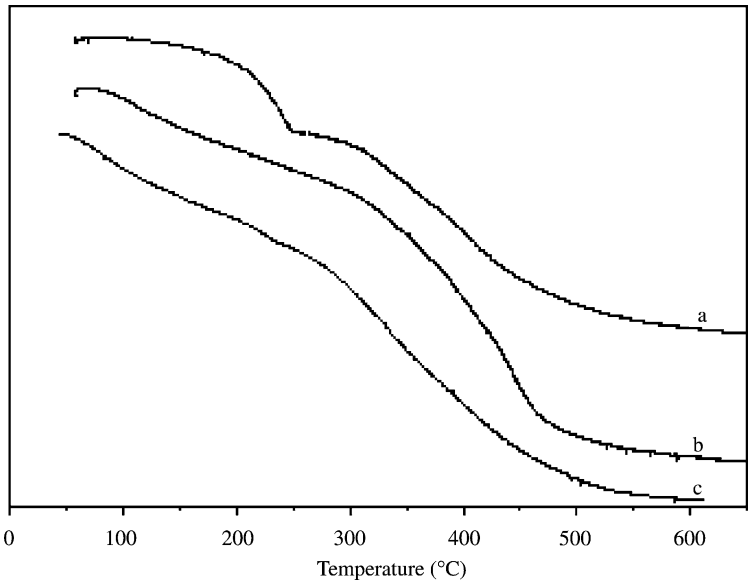

Fig. 3. TG curves for (a) Mg/Al-carbonate LDH, (b) glutamate-pillared LDH and (c) penicillin acylase immobilized on (b) by introduction of glutaraldehyde linker. The mass losses for (b) and (c) are similar, indicating that the amount of enzyme immobilized on the support is small.

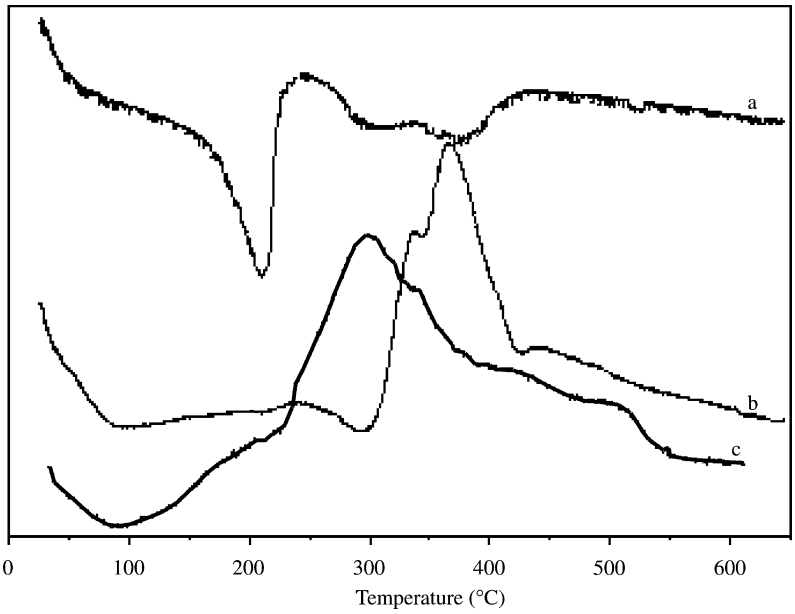

Fig. 4. DTA curves for (a) Mg/Al-carbonate LDH, (b) glutamate-pillared LDH and (c) penicillin acylase immobilized on (b) by introduction of glutaraldehyde linker. The presence of the exotherms in (b) and (c) is associated with the combustion of the intercalated organic material.

attributed [15] to the C=N bond of the Schiff's base linkage formed between glutaraldehyde and amino groups. The mass loss observed in the TG for the immobilized enzyme was almost the same as that for the glutamate-pillared LDH indicating that the amount of PGA immobilized on the glutamate-pillared LDH was low. The corresponding DTA traces for the glutamate-pillared LDHs and immobilized enzyme (Fig. 4(b) and (c)) showed exotherms at 365 and 295 °C, respectively, associated with decomposition of the organic material. Although the amount of enzyme immobilized on glutamate-LDH was low, the area of the DTA peak for the IME (Fig. 4(c)) was larger than that for the glutamate-LDH (Fig. 4(b)), showing that some additional organic material had been incorporated.

The immobilization of PGA on glutamate-pillared LDHs is illustrated schematically in Fig. 5. Reaction of the primary amino groups of the intercalated glutamate ions with glutaraldehyde through Schiff's base formation is followed by covalent coupling of the residual aldehyde groups with the free amino groups of the enzyme. Since the carboxylate groups of the glutamate ions are strongly attracted by the layers and the glutamate ions are covalently bonded to the enzyme, it is hoped that the enzyme will thereby be strongly immobilized on the support.

