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Some factors affecting the immobilization of penicillin G acylase on calcined layered double hydroxides

Lingling Ren [a], Jing He [a], D.G. Evans [a], Xue Duan [a,*], Runyu Ma [b]

[a] *The Key Laboratory of Science & Technology of Controllable Chemical Reactions, Beijing University of Chemical Technology, Ministry of Education, 15 Beisahuan Dong Lu, P.O. Box 32, Beijing 100029, China*

[b] *Department of Biochemical Technology, Beijing University of Chemical Technology, Beijing 10029, China*

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Abstract

A new type of support, calcined layered double hydroxides (CLDH), has been used to immobilize penicillin G acylase. The effect of varying the composition of the layered double hydroxide precursor and the calcination temperature on the activity of the immobilized enzyme has been studied. The activity of the immobilized enzyme decreased with increasing Mg/Al molar ratio of the CLDH. Mg/Al-CLDH had a higher affinity for the enzyme than Zn/Al-CLDH, but a lower percentage expressed activity. The activity of the immobilized enzyme was a maximum when the calcination temperature was between 450 and 550°C. The amount of enzyme adsorbed by the support shows a close correlation with its surface area. Immobilization of the enzyme on the support increases the acid resistance of the former.

Keywords: Immobilized enzyme; Penicillin acylase; Layered double hydroxides; Calcination; Hydrotalcite

1. Introduction

Penicillin G acylase (EC3.5.1.11) (abbreviated as PGA) is often used to produce 6-aminopenicillanic acid (6-APA). When the enzyme is immobilized, products of high purity and high concentration with a minimum amount of side products can be obtained. Furthermore, use of the immobilized enzyme allows the destructive effect of ballast proteins, saccharides and other compounds on the labile β-lactam nucleus of penicillin G and 6-APA to be mitigated.

Many carriers, both organic and inorganic, have been used to immobilize PGA, but efforts to immobilize PGA on newer type of carriers are still underway in many laboratories with the goals of improving the catalytic efficiency of the enzyme and reducing the cost of the supports.

Calcination of layered double hydroxides (LDH or hydrotalcite-like materials, which have the general formula $[M_{1-x}{}^{2+}M_x{}^{3+}(OH)_2]^{x+}(X^{n-})_{x/n} \times mH_2O$) affords calcined layered double hydroxides (CLDH), sometimes known as layered double oxides (LDO), which have porous structures, large specific surface areas and abundant basic sites to bind with an enzyme. We have investigated these latter materials as a potential support for PGA [1]. Of key importance, is the ability to control both the particle size and the particle size distribution of the LDH precursor by varying the synthesis conditions according to the requirements of the PGA immobilization process, using a new synthetic procedure developed in our laboratory [2].

* Corresponding author. Tel.: +86-1-64425395; fax: +86-1-6445385.
E-mail address: duanx@buct.edu.cn (X. Duan).

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The aim of the present work is to investigate the effect of varying the composition of the layered double hydroxide precursors and the calcination temperature on the activity of the CLDH-immobilized PGA.

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 LDH containing carbonate as the interlayer anion was synthesized by a modified co-precipitation method as described elsewhere [2]. The method involves very rapid mixing under vigorously stirred conditions to complete the nucleation process, followed by a separate aging process. $Mg(NO_3)_2{\cdot}6H_2O$ (92.3 g, 0.36 mol) and $Al(NO_3)_3{\cdot}9H_2O$ (67.5 g, 0.18 mol) were dissolved in deionized water. A second solution containing NaOH (34.6 g, 0.86 mol) and Na_2CO_3 (38.2 g, 0.36 mol) in deionized water was prepared. The two solutions were simultaneously added to a colloid mill rotating at 5000 rpm and stirred for 2 min. The resulting slurry was removed from the colloid mill and aged at 100°C for a specified period. The final precipitate was filtered, washed thoroughly with water and dried.

CLDH was subsequently produced by calcination of LDH at different temperatures for 3 h.

