Volume 258, number 2, 185–189 FEB 07853 December 1989

Stabilisation and immobilisation of penicillin amidase

N. Burteau, S. Burton and R.R. Crichton

Université Catholique de Louvain, Unité de Biochimie, Place Louis Pasteur, 1, B-1348 Louvain-la-Neuve, Belgium

Received 29 September 1989

Penicillin amidase was coupled to a periodate-oxidised dextran by reductive alkylation in the presence of sodium cyanoborohydride A loss of activity (25%) was observed but the conjugate enzyme dextran was more thermostable than the native enzyme Native and dextran-conjugated penicillin amidase were immobilised on amino activated silica (Promaxon, Spherosil, Aerosil) by a classical method using glutaraldehyde for the native enzyme and reductive alkylation for the modified enzyme Good relative activity of the enzymes was obtained after insolubilisation Immobilisation of both native and modified enzymes resulted in the thermostabilisation of the penicillin amidase

Enzyme stabilisation, Enzyme immobilisation, Penicillin amidase, (*Escherichia coli*)

1. INTRODUCTION

The use of enzymes at elevated temperatures has a number of advantages in industrial applications including enhanced productivity, reduction of microbial contamination and higher solubility of the substrates [1]. Since most enzymes are rapidly inactivated at high temperatures, it is important to obtain their stabilisation to make them practically useful.

In previous papers, we have developed a method of enzyme stabilisation by covalent coupling of protein to a periodate-oxidised polysaccharide in the presence of sodium cyanoborohydride [2–6].

In addition, the immobilization of enzymes on our insoluble supports offers the possibility of using them in automatic processes, to separate products from catalysts and to reuse them [7].

In this paper, we try to combine the advantages of stabilisation and immobolisation, thus producing a conjugate of penicillin amidase. This enzyme is very interesting in that it catalyses the transformation of benzylpenicillin into 6-aminopenicillanic acid (6-APA), the precursor of most of the semisynthetic penicillins [8].

Correspondence address. N Burteau, Université Catholique de Louvain, Unité de Biochimie, Place Louis Pasteur, 1, B-1348 Louvain-la-Neuve, Belgium

Abbreviations NIPAB, 6-nitro, 3-phenylacetaminobenzoic acid, *Abbreviations.* NIPAB, 6-nitro, 3-phenylacetaminobenzoic acid; APTS, 3-aminopropyltriethoxysilane; PAN, native penicillin amidase, PAD, conjugated penicillin amidase dextran; PANIS, Native penicillin amidase immobilized on Spherosil, PADIS, Conjugated penicillin amidase dextran immobilised on Spherosil; PANIP, Native penicillin amidase immobilised on Promaxon, PADIP, Conjugated penicillin amidase dextran immobilized on Promaxon, PANIA, Native penicillin amidase immobilised on Aerosil, PADIA, Conjugated penicillin amidase dextran immobilised on Aerosil

2. MATERIALS AND METHODS

2 1 *Materials*

Penicillin amidase (EC 3 5 1 11) from *Escherichia coli,* was a gift of Beecham Pharmaceuticals (Worthing, UK) The 6-nitro, 3-phenylacetamido-benzoic acid (NIPAB) from Sigma Chemical Co (St Louis, USA) was used as substrate

Dextran T 70 was a product from Pharmacia (Uppsala, Sweden) Sodium periodate and glutaraldehyde were from Merck (Darmstadt, F R G) Sodium cyanoborohydride and 3-aminopropyltriethoxysilane were from Aldrich Chemical Co (Beerse, Belgium)

Promaxon CFT 12 was a gift of Promat (Hilversum, The Netherlands). Aerosil 200V was a product of Degussa (Frankfurt, F R G) Spherosil XOA 200 was from Rhône-Poulenc (Lyon, France)

Protein concentration was determined using the reagent for protein assays from Biorad (Richmond, USA) The standard protein was bovine serum albumin from Sigma Chemical Co (St Louis, USA)

Other chemicals were reagent grade

2 2 *Methods*

2 2 1 Essay of penicillin amidase activity

Penicillin amidase activity was routinely essayed on a synthetic substrate, 6-nitro, 3-phenylacetaminobenzoic acid (NIPAB) The reaction was followed by measuring the absorbance of the solution at 405 nm [9] The activity unit of penicillin amidase (U) was determined as the amount (nanomol) of product formed per ml during 1 min During immobilisation of the enzyme on an insoluble support, the solution was shaken and filtered before the absorbance was measured