3.2. Activity of immobilized penicillin acylase

3.2.1. Activity assay

The activity of the LDH-immobilized PGA towards the hydrolysis of penicillin G was assayed in a batch process. Based on the residual activity of the immobilization solution, the immobilization yield of PGA on glutamate–glutaraldehyde-LDH was 79.6%, when a total of 1503 U of enzyme per gram of carrier (dry weight) was employed in the immobilization reaction. In the hydrolysis of penicillin G solution, the expressed activity of the IME was 486.7 U/g, equivalent to an expressed activity of 40.6% of the total immobilized enzyme.

3.2.2. Thermal stability

The thermal stability of both free PGA and PGA immobilized on glutamate-pillared LDHs were compared by storing samples for 4 h at temperatures of 35, 40, 45 and 50 °C in a pH 7.95 buffer solution.

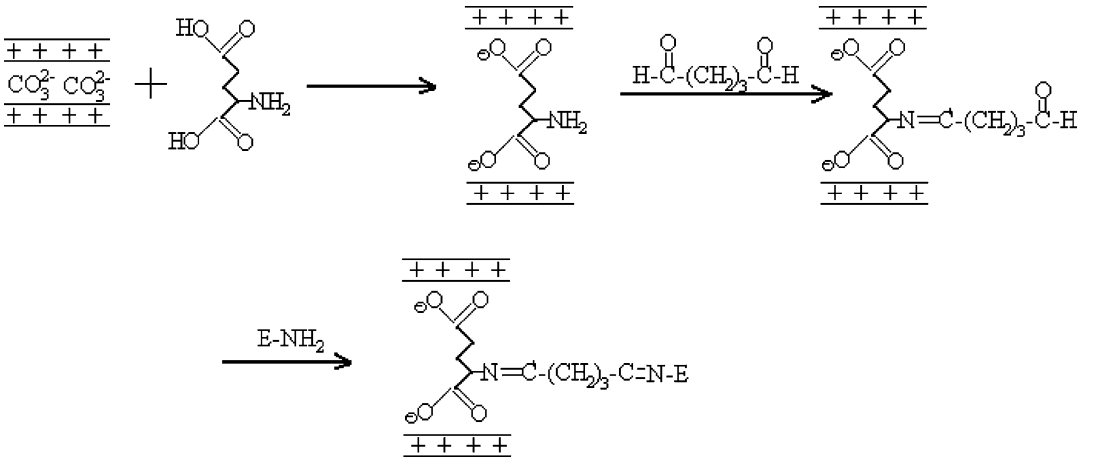

Fig. 5. Schematic illustration of the immobilization of PGA on glutamate-pillared LDHs. Reaction of the primary amino groups of the intercalated glutamate ions with glutaraldehyde through Schiff's base formation is followed by covalent coupling of the residual aldehyde groups with the free amino groups of the enzyme.

As shown in Fig. 6, the activity of free PGA decreased slowly with increasing storage temperature below 45 °C but showed a marked loss in activity after heating at 50 °C. In contrast, the activity of the immobilized PGA showed no significant decrease even when the storage temperature reached 50 °C. Thus, it can be concluded that intercalation between the layers has a significant beneficial effect on the thermal stability of the enzyme.

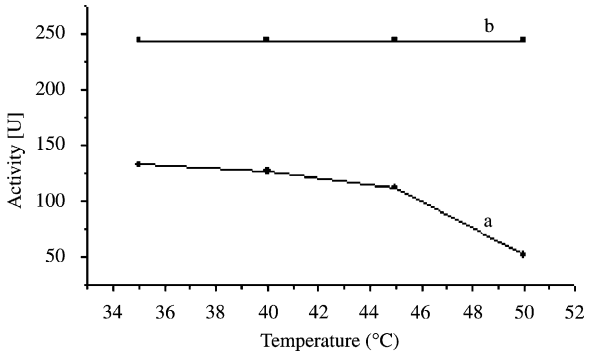

Fig. 6. Activities toward hydrolysis of penicillin G of (a) free and (b) LDH-immobilized penicillin acylase after storing for 4 h at various temperatures in a pH 7.95 buffer solution. About 1 ml of free enzyme and 0.5 g of immobilized enzyme were used for the assays. Immobilization of the enzyme enhances its thermal stability.