A phosphate buffer solution of pH 5.87 was added to CLDH under N_2 protection until the pH value was below 7.0. Free PGA (164.2 IU/ml) was added after 3 min (1 IU corresponds the amount of enzyme which cleaves 1 μmol of penicillin G to 6-APA per minute at pH7.9 and 37°C). The suspension was shaken for 30 h at 30°C. The immobilized PGA was recovered by centrifugation, washed and dried at ambient temperature. A pink solid was obtained.

Activities of both soluble and immobilized enzymes expressed in the hydrolysis of penicillin G solution (4%, w/w) in 0.05N pH 7.9 phosphate buffer were measured using the pH-stat method at 37°C for 12 min [3].

X-Ray powder diffraction (XRD) data were collected on a Siemens D5005 powder diffractometer, using Cu Kα radiation between 3 and 70° using step-scans of 0.02° (2θ).

3. Results and discussion

3.1. Blank experiments

The activity of the enzyme was determined by titration (with aqueous sodium hydroxide) of the phenylacetic acid produced along with 6-APA in the hydrolysis of penicillin G. Blank experiments with substrate (penicillin G) alone and immobilized enzyme alone were performed. It was found that in each case negligible quantities of sodium hydroxide were required to reach the titration endpoint, confirming that the alkali consumed in the assay is an accurate method of determining the amount of phenylacetic acid produced in the hydrolysis reaction.

3.1.1. Effect of Mg/Al molar ratio on the activity of the immobilized enzyme

LDHs with Mg/Al molar ratios of 1.6, 2.0, 3.0, 4.0 were calcined at 500°C for 3 h and subsequently used as supports to immobilize the enzyme as described in Section 2.

As shown in Fig. 1a, for the case of Mg/Al = 2, the LDH precursors have the characteristic X-ray powder

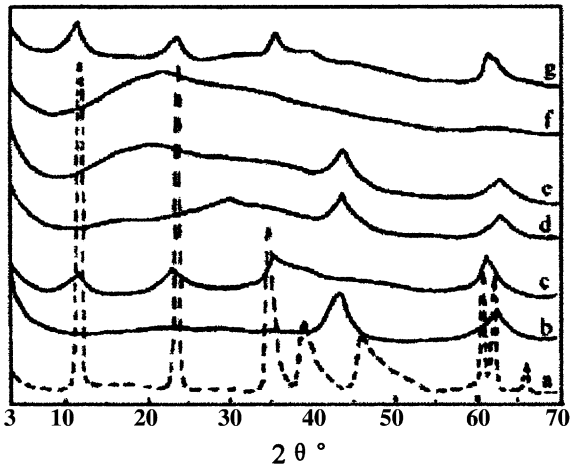

Fig. 1. X-ray powder diffraction patterns of (a) LDH (Mg/Al = 2); (b) CLDH (Mg/Al = 2); (c) CLDH (Mg/Al = 2)-buffer; (d) CLDH (Mg/Al = 1.6)-immobilized PGA; (e) CLDH (Mg/Al = 2.0)-immobilized PGA; (f) CLDH (Mg/Al = 3.0)-immobilized PGA; and (g) CLDH (Mg/Al = 4.0)-immobilized PGA.

Table 1
Effect of Mg/Al molar ratio on the activity of the immobilized PGA

Mg/Al molar ratio	Free enzyme loaded (IU/g)	Enzyme adsorbed[a]		IME assay[b]			
		(IU/g)	Adsorbed (%)	1st cycle		2nd cycle	
				Activity (IU/g)	Expressed (%)	Activity (IU/g)	Expressed (%)
1.6	1647.5	1629.3	98.9	511.6	31.4	402.3	24.7
2.0	1627.4	1617.2	99.4	446.3	27.6	435.2	26.9
3.0	1629.5	1576.2	96.7	324.7	20.6	317.5	20.1
4.0	1628.4	1435.7	88.2	147.0	10.2	147.0	10.2

[a] Based on the residual activity of the immobilization solution.
[b] Based on the activity of the bound enzyme.

