Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Molecular Catalysis B: Enzymatic 27 (2004) 29–35
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Activity and stability of immobilized penicillin amidase at low pH values

Juliana S. Ferreira [a,b], Adrie J.J. Straathof [a,*], Telma T. Franco [b], Luuk A.M. van der Wielen [a]

[a] *Department of Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands*
[b] *Chemical Engineering School, State University of Campinas, P.O. Box 6066, 13081-970 Campinas, SP, Brazil*

Received 3 February 2003; received in revised form 12 August 2003; accepted 2 September 2003

Abstract

Penicillin amidase is being applied widely in the production of semi-synthetic β-lactam antibiotics. Usually the processes are at pH 7–8, but for many new applications the range of pH 3–6 is of interest too. Therefore, we studied the activity of penicillin amidase at 25 °C in potassium phosphate buffer of pH 3.7–9, as well as its stability in potassium phosphate buffer of pH 3–6. At each pH, the enzyme was stable during at least 32 days. On the other hand, immobilized penicillin amidase incubated in butyl acetate lost its stability, showing after 32 days a decrease of 52% in relation to its initial enzymatic activity value. In phosphate buffer, the enzyme showed the highest activity at pH 8–9. A gradual decrease to about 20% of this activity occurred when the pH was decreased to 3.7. At even lower pH, the enzyme activity could not be determined with the assay that was used due to a very low stability of penicillin G (PenG). The course of penicillin G conversion and 6-aminopenicillanic acid (APA) production, during enzymatic hydrolysis at pH 4, could be quantitatively described by a simple model when the thermodynamic equilibrium of the hydrolysis was taken into account.

Keywords: Penicillin acylase; Immobilized enzyme; Two-phase reaction; Penicillin G hydrolysis; pH optimization

1. Introduction

Enzymes are extensively used in the food, pharmaceutical, and chemical industries, as well for analytical purposes. One particularly important enzyme is penicillin amidase (E.C. 3.5.1.11) [1,2]. Enzymatic hydrolysis of penicillins to 6-aminopenicillanic acid (APA) and phenylacetic acid (PAA) by penicillin amidases is applied widely in the industry. Therefore, much research has been devoted to the kinetics of the hydrolysis of penicillin G (PenG) by penicillin amidase as a function of the concentration of substrate and products, pH, temperature, buffer type, and concentration, and cosolvent type and concentration [3–7]. Most of these papers focus on pH 7–8, the optimum range for PenG hydrolysis in purely aqueous systems and do not provide systematic studies at lower pH.

Novel reactor configurations are being investigated for enzymatic conversions, to enhance their yield and rate, which is in many cases low or even too low for feasible processes. In particular, integrated reactor–separator systems allowing for in situ product removal (ISPR) offer potential improvements, i.e., two-phase reactors [8]. These systems are tuned at high volumetric capacity, and will hence operate under extreme conditions relative to the “normal” physiological operation of enzymes [2].

The biphasic system allows the separation of both reaction products due to extraction of PAA to the organic phase and precipitation of APA [9,10]. Nevertheless, in a two-phase system, a pH increase above pH 4.4 hampers extraction of phenylacetic acid into the organic phase, prevents APA precipitation, and in contrast to the homogeneous reaction, does not improve hydrolysis [11].

For penicillin hydrolysis, a counter-current multi-stage reactor without pH control is being developed [12]. In this reactor, the pH drops to values as low as 3, depending on the progress of the reaction and concentration of substrate. Therefore, it is worthwhile to study enzyme stability and

* Corresponding author. Tel.: +31-15-278-2330; fax: +31-15-278-2355.
E-mail address: a.j.j.straathof@tnw.tudelft.nl (A.J.J. Straathof).