2 2 2 Penicillin amidase purification

The enzyme was purified following the method of Oliver et al. [10] After precipitation at pH 5 and a second precipitation with $(NH_4)_2SO_4$, the penicillin amidase solution had a specific activity of 3500 U/mg proteins

2 2 3 Preparation of penicillin amidase dextran conjugates (PAD)

Dextran T 70, 0 1 g, was dissolved in 2 ml distilled water and 0 133 g sodium periodate was added The mixture was stirred for 75 min at 20°C and dialysed 4 times for 1 h against distilled water. A volume of 0 3 ml of this activated dextran solution was then mixed with 0 2 ml 0 5 M sodium phosphate buffer, pH 7 0, 10 mg $NaBH_3CN$ and 0 4 ml 1 mg/ml protein solution After 17 h at 20°C, the mixture was dialys-

Published by Elsevier Science Publishers B V (Biomedical Division)
00145793/89/$3 50

ed 4 times for 1 h against 0 01 M sodium phosphate buffer, pH 7 0, at 4°C

2 2 4 Immobilisation procedure of native penicillin amidase (PAN) on Promaxon (PANIP), on Spherosil (PANIS) and on Aerosil (PANIA)

Silica, 0 5 g, was washed 5 times with 5 ml acetone and mixed with 4 ml 0 5% (v/v) APTS in acetone for 16 h at 20°C The aminated supports were washed and dried

To quantify the amino groups on the aminated silica, the basicity was measured by back titration using HCl, NaOH and bromothymol blue as the acid -base indicator

Then 0 2 g of aminated silica was mixed with 4 ml 2 5% (v/v) glutaraldehyde in 0 1 M sodium phosphate buffer pH 7 for 4 h at 20°C Thereafter, the support was washed with the same buffer and added to 4 ml native penicillin amidase solution dissolved in 0 1 M sodium phosphate buffer The coupling was carried out at 20°C, for 16 h with shaking

2 2 5 Immobilisation procedure of conjugated penicillin amidase (PAD) on Promaxon (PADIP), on Spherosil (PADIS) and on Aerosil (PADIA)

For the immobilisation of stabilised penicillin amidase, 0 2 g aminated support were mixed with 10 mg of $NaBH_3CN$ and 1 ml of conjugate enzyme-dextran solution in 0 1 M sodium phosphate buffer The coupling was carried out at 20°C for 16 h with shaking

3. RESULTS AND DISCUSSION

3.1. *Stabilisation of penicillin amidase*

3.1.1. Residual activity of modified enzymes

Penicillin amidase was first coupled to periodate-oxidised dextran by reductive alkylation (see section 2). The residual activity is defined as the fraction of the activity recovered after chemical modification compared to the total activity within the process. A 75% residual activity was obtained. The loss of activity could result from a chemical denuration of the enzyme due to reaction conditions (the nature of support, the reductant used, etc. [11]. It could also be due to a lower accessibility at the catalytic site and steric hindrance between substrate and support [7]; however, this hypothesis is unlikely due to the low molecular mass of the substrate ($PM_{NIPAB} = 300$). Another explanation could be that a lysine next ot the catalytic site could be chemically modified by the dextran and cause a distortion, thus diminishing catalytic activity.

3.1.2. Determination of the K_M values of enzymes

An increase of the apparent Michaelis-Menten constant of an enzyme can be attributed to steric hindrances that create an internal diffusion limit [7]. The determination of the K_M of native and immobilised penicillin amidase is presented in fig.1 using Lineweaver-Burke plots. The activities of the enzymes were determined by the classical test described in section 2 using variable concentrations of substrate [12]. The K_M values obtained and expressed in mM of NIPAB were $0.033 \pm 3 \times 10^{-4}$ for native penicillin amidase and $0.039 \pm 4 \times 10^{-4}$ for the stabilised enzyme. The increase of the K_M value after chemical modification was not very significant; the low molecular weight of the substrate (NIPAB) does not lead, after the chemical modification, to an important diffusion limit.