diffraction pattern of a layered hydrotalcite-like material. After calcination at 500°C, the layered structure is lost and the XRD pattern (Fig. 1b) resembles that of poorly crystalline magnesium oxide as has been reported elsewhere [4]. If the CLDH is treated with an aqueous solution of phosphate buffer, as shown in Fig. 1c, a poorly crystalline LDH material is regenerated. This phenomenon has been widely documented [5]. The reaction was carried out in air, so that although in principle a phosphate ion intercalated LDH phase might have been expected, in fact a carbonate ion intercalated phase produced by reaction with atmospheric carbon dioxide results. It is well-known that LDH materials have a very high selectivity for carbonate at the expense of other ions so that unless the regeneration is carried out under a nitrogen atmosphere, carbonate phases often result. Interestingly, when the CLDH is reacted with an aqueous solution containing the phosphate buffer and PGA under the same conditions, reconstruction of the LDH structure to different extents is observed. As shown in Fig. 1d and e, with Mg/Al molar ratios of 1.6 and 2.0, the XRD patterns are similar to those of CLDH itself indicating that adsorption of PGA on the surface of CLDH prevents regeneration of the LDH by dissolution of the solid and re-precipitation of an LDH phase or other pathways [6]. This may be because the base strength is lower when the Mg/Al molar ratio is small. However, when the Mg/Al molar ratio is 3.0, as shown in Fig. 1f, the XRD pattern is that of an amorphous material. When the Mg/Al molar ratio is 4.0, as shown in Fig. 1g, the XRD pattern is that of a poorly crystalline LDH structure indicating that adsorption of PGA on the surface of CLDH does not prevent regeneration of the LDH. This may be a consequence of the higher base strength associated with increasing Mg/Al molar ratio.

As shown in Table 1, the amount of enzyme adsorbed and the activity of the immobilized enzyme decreases with increasing Mg/Al molar ratio. It is known [7] that reconstruction of LDH by hydration of CLDH is associated with a decrease in surface area, consistent with these results. When subsequently filtered, washed and reused in a second reaction, however, the activity of the immobilized enzyme showed a smaller decrease with increasing Mg/Al molar ratio. It may be that the increasing strength of the interaction between the support and enzyme is a result of the increasing base strength associated with increasing Mg/Al ratio.

3.1.2. Effect of calcination temperature on the activity of the immobilized enzyme

The effect of calcination temperature on the activity of the immobilized enzyme (IME) was investigated by adding 80 ml of 0.5N pH 5.87 phosphate buffer to 0.5 g CLDH (Mg/Al = 2) calcined at different temperatures followed by addition of 3.5 ml PGA and shaking for 30 h as described in Section 2.

As shown in Fig. 2a, the LDH precursor with molar ratio Mg/Al = 2 has the characteristic X-ray powder diffraction pattern of a layered hydrotalcite-like material. After calcination at 230°C, the layered structure is retained (Fig. 2b). However, after calcination between 350 and 650°C, the layered structure is lost and the XRD patterns (Fig. 2c, d, f and g) resemble that of poorly crystalline magnesium oxide as has been reported previously [4]. After calcination at 850°C, segregation of magnesium oxide and spinel phases is observed as reported elsewhere [8].

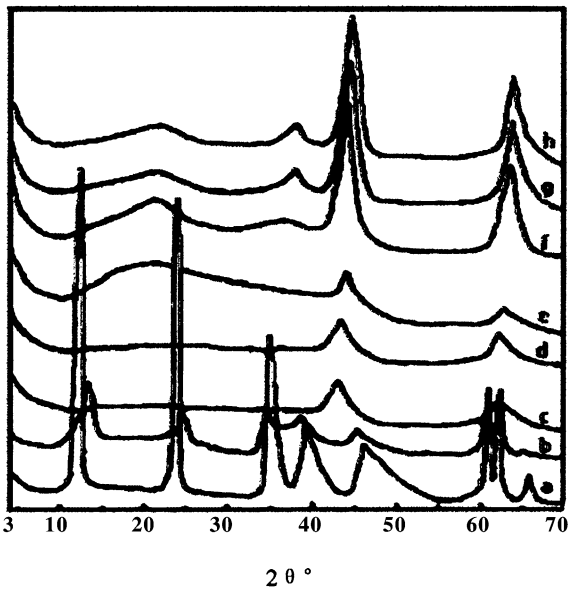

Fig. 2. X-ray powder diffraction patterns of (a) LDH (Mg/Al=2); (b) CLDH (230°C); (c) CLDH (350°C); (d) CLDH (450°C); (e) CLDH (450°C)-immobilized PGA; (f) CLDH (550°C); (g) CLDH (650°C); and (h) CLDH (850°C).