1381-1177/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2003.09.003

Nomenclature

A	enzyme activity (μmol/(g_{enz} min))
C	concentration of PenG (mM)
C_{enz}	concentration of enzyme (g/l)
F	fraction of the specie
k_d	degradation rate constant (h^{-1})
k_{enz}	enzymatic rate constant (l/(g min))
K_{app}	apparent equilibrium constant (mol/l)
K_{eq}	equilibrium constant (mol/l)
m_{enz}	mass of enzyme (g)
n	amount (mol)
r_{enz}	enzymatic reaction rate (mol/(l min))
T	temperature (°C)
$t_{half\text{-}time}$	half-life time (h)

Subscripts

0	initial situation
APA	6-aminopenicillanic acid
minus	negative state of ionization
minus/plus	zwitterionic state of ionization
PAA	phenylacetic acid
PenG	penicillin G
plus	positive state of ionization
uncharged	neutral state of ionization

activity under these extreme conditions including the pH range 3–4. Furthermore, a relatively low pH has been used for many synthesis reactions catalyzed by penicillin amidase for which commercial processes are under development [13–18]. Such as, the syntheses of loracarbef at pH 6, amoxicillin at pH 5.5–7, xemilofiban intermediate at pH 5.25–6.25, cefamandole at pH 4.25, and ampicillin at pH 6.3. A pH below 5 is believed to have a detrimental effect on penicillin amidase stability and activity [16,17].

For an evaluation of enzymatic hydrolysis of PenG at low pH, the stability of PenG and the main product, APA, have to be evaluated. Phenylacetic acid is known to be stable. Penicillin G is unstable in aqueous solution at acid or alkaline conditions [19–27] and the half-life may change if the aqueous phase becomes saturated by butyl acetate [20]. The decomposition kinetics is first order with maximum stability about pH 6 and the degradation rate of penicillin is much higher at a low pH than at a high pH. There is not much information about degradation of APA in the literature. Studying APA degradation in the pH and temperature ranges of 5.8–6.6 and 35–90 °C, respectively, Dennen [28] showed that the degradation follows pseudo first-order kinetics. At higher pH, higher orders of degradation rates were determined. Furthermore, the author found in all temperature ranges the highest stability at approximately pH 8.

In this work, we will only consider a temperature of 25 °C which is used for counter-current reactor development [10,12]. In the counter-current reactor, the aqueous phase will be saturated with butyl acetate, and the influence of this solvent will be studied as well.

2. Material and methods

2.1. Materials

Potassium di-hydrogen phosphate (anhydrous extra pure), di-potassium hydrogen phosphate, 85% ortho-phosphoric acid, potassium hydroxide pellets, butyl acetate (BuAc), and acetonitrile were purchased from Merck (Darmstadt, Germany). Phenylacetic acid (PAA) was purchased from Fluka Chemika (Steinheim, Switzerland). All reagents were of analytical grade. Penicillin G potassium salt (PenGK), 6-aminopenicillanic acid (APA), and Assemblase® were kindly provided by DSM, Delft, The Netherlands. Assemblase® is an immobilized penicillin G amidase from *Escherichia coli*, an in-house enzyme of DSM anti-infectives. The enzyme is supplied in a propylene glycol–water solution. The enzyme was washed with Milli-Q water prior use and filtered to dryness.

2.2. Standard enzyme assay

The standard assay was performed at pH 8.0 in a magnetically stirred glass reactor at 25 °C. PenGK salt (1.00 g) was dissolved in 50 ml potassium phosphate buffer (40 mM) saturated with butyl acetate (BuAc). The reaction was started by the addition of 450–500 mg of enzyme. The pH was kept constant, using 0.250 M KOH in a digital burette.

During the initial part of the reaction (5 min), the burette reading was recorded. At pH 8.0, the number of the moles of KOH addition corresponds to the number of moles of PenG (n_{PenG}) converted into in APA and PAA. These assays were carried out in duplicate. The initial reaction rate was determined from linear regression of time-course profile and the enzyme activity (A) was calculated from:

$$A = \frac{n_{PenG0} - n_{PenG}}{m_{enz} time} \quad (1)$$

2.3. Degradation of penicillin G and 6-aminopenicillanic acid

The degradation of PenG was evaluated at pH 3.0, 3.5, 4.0 and 4.5, in a magnetically stirred glass reactor at 25 °C. PenGK salt (1.00 g) was dissolved in 50 ml potassium buffer (40 mM) saturated with BuAc. The pH was controlled using a pH stat system with H_3PO_4 (40 mM) as titrant. To prevent significant dilution, the pH control procedure was preceded by a pH adjustment with some drops of H_3PO_4 (85%) for pH 3.0, H_3PO_4 (0.5 M) for pH 3.5 and H_3PO_4 (0.1 M) for pH 4.5 and 4.0.