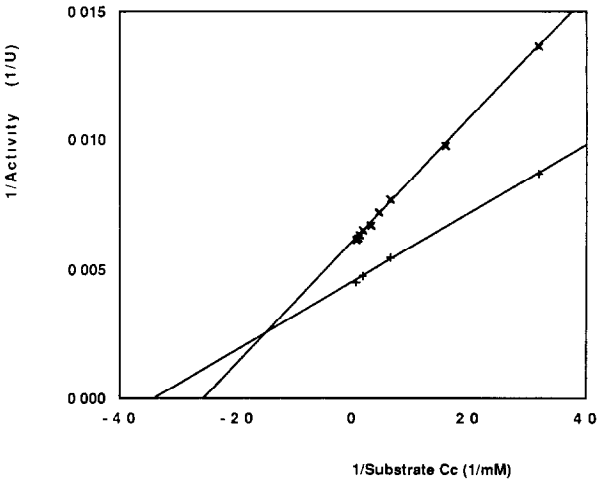

Fig 1. Lineweaver-Burke plots of native penicillin amidase, PAN (+) and stabilized enzyme, PAD (×) The same concentration of penicillin amidase was used with different levels of NIPAB Reaction rates were determined measuring activities by the classical method (see section 2)

3.1.3. Thermal properties of enzymes

To determine if the conjugate met the objective of the chemical modification, namely the thermostabilisation of penicillin amidase, a heat inactivation study was carried out (fig.2). Enzymes were incubated without substrate in 50 mM sodium phosphate buffer pH 7.8 at 55°C for different periods of time. Activity was then measured by the described method. At this temperature, the native penicillin amidase, PAN, was totally inactivated after 30 min, wheras the conjugate PAD showed 75% residual activity (defined as the activity after inactivation compared to the initial activity) after a 60 min incubation at the same temperature.

The criteria for defining a good penicillin amidase must be the efficacity of the enzyme. The efficacity is defined as the time course of heat inactivation where activity is expressed as a percentage of the initial activity before chemical modification at a given temperature (fig.3). Initially, the native enzyme had more activity than the conjugated penicillin amidase, however, after 5 min incubation at 55°C, the residual activity of the stabilised enzyme was higher: 68% compared to 58% for the native catalyst. The latter was completely inactivated after 30 min incubation whereas the conjugated enzyme still maintained a 58% activity.

3.2. *Immobilisation of penicillin amidase on silica*

3.2.1. Residual activity of immobilised enzymes

The first step was to functionalise silica with different concentrations of APTS (0,5,10,20% v/v in acetone). The amino groups introduced were quantified with acid-base titration (fig.4). Promaxon fixed the greater

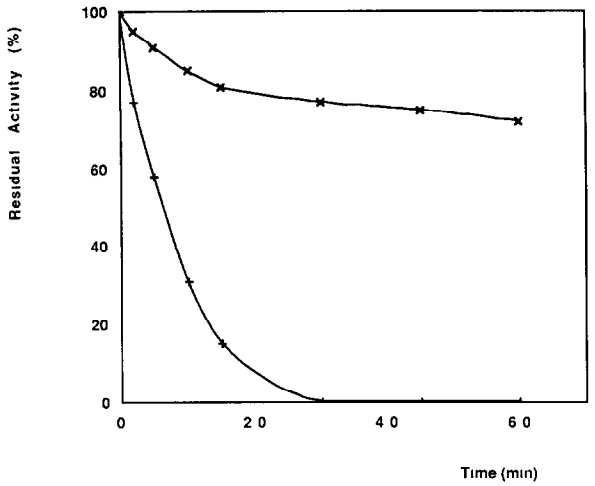

Fig 2. Heat inactivation of native penicillin amidase, PAN (+) and stabilized enzyme, PAD (×). Penicillin amidase in 50 mM sodium phosphate buffer pH 7.8, were heated at 55°C for different lengths of time and the remaining activity was determined.

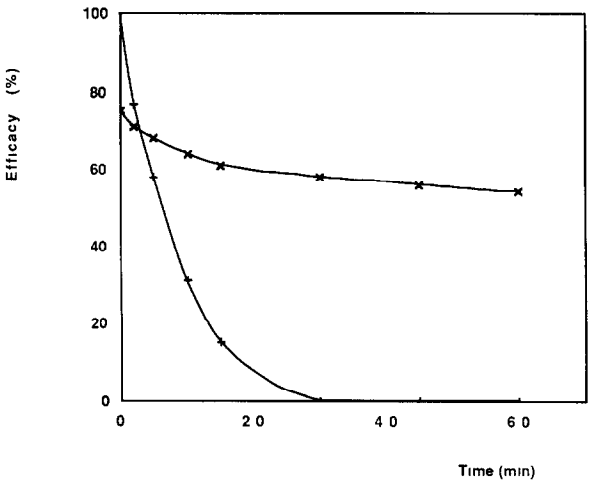

Fig 3 Efficacy of native penicillin amidase, PAN (+) and stabilised enzyme, PAD (×) Efficacy was expressed as the residual activity obtained as a function of incubation time

concentration of amino groups with 0.4 mmol/g support for 5% APTS. Promaxon has a large specific area (400 m^2/g) compared with Spherosil and Aerosil (about 200 m^2/g) which had amino group levels of 0.25 and 0.17 mmol/g support (5% APTS) respectively. For the following experiment, supports activated with 5% APTS were chosen. Under these conditions, a good level of amino groups was obtained; at higher APTS concentrations the amination yield is lower.

Immobilisation took advantage of the reactive groups (aldehydes) present on the dextran-penicillin amidase conjugate and was performed by reductive alkylation in the presence of sodium cyanoborohydride on 3-aminopropyltriethoxysilane functionalised porous silica. Native enzyme was covalently immobilised by coupling on the same functionalised supports activated with glutaraldehyde.

The non-reversiblility of the linkage between enzymes and insoluble support was tested using a procedure described previously [3]. The results obtained showed that there was no release of soluble active enzyme from the matrix during incubation of the immobilized enzyme (not shown).

The influence of native penicillin amidase concentration on the enzymatic activity of the immobilised preparation was also investigated (table 1). Several conjugates were synthesised introducing different concentrations of enzymes on Aerosil ($PANIA_{1-4}$), on Promaxon ($PANIP_{1-4}$) and on Spherosil ($PANIS_{1-4}$). When the quantity of bound enzyme was increased, the relative activity (defined as the ratio between activity of the immobilised enzyme and the theoretical activity bound to the silica) of penicillin amidase was lower. A likely hypothesis is that the reaction using a high concentration of bound enzyme is controlled by factors such as the transport of substrate and product in the matrix pores leading to a partial lack of substrate. The reduction in relative activity was lower for the enzyme on Aerosil which has larger pores (350 Å) than Spherosil (160 Å). Promaxon has small pores (64 Å), therefore its specific area is larger; the reduction of relative activity was intermediate.

Aerosil fixed the greatest protein concentration (A–B) and moreover presents the best relative activity. Promaxon followed and Spherosil showed the lowest relative activity and the smallest amount of protein fixed.

For the immobilisation of stabilised enzyme, 1155 U of native penicillin amidase were first coupled on a soluble dextran and then immobilised on 1 g of silica by reductive alkylation (table 2). The conjugates $PADIA_2$, $PADIP_2$ and $PANIS_2$ were obtained.

It is important to remember that after linking the enzyme and dextran, penicillin amidase showed 75% residual activity. A very small amount of activity was lost during the immobilisation on the insoluble silica.

3.2.2. Thermal properties of immobilised enzymes

Fig.5 shows the heat inactivation curves of PAN, $PANIA_2$, $PANIP_2$, $PANIS_2$ and PAD, $PADIA_2$, $PADIP_2$ and $PADIS_2$. These conjugates were incubated without substrate in 50 mM sodium phosphate buffer pH 7.8 at 55°C for different periods of time. The activity was measured using the method described in section 2.

Immobilisation of native penicillin amidase stabilises it against high temperatures. After 30 min incubation PAN showed 1% residual activity compared to 34, 37 and 38%, respectively, for $PANIS_2$, $PANIP_2$ and $PANIA_2$. The immobilisation process involves the formation of links between the enzyme and the silica. These links contribute to the rigidification of the pro-

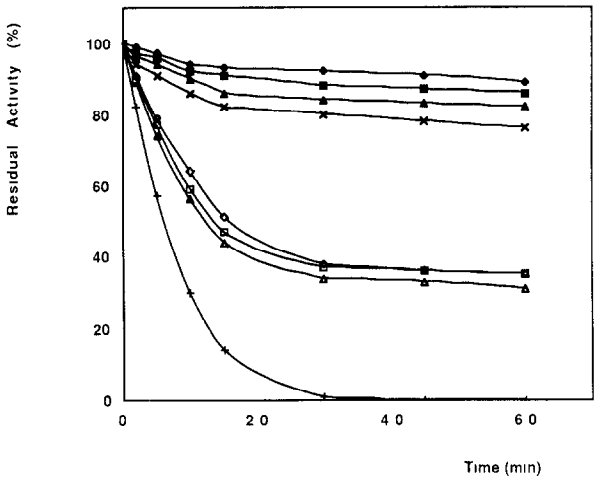

Fig 4. Amination levels of Promaxon (□), Spherosil (△) and Aerosil (◇) The silica was functionalized for 16 h at 20°C with shaking, using different concentrations of APTS in acetone Then amino levels were determined

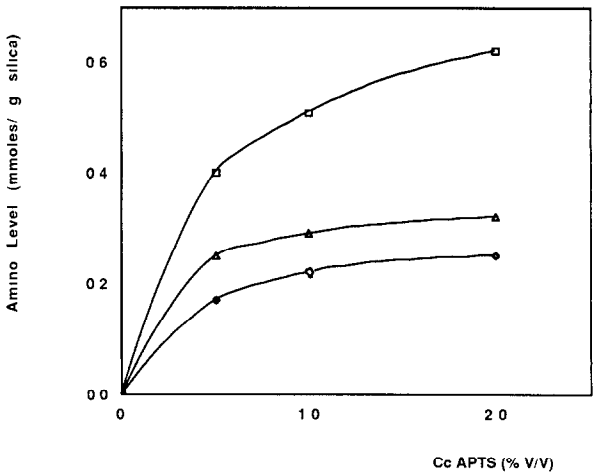

Fig 5 Heat inactivation of PAN (+), PAD (×), PANIS (△), PADIS (▲), PANIP (□), PADIP (■), PANIA (◇) and PADIA (◆) Penicillin amidase, in 50 mM sodium phosphate buffer pH 7.8, was heated at 55°C for different lengths of time and the remaining activity was determined

Table 1

Immobilisation of native and stabilised penicillin amidase on Aerosil (PANIA, PADIA), Promaxon (PANIP, PADIP) and Spherosil (PANIS, PADIS)

Conjugate	Activity (U)				Relative activity (%)
	A	B	A-B	C	C×100/(A-B)
$PANIA_1$	289	0	289	288	100
$PANIA_2$	1155	0	1155	1148	99
$PANIA_3$	2310	0	2310	2167	94
$PANIA_4$	11551	30	11521	7334	63
$PANIP_1$	289	0	289	296	100
$PANIP_2$	1155	0	1155	1154	100
$PANIP_3$	2310	0	2310	2094	91
$PANIP_4$	11551	500	11021	6876	61
$PANIS_1$	289	0	289	291	100
$PANIS_2$	1155	0	1155	1083	93
$PANIS_3$	2320	10	2300	1980	86
$PANIS_4$	11551	1925	9626	4919	51

A, # activity in the immobilisation reaction, B, # activity in the washings, A-B, # theoretical activity bound to the silica, C # activity immobilised on the silica The quantities of enzyme in this table are per g of dry silica

Table 2

The relative activities of $PANIA_2$, $PANIP_2$, $PANIS_2$, $PADIA_2$, $PADIP_2$, and $PADIS_2$

Conjugate	Relative activity
$PANIA_2$	99%
$PANIP_2$	100%
$PANIS_2$	93%
$PADIA_2$	75%
$PADIP_2$	75%
$PADIS_2$	72%

teins three-dimensional structure and thus to the stabilisation of the enzyme [6].

The results demonstrate the enhanced thermal resistance of chemically modified penicillin amidase immobilised on silica compared with the native form immobilised on the same supports. After 30 min incubation at 55°C, residual activities were 34% for $PANIS_2$, 37% for $PANIP_2$, 38% for $PANIA_2$, 84% for $PADIS_2$, 88% for $PADIP_2$ and 92% for $PADIA_2$. Immobilisation of the enzyme on a soluble support like dextran probably involves a better rigidification of the three-dimensional structure of the protein and therefore a better thermostabilisation compared with immobilisation on an insoluble support like silica.

Nevertheless it should be noted that the higher resistance to the thermoinactivation of immobilised penicillin amidase may in part be an artefact resulting from a substrate diffusional effect [13]. This observation is in agreement with the relative activity observed for the different compounds (see tables 1 and 2).

The nature of the silica does not seem to influence the thermostability of the conjugate. The low differences observed were not very significant but could perhaps be explained by the different average numbers of links between the enzyme and the support [6].

Acknowledgements. The authors are grateful to Dr O Anton (Promat) and Dr G Poncelet (U C.L. Unité CATA) for giving information and different analysis concerning the silica used and to Dr P Germain for his helpful and stimulating discussions

REFERENCES

[1] Zale, S E and Klibanov, A M (1983) Biotechnol Bioeng , 25, 2221

[2] Lenders, J.P. and Crichton, R R (1984) Biotechnol Bioeng , 26, 1343
[3] Lenders, J.P., Germain, P and Crichton, R R. (1985) Biotechnol Bioeng , 27, 572.
[4] Germain, P , Makaren, J.S. and Crichton, R R (1988) Biotechnol Bioeng , 32, 249.
[5] Germain, P and Crichton R.R (1988) J. Chem