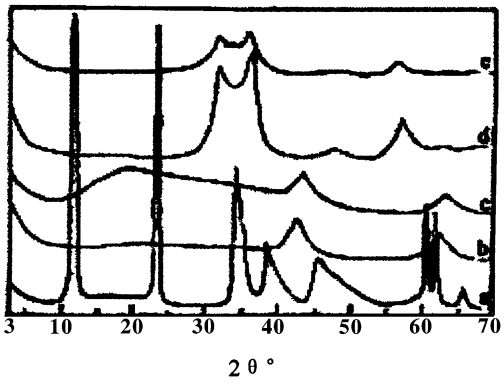

Fig. 3. X-ray powder diffraction patterns of (a) Zn/Al-LDH (Zn/Al = 2); (b) CLDH (Mg/Al) (500°C); (c) CLDH (Mg/Al) (500°C)-immobilized PGA; (d) CLDH (Zn/Al) (500°C); and (e) CLDH (Zn/Al) (500°C)-immobilized PGA.

The data reported in Table 2 show that the amount of enzyme adsorbed increases as the calcination temperature is increased from 230–450°C and then remains essentially constant between 450 and 650°C. Reichle et al. [9] have shown that the surface area of the CLDH reaches a maximum after calcination at 400°C and remains essentially constant between 400 and 600°C, which is consistent with these results since solids with highest surface area can be expected to adsorb the maximum amount of enzyme. Above 650°C, the amount of enzyme adsorbed decreases markedly. Segregation of the spinel phase is known [9] to be accompanied by a significant decrease in surface area so that a decrease in affinity for the enzyme is expected on the basis of the above arguments.

3.1.3. Effect of the composition of the LDH precursor on the activity of the immobilized enzyme

To determine the effect of the composition of the LDH precursor on the immobilized PGA, LDHs with molar ratio Zn/Al = 2 and Mg/Al = 2 were calcined at 500°C for 3 h and subsequently used to immobilize PGA as described in the Experimental section.

As shown in Figs. 2a and 3a, the Mg/Al and Zn/Al-LDH precursors have the characteristic X-ray powder diffraction pattern of a layered hydrotalcite-like material. After calcination at 500°C, the layered

Table 2
Activities of the immobilized enzyme on CLDH calcined at different temperatures

CLDH calcination temperature (°C)	Free enzyme loaded (IU/g)	Enzyme adsorbed[a]		IME assay[b]	
		Activity (IU/g)	Adsorbed (%)	Activity (IU/g)	Expressed (%)
230	1624.2	1468.3	90.4	312.7	21.3
350	1623.5	1508.2	92.9	398.2	26.4
450	1625.6	1606.1	98.8	432.0	26.9
550	1624.7	1611.7	99.2	438.4	27.2
650	1626.3	1597.0	98.2	236.4	14.8
850	1624.8	616.3	37.9	235.0	38.1

[a] Based on the residual activity of the immobilization solution.
[b] Based on the activity of the bound enzyme.

Table 3
Effect of the composition of the LDH precursor on the activity of the immobilized PGA

Composition of LDH	Free enzyme loaded (IU/g)	Enzyme adsorbed[a]		IME assay[b]			
		IU/g	Adsorbed (%)	1st cycle		2nd cycle	
				Activity (IU/g)	Expressed (%)	Activity (IU/g)	Expressed (%)
Zn/Al = 2	1628.3	1097.5	67.4	497.2	45.3	259.0	23.6
Mg/Al = 2	1627.4	1617.2	99.4	446.3	27.6	435.2	26.9

[a] Based on the residual activity of the immobilization solution.
[b] Based on the activity of the bound enzyme.

structures are lost (as shown by the XRD patterns in Fig. 3b and d). The XRD pattern of the Zn/Al-LDH resembles that of a poorly crystalline zinc oxide as has been reported elsewhere [10]. As shown in Fig. 3c and e, the XRD patterns of the CLDH-immobilized PGA materials are similar to those of the parent CLDH itself for both Mg/Al and Zn/Al-CLDHs indicating that adsorption of PGA on the surface of the CLDH prevents regeneration of the LDH by dissolution of the solid and re-precipitation of an LDH phase or other pathways [6].