Samples were withdrawn, filtered (Millex by Millipore, 0.45 μm pore size), and added to Eppendorf tubes filled with 1.5 ml of cold (4 °C) KOH solution in a way to adjust the pH

of the sample to approximately 6.5–7.5. The samples were analyzed for PenG in duplicate by HPLC.

For the degradation of APA, experiments were performed at pH 3.5, 4.0, 4.5, and 5.0 in similar procedure. However, after samples were withdrawn and filtered, 1 ml was diluted into 25 ml before HPLC analysis for APA.

2.4. Influence of pH on enzyme activity

Reactions at pH 4.0, 4.5, 5.0, 6.0, 7.0, 8.0, and 9.0 were carried out to determine the enzyme activity. The standard assays were allowed to take place in 40 mM potassium phosphate buffers saturated with BuAc. The buffers were prepared by mixing different volumes of aqueous solutions of K_2HPO_4, KH_2PO_4, and H_3PO_4. The titration solutions were KOH (0.25 M) for pH 5.0, 6.0, 7.0, 8.0, and 9.0 and H_3PO_4 (40 mM) for pH 4.0 and 4.5. Especially, for 4.0 and 4.5, the pH of the reaction medium had to be corrected with H_3PO_4 solution (0.1 M), right after adding PenGK salt, since the pH increased as soon as PenGK was added.

At pH values below 7.0, the number of moles of acid or base added in the titration method does not correspond to the number of moles of PenGK transformed in products. The dissociation state of the reactants should be taken into account. As the pH decreases, the fractions of the negative forms decrease ($F_{\text{PenG minus}}$, $F_{\text{PAA minus}}$, and $F_{\text{APA minus}}$) while fractions of the positive ($F_{\text{APA p1us}}$), zwitterionic ($F_{\text{APA minus/plus}}$), or uncharged ($F_{\text{PenGuncharged}}$, $F_{\text{PAA uncharged}}$, and $F_{\text{APA uncharged}}$) forms start to be produced [10]. Therefore, at low pH the reaction is not acidifying anymore, but producing base. The net charged fractions of each component are shown in Fig. 1.

For these species, the charged amounts are related to the overall amounts as follows:

$$n_{\text{APA plus}} = F_{\text{APA plus}} \times n_{\text{APA}} \tag{2}$$

$$n_{\text{APA minus}} = F_{\text{APA minus}} \times n_{\text{APA}} \tag{3}$$

$$n_{\text{PenG minus}} = F_{\text{penG minus}} \times n_{\text{PenG}} \tag{4}$$

$$n_{\text{PAA minus}} = F_{\text{PAA minus}} \times n_{\text{PAA}} \tag{5}$$

These charged amounts can be substituted in the charge balance as

$$\begin{aligned} n_{\text{K plus}} + n_{\text{APA plus}} + n_{\text{H plus}} &= n_{\text{H}_2\text{PO}_4\text{ minus}} + 2n_{\text{HPO}_4\text{ minus2}} + n_{\text{PenG minus}} \\ &\quad + n_{\text{APA minus}} + n_{\text{PAA minus}} + n_{\text{OH minus}} \end{aligned} \tag{6}$$

The stoichiometric balances are:

$$n_{\text{PenG0}} = n_{\text{PenG}} + n_{\text{APA}} = n_{\text{PenG}} + n_{\text{PAA}} \tag{7}$$

after eliminating n_{APA} and n_{PAA} Eq. (8) is obtained.

$$n_{\text{PenG}} = \frac{-n_{\text{K plus}} + (-F_{\text{APA plus}} + F_{\text{APA minus}} + F_{\text{PAA minus}}) \times n_{\text{PenG0}} - n_{\text{H plus}} + n_{\text{H}_2\text{PO}_4\text{ minus}} + 2(n_{\text{HPO}_4\text{ minus2}} + n_{\text{OH minus}})}{-F_{\text{APA plus}} - F_{\text{PenG minus}} + F_{\text{APA minus}} + F_{\text{PAA minus}}} \tag{8}$$

Knowing the values of the added amounts of potassium, phosphate, and penicillin, the activity can be calculated.