3.2.3. pH stability

The activities of both free PGA and immobilized PGA were assayed after being kept for 4 h in various buffer solutions at 37 °C. As shown in Fig. 7, the activity of free PGA showed a maximum after storing at pH 6 and decreased after storing at higher or lower pH. The activity of the immobilized PGA also decreased after storing at pH values above 6 but in contrast to the free enzyme was much more stable at lower pH values. Thus, it can be concluded that immobilizing

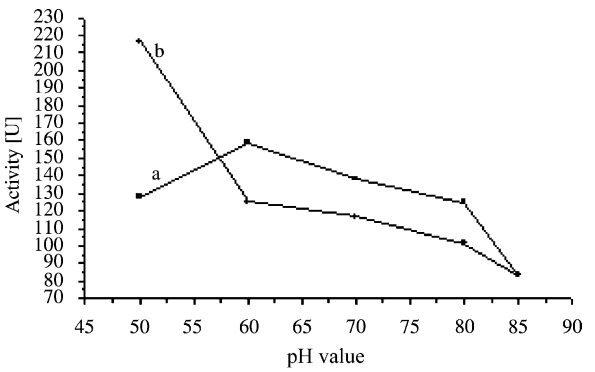

Fig. 7. Activities toward hydrolysis of penicillin G of (a) free and (b) LDH-immobilized penicillin acylase after storing for 4 h at 37 °C in different buffer solutions. About 1 ml of free enzyme and 0.5 g of immobilized enzyme were used for the assays. Immobilization of the enzyme enhances its acid resistance.

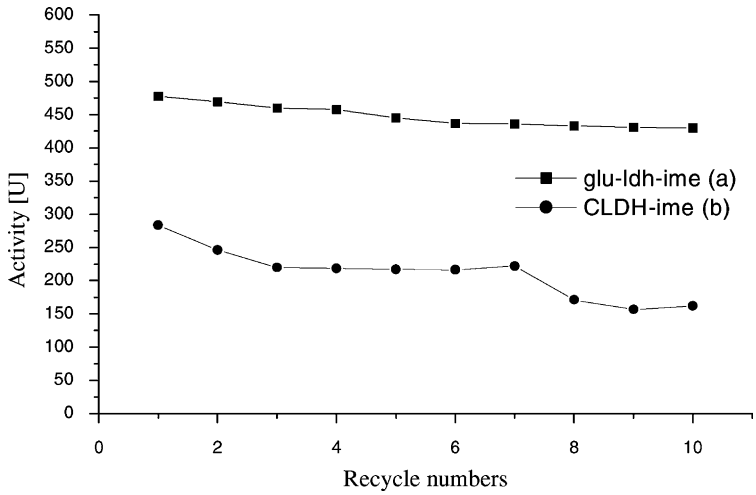

Fig. 8. Operational stability of penicillin acylase immobilized on (a) glutamate-pillared LDH and (b) calcined LDH. Assays were carried out at 37 °C in pH 7.95 buffer solution in a discontinuous batch reactor with a 12 min assay period for each cycle. A total of 1.0 g of immobilized enzyme was used to hydrolyze the penicillin G solution (4%, w/w). Immobilization of the enzyme in the interlayer galleries of LDH gives a superior operational stability compared with immobilization on the calcined LDH support.

the enzyme between the basic sheets of the LDH enhances its acid resistance.

3.2.4. Operational stability

The immobilized PGA was repeatedly used in a discontinuous batch reactor, with filtration and washing between cycles. Assays were carried out at 37 °C in pH 7.95 buffer solution with a 12 min assay period for each cycle. A total of 1.0 g of immobilized enzyme was used to hydrolyze the penicillin G solution (4%, w/w). After 10 recycles the IME displayed 90% activity retention, with the expressed activity remaining above 430 U/g as shown in Fig. 8.

We have previously shown [16] that the materials produced by calcination of layered double hydroxides (which we abbreviate as CLDH), in which the layer structure has been lost, can also be used as a support for immobilization of PGA. As shown in Fig. 8 however, both the expressed activity and the operational stability of the CLDH-supported material are significantly lower than the corresponding values for the glutamate–glutaraldehyde-LDH support. This indicates that by careful design of the chemical nature of the interlayer galleries in LDH it is possible to synthesize a more effective support for the enzyme.

4. Conclusions

Immobilized penicillin G acylase has been assembled in the interlayer galleries of layered double hydroxides by a process involving (i) intercalation of glutamate ions, which are strongly held by interaction between the carboxylate groups and the layers, (ii) reaction of the amino groups of the immobilized glutamate ions with a glutaraldehyde linker followed by (iii) incorporation of the enzyme. The IME possess good thermal stability and excellent acid resistance. After 10 recycles, the IME displayed 90% activity retention, with the expressed activity remaining above 430 U/g.

Acknowledgements

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