The results given in Table 3 show that the Mg/Al-CLDH has a higher adsorption capacity for the enzyme than the Zn/Al-CLDH, but the expressed activity is lower. The surface area of Zn/Al-CLDH is known to be much lower than that of the Mg/Al analogue [11] consistent with this result. When subsequently reused however, the Zn/Al-CLDH-immobilized enzyme shows a marked drop in activity whereas that of the Mg/Al-CLDH is essentially unchanged. This is consistent with the base strength of the Zn/Al-CLDH being significantly weaker than that of the Mg/Al analogue [11].

3.1.4. pH stability

The activities of the both the free enzyme and the immobilized enzyme (Mg/Al = 2, calcined at 500°C) were assayed after being kept for 4 h in various buffer solutions at 37°C. As shown in Fig. 4, the activity of the free enzyme decreased markedly after being stored below pH 5, but the immobilized enzyme retained its activity even after keeping it at pH 3. The basic nature of the CLDH support presumably stabilizes the enzyme at low pH, thus enhancing its acid resistance.

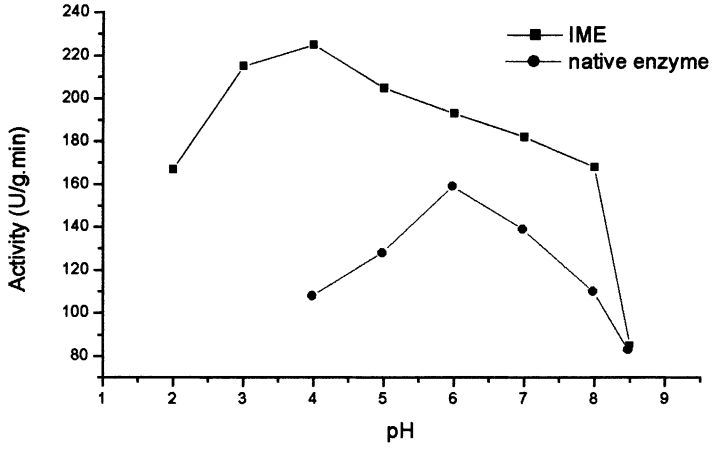

Fig. 4. Activities of free and immobilized enzyme after storage for 4 h at different pH values. About 1 ml of free enzyme and 0.5 g dry weight of IME, respectively were used for the assays.

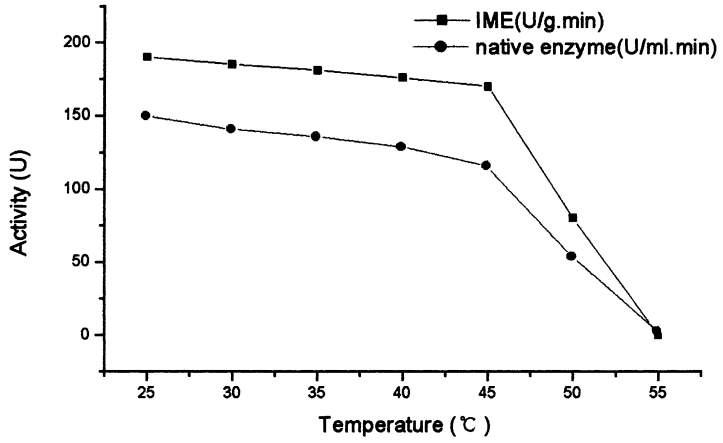

Fig. 5. Activities of free and immobilized enzyme after storage for 4 h at different temperatures. About 1 ml of free enzyme and 0.5 g dry weight of IME, respectively were used for the assays.