Besides the recording of the burette reading, in one of the duplicate series of experiments, four to six samples (500 μl) were withdrawn from the reaction mixture after the start of the reaction with 1 min intervals and filtered (Millex by Millipore, 0.45 μm pore size) to remove the enzyme and stop the reaction. As the reaction occurred, APA and PAA were formed and they were detected by HPLC. The initial reaction rate was determined based on the amount of APA produced. For the assays at pH 4.0 and 4.5, the filtrate was collected in Eppendorf tubes filled with 1.5 ml of cold (4 °C) KOH, in order to correct the pH to 6.5–7.5 and avoid decomposition of the compounds.

2.5. Influence of pH on enzyme stability

Potassium phosphate buffer (40 mM) at pH 3.0, 4.0, 5.0, and 6.0, was mixed with BuAc. The saturated phases were

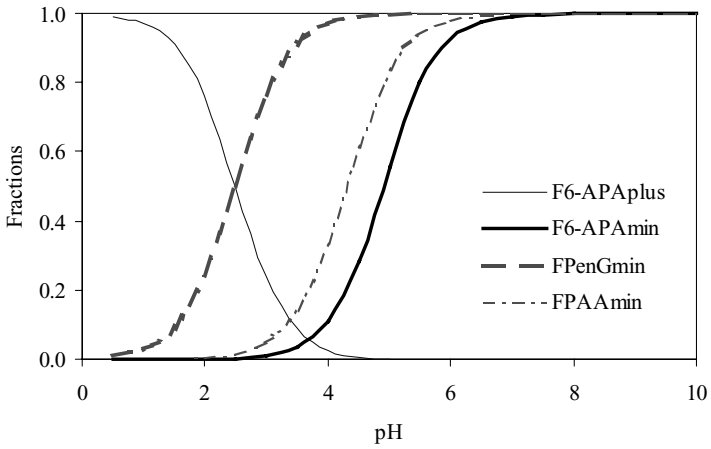

Fig. 1. Fraction of the charged species of the reactants as part of each reactant's total concentration. Calculation performed with the dissociation constants in [14].

separated with a separation funnel. After washing with water and filtration to dryness, the enzyme (450–500 mg) was incubated in 1 ml of five different solutions, namely the four potassium phosphate buffers of pH 3.0, 4.0, 5.0, and 6.0 saturated with BuAc and the BuAc solution saturated with potassium phosphate buffer of pH 6.0. The incubation was performed in closed Eppendorf tubes at 25 °C, for different intervals. After this interval, the remaining activity was measured. The content of the Eppendorf tube was transferred to the reaction vessel, mixed with 49 ml of potassium phosphate buffer of pH 8.0 saturated with BuAc, and the standard enzyme assay was performed.

2.6. HPLC analysis

The samples were analyzed by HPLC (Waters), using a C18-Delta Pak column (particle size: 5 μm, pore size: 300 Å, column size: 3.9 mm × 150 mm), and C18-Platinum EPS column (particle size: 5 μm, pore size: 100 Å, column size: 4.6 mm × 250 mm), and UV detector. The mobile phase was 28:72 (v/v) of acetonitrile and 0.64 g/l KH_2PO_4 aqueous solution, the pH was adjusted to 2.75 with H_3PO_4. The flow was 0.7 ml/min, in the Delta Pak column and 1.0 ml/min in the Platinum EPS column. The elution times of APA, PAA, and PenG were 2.2, 5.3, and 9.0 min, respectively, when analyzed in the Delta Pak column and 3.3, 7.7, and 13.9 min in the Platinum EPS column.

3. Results and discussion

3.1. Influence of pH on degradation of penicillin G and 6-aminopenicillanic acid

During enzymatic activity, assays at low pH the chromatograms showed peaks not identified as APA, PAA or PenG, indicating that spontaneous degradation of some of the reactants occurred. Therefore, this background degradation of PenG and APA was investigated.

At pH 4.5, in the absence of enzyme, there was no degradation of PenG within 5 min of reaction, the duration of the enzymatic assay. However, a peak with retention time of 4.8–5.0 min was present in the chromatograms of pH 3.0–4.0. No APA or PAA was formed.

Logarithmic plots of PenG concentration against time were linear for both pH values indicating first-order degradation. The degradation rate constants were determined from the slope of these graphs. The values of the degradation rate constants (see Table 1) correspond to half-life times of 21, 210, and 780 min at pH 3.0, 3.5, and 4.0, respectively. Table 1 also presents the data reported in the literature. The results are within a wide range of values, probably due to the different methods employed to determine the concentration of PenG. We assume that the HPLC method used by us is the most accurate method. According to the last two entries in Table 1, butyl acetate does not influence the PenG degradation.

Table 1
Values of degradation rate constant of PenG in aqueous solution

pH	T (°C)	k_d (h^{-1})	$t_{half\text{-}life}$ (h)	Reference
3.0	25	1.932	0.35	This work[a]
3.0	25	1.002	0.68	[24]
3.0	35	1.140	0.60	[25]
3.0	24	0.294	2.33	[21]
3.5	25	0.198	3.50	This work[a]
3.5	25	0.252	2.75	[24]
3.5	35	0.450	1.53	[25]
4.0	25	0.053	13	This work[a]
5.0	25	0.011	64.2	[20]
5.0	25	0.096	72.2	[20][a]

[a] Aqueous solution saturated with BuAc.

Degradation of APA was determined at pH 3.5, 4.0, 4.5, and 5.0 and it turned out to be much slower than degradation of PenG. The values of first-order degradation rate constants are shown in Table 2.

3.2. Influence of pH on enzyme activity

In order to check the reliability of reaction set up, some preliminary assays were carried out in aqueous and in phosphate buffer solution (results not shown). The most reliable results were obtained with phosphate buffer solution, since the variation of pH is large in unbuffered aqueous solution and the accuracy of the results was low.

Concerning the influence of BuAc on the enzyme activity, no difference was found between the data obtained from preliminary assays involving potassium phosphate buffer and from assays involving potassium phosphate buffer saturated with BuAc. This is in agreement with previous research on the influence of organic solvents on penicillin amidase [26,27].

The influence of pH on penicillin amidase activity is shown in Fig. 2, which presents results from both the titration method and HPLC analysis. The highest activity is about 280 μmol/(g_{enz} min) and occurs in the range of pH 8.0–9.0. It is worth to point out that enzyme activity is still about 50 μmol/(g_{enz} min) at pH 3.7. At even lower pH, the enzyme activity could not be determined due to the instability of PenG. For pH above 5.0, the data obtained from both methods were similar (Fig. 2), although the HPLC results showed higher errors taking into account a 95% confidence

Table 2
Values of degradation rate constant of APA at $T = 25°$

pH	k_d (h^{-1})	$t_{half\text{-}life}$ (h)
3.5	0.0084	82
4.0	0.0058	120
4.5	0.0053	131
5.0	0.0036	192

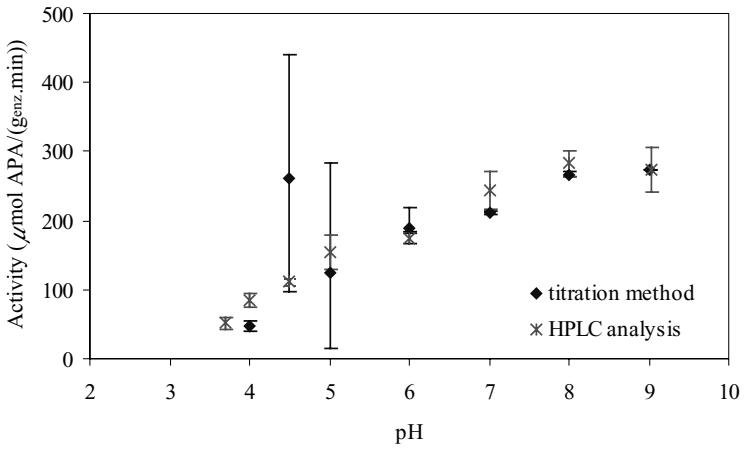

Fig. 2. Enzymatic activity of penicillin amidase including 95% confidence range.

limit. Nevertheless, the application of the titration method is not recommended for pH values close to pK_a of APA. Like in earlier work [7,10,14], the value used to calculate the fraction of the Eq. (3) was 4.9, however, the literature also reports values from 4.6 to 5.4 [29,30]. As this value is not precisely known, an error can occur in the calculation of the APA fractions and consequently, in the determination of enzyme activity by Eqs. (1) and (8).

In order to determine if diffusion limitation of PenG in the immobilization matrix might have influenced the data, the efficiency was calculated. By assuming that the average diameter was 0.42 mm and the diffusion coefficient of PenG into the catalyst was 0.2×10^{-9} m^2/s [31], the characteristic time for diffusion was calculated to be 222 s. At the highest initial reaction rate, the first-order reaction characteristic time was 22 min. The ratio of these values provided a Damköhler$_{(II)}$ number of 0.18, which means that the efficiency was 100%. The buffer concentration was approximately equal to the reactant concentration, so that, the pH gradients in the particles were absent [31].

3.3. Stability of penicillin amidase

Fig. 3 presents the results of residual activity of penicillin amidase after incubation in phosphate buffer solution saturated with BuAc at pH values 3.0, 4.0, 5.0, and 6.0 and incubation in BuAc saturated with potassium phosphate buffer of pH 6.0. The plot shows that the enzyme remained stable in aqueous solution at each pH during at least 32 days, despite of the presence of BuAc. This provides large opportunities for developing a counter-current enzymatic hydrolysis of PenG in a two-phase system of BuAc and water [12]. The incubation would have to be extended or carried out under more stressing conditions to determine at which pH the enzyme would show maximum activity, but this was beyond the scope of this study. A 58% decrease of the enzyme activity in the standard assay at pH 8.0 (280 μmol/(g_{enz} min)) occurred upon incubation in BuAc saturated with potassium phosphate buffer of pH 6.0 during 32 days corresponding to enzyme activity of 117 μmol/(g_{enz} min). This may be explained by denaturation. The literature [32–34] reports

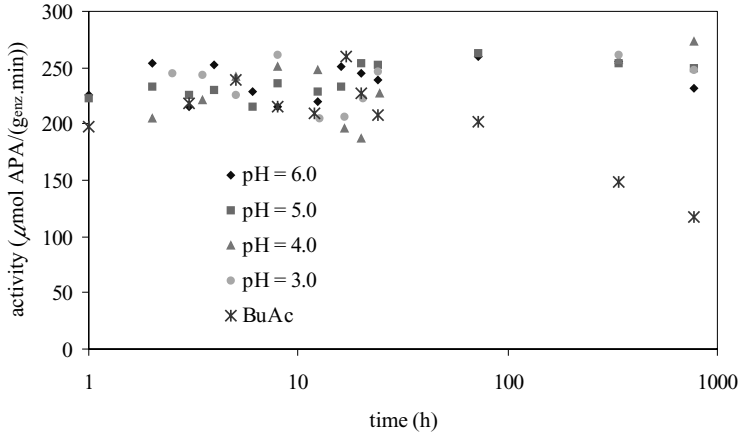

Fig. 3. Stability of penicillin amidase incubated in buffer solution saturated with BuAc at pH values 3.0, 4.0, 5.0, and 6.0 and in BuAc saturated with potassium phosphate buffer of pH 6.0 for 32 days. The data were determined by titration method. Similar results were obtained by HPLC analysis.

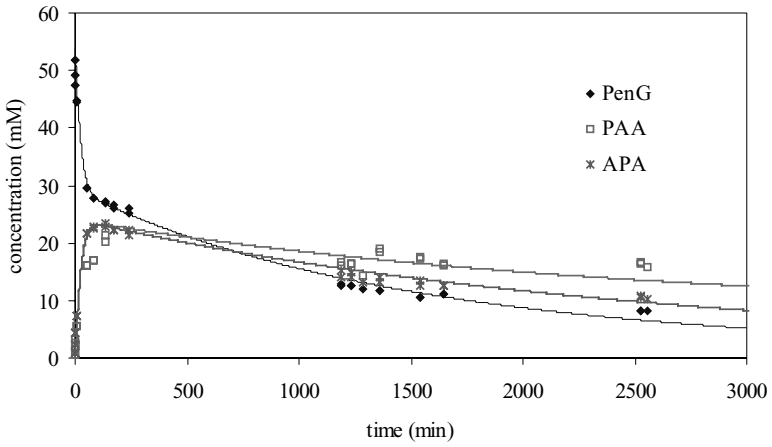

Fig. 4. Hydrolysis of PenG by penicillin amidase at pH 4.0. Markers are experimental data, lines are simulations using $k_{dpenG} = 0.053\,h^{-1}$, $k_{dAPA} = 0.0058\,h^{-1}$, $k_{enz} = 1.5575 \times 10^{-3}$ l/(g_{enz} min), $C_{PenG0} = 54$ mM, and $C_{enz} = 9.47$ g/l.

inactivation of most of the industrial enzymes (free or immobilized) when they are incubated in organic solvents with the polarity of butyl acetate.

3.4. Hydrolysis of PenG by penicillin amidase at pH 4.0

The rate equation that is obtained when considering the mechanism of the enzyme [35] contains many kinetic parameters that are unknown. For simplicity, it was assumed that the substrate and product concentrations would be much lower than the Michaelis and inhibition constants. The result is Eq. (9).

$$r_{enz} = k_{enz}\, C_{enz}[C_{PenG} - (C_{PAA} C_{APA}/K_{app})] \quad (9)$$

The apparent equilibrium constant, K_{app}, can be calculated at pH 4.0 using Eq. (10)

$$K_{app} = \frac{K_{eq}\, F_{PenG\,minus}}{F_{APA\,minus}\, F_{PAA\,minus}\, C_{Hplus}} \quad (10)$$

according to the reference reaction with equilibrium constant K_{eq}:

$$PenG^- \Leftrightarrow APA^- + PAA^- + H^+$$

The value of equilibrium constant adopted was 7.35×10^{-8} mo1/l, given by Tewari and Goldberg [30].

Knowing the initial enzymatic reaction rate and using the degradation rate constants for PenG and APA, results obtained from a long-term experiment for hydrolysis of PenG at pH 4.0 were compared with a model simulation in which the enzymatic reaction rate was assumed to follow Eq. (9).

Fig. 4 shows that the model which contains no adjustable parameters agrees to the experimental data. Although the literature [3,35,36] reports that inhibition occurs, and mainly due to PAA as a competitive inhibitor, our results show that PenG hydrolysis at pH 4.0 is limited exclusively by the thermodynamic equilibrium. It should be pointed out that the pH values reported in the literature were higher and the product concentrations were different. In Fig. 4, the enzymatic reaction rapidly proceeds towards equilibrium, but degradation occurs of PenG, and to a smaller extent, of APA. This leads to a reversal of the enzymatic hydrolysis equilibrium and consequently to a decrease in PAA concentration.

4. Conclusions

This work allowed the determination of the optimum pH and stability of penicillin amidase in PenG hydrolysis by a titration method as well as HPLC analysis. The data were in agreement with each other. The titration method was more economical and practical, but its application is not recommended for pH 4.5–5.0, because of a low accuracy in this range. The enzyme presented maximum activity in the pH range 8.0–9.0. In contrast to the common belief, penicillin amidase remains stable if the pH is decreased from 8.0 to 3.0. Although the enzyme activity decreased by about 80%, this does not confine processes to high pH values. The major limitation at low pH seems to be the low chemical stability of PenG and, to a less extent, APA. Based on this information, conditions can be found that will be suitable for the hydrolysis of PenG in a butyl acetate–water continuous process.

Acknowledgements

The authors gratefully acknowledge Brazilian government (CNPq and CAPES), for the financial support and DSM-Delft, The Netherlands, for donating the enzyme and reactants.

References

[1] M. Arroyo, I. De la Mata, C. Acebal, M.P. Castillón, Appl. Microbiol. Biotechnol. 60 (2003) 507.

[2] R. Fernández-Lafuente, C.M. Rosell, B. Piatkowska, J.M. Guisán, Enzyme Microb. Technol. 19 (1996) 9.
[3] J. Danzig, W. Tischer, C. Wandrey, Ind. J. Chem. 32B (1993) 40.
[4] A. Illanes, C. Altamirano, M.E. Zuñiga, Biotechnol. Bioeng. 50 (1996) 609.
[5] A.M. Azevedo, L.P. Fonseca, D.M.F. Prazeres, J. Chem. Technol. Biotechnol. 74 (1999) 1110.
[6] A. Erarslan, A. Güray, J. Chem. Tech. Biotechnol. 51 (1991) 181.
[7] M.B. Diender, A.J.J. Straathof, J.J. Heijnen, Biocatal. Biotransform. 16 (1998) 275.
[8] H.M. Van Sonsbeek, H.H. Beeftink, J. Tramper, Enzyme Microb. Technol. 15 (1993) 722.
[9] L.A.M. van der Wielen. M. Ottens, A.J.J. Straathof, Process technology and process integration in the preparation of penicillins, in: A. Bruggink (Ed.), Synthesis of β-lactam Antibiotics. Chem. Biocatal. Process Integr., Kluwer Academic Publishers, Dordrecht, 2001, p.150.
[10] G.O. Chilov, V. Švedas, Can. J. Chem. 80 (2002) 699.
[11] J.L. den Hollander, M. Zomerdijk, A.J.J. Straathof, L.A.M. van der Wielen, Chem. Eng. Sci. 57 (2002) 1591.
[12] M.B. Diender, A.J.J. Straathof, T. van der Does, C. Ras, J.J. Heijnen, Biotechnol. Bioeng. 78 (2002) 395.
[13] M.J. Zmijewski, B.S. Briggs, A.R. Thompson, I.G. Wright, Tetrahedron Lett. 32 (1991) 1621.
[14] M.B. Diender, A.J.J. Straathof, T. van der Does, M. Zomerdijk, J.H. Heijnen, Enzyme Microb. Technol. 27 (2000) 576.
[15] R.S. Topgi, J.S. Ng, B. Landis, P. Wang, J.R. Behling, Bioorg. Medic. Chem. 7 (1999) 2221.
[16] C.G.P.H. Schroën, V.A. Nierstrasz, P.J. Kroon, R. Bosma, A.E.M. Janssen, H.H. Beeftink, J. Tramper, Enzyme Microb. Technol. 24 (1999) 498.
[17] V.A. Nierstrasz, C.G.P.H. Schröen, R. Bosma, P.J. Kroon, H.H. Beeftink, A.E.M. Janssen, J. Tramper, Biocatal. Biotransform. 17 (1999) 209.
[18] M.I. Youshko, L.M. van Langen, E. de Vroom, F. van Rantwijk, R.A. Sheldon, V.K. Švedas, Biotechnol. Bioeng. 73 (2001) 426.
[19] I.A. Arnott, L.R. Weatherley, Proc. Biochem. 30 (1995) 447.
[20] M. Reschke, K. Schügerl, Chem. Eng. J. 28 (1984) B1.
[21] R.G. Benedict, W.H. Schmidt, R.D. Coghill, A.P. Oleson, J. Bacteriol. 49 (1945) 85.
[22] A. Linás, B. Vilanova, F. Muñoz, J. Donoso, J. Mol. Catal. A: Chem. 175 (2001) 3.
[23] B.B. Levine, Arch. Biochem. Biophys. 93 (1961) 50.
[24] A. Kheirolomoom, A. Kazemi-Vaysari, M. Ardjmand, A. Baradar-Khoshfetrat, Proc. Biochem. 35 (1999) 205.
[25] A. Llinás, B. Vilanova, J. Frau, F. Muñoz, J. Donoso, M.I. Page, J. Org. Chem. 63 (1998) 9052.
[26] M.G. Kim, S.B. Lee, J. Mol. Catal. B: Enzym. 1 (1996) 71.
[27] A. Illanes, A. Fajardo, J. Mol. Catal. B: Enzym. 11 (2001) 587.
[28] D.W. Dennen, J. Pharm. Sci. 56 (1967) 1273.
[29] V.K. Švedas, A.L. Margolin, I.V. Berezin, Enzyme Microb. Technol. 2 (1980) 138.
[30] Y.B. Tewari, R.N. Goldberg, Biophys. Chem. 29 (1988) 245.
[31] L.A.M. van der Wielen, Ph.D. thesis, Delft University of Technology, Delft, The Netherlands.
[32] A.M. Klibanov, Trends Biotechnol. 15 (1997) 97.
[33] C. Ebert, L. Gardossi, P. Linda, J. Mol. Catal. B: Enzym. 5 (1998) 241.
[34] R. Fernandez-Lafuente, C.M. Rosell, L. Caanan-Haden, L. Rodes, J.M. Guisán, Enzyme Microb. Technol. 24 (1999) 96.
[35] L.A.M. van der Wielen, M.J. van Buel, A.J.J. Straathof, K.Ch.A.M. Luyben, Biocatal. Biotransform. 15 (1997) 121.
[36] G. Duan, J.Y. Chen, Proc. Biochem. 31 (1996) 27.