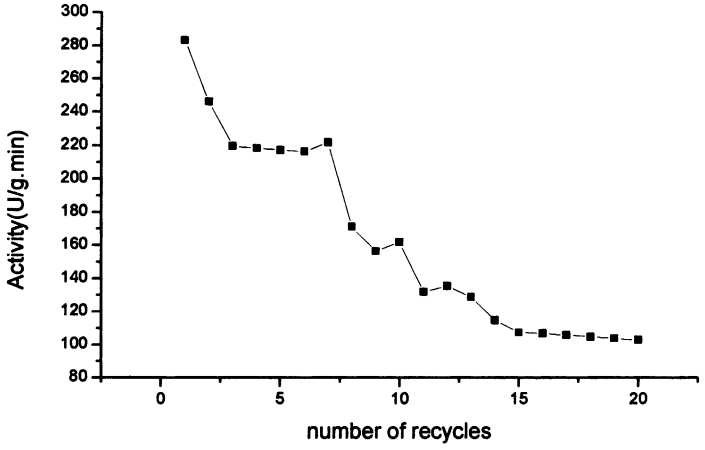

Fig. 6. Operational stability of immobilized PA at 37°C in a discontinuous batch reactor.

3.1.5. Thermal stability

The activities of the both the free enzyme and the immobilized enzyme (Mg/Al = 2, calcined at 500°C) were assayed after being kept at various temperatures in a pH 7.95 buffer solution. As shown in Fig. 5, the activity of both free and immobilized enzymes decreased on storing above 40°C. Thus, it appears that the support has no effect on the thermal stability of the enzyme.

3.1.6. Operational stability

The operational stability in repeated reactions of the immobilized enzyme (Mg/Al = 2, calcined at 500°C) was determined by means of reactions carried out in a discontinuous batch reactor. As shown in Fig. 6, after 15 cycles the immobilized enzyme displayed 36% activity retention. Between 15 and 20 cycles there was no further loss in activity, with the expressed activity remaining above 100 U/g.

4. Conclusions

Calcined layered double hydroxides are effective supports for penicillin G acylase. It was found that the activity of the immobilized enzyme decreased with increasing Mg/Al molar ratio of the CLDH. Mg/Al-CLDH had a higher affinity for the enzyme

than Zn/Al-CLDH, but a lower percentage expressed activity. The activity of the immobilized enzyme was the highest when the calcination temperature was 450–550°C. The immobilization of other enzymes on CLDHs will be examined in the future in order to extend the range of its application as a support.

Acknowledgements

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References

[1] L.L. Ren, J. He, D.G. Evans, X. Duan, in: Y.H. Sun, B. Zhong, J.G. Wang (Eds.), New Century Catalytic Science and Technology, 2000, p. 1087.
[2] X. Duan, L. Li, Q.Z. Jiao, Chinese Patent CN9,911,385, 1999.
[3] D. Bianchi, R. Golini, R. Bortolo, P. Cesti, Enzyme Microb. Technol. 18 (1996) 592.
[4] W.T. Reichle, J. Catal. 94 (1985) 547.
[5] A. Legrouri, M. Badreddine, A. Barroug, A. De Roy, J.P. Besse, J. Mater. Sci. Lett. 18 (1999) 1077.
[6] F. Millange, R.I. Walton, D. O'Hare, J. Mater. Chem. 10 (2000) 1713.
[7] F. Malherbe, C. Forano, J.P. Besse, Microporous Mater. 10 (1997) 67.
[8] M.A. Aramendia, Y. Aviles, V. Borau, J.M. Luque, J.M. Marinas, J.R. Ruiz, F.J. Urbano, J. Mater. Chem. 9 (1999) 1603.
[9] W.T. Reichle, S.Y. Kang, D.S. Everhardt, J. Catal. 101 (1986) 352.
[10] V. Prevot, C. Forano, J.P. Besse, F. Abraham, Inorg. Chem. 37 (1998) 4293.
[11] W. Kagunya, Z. Hassan, W. Jones, Inorg. Chem. 35 (1996) 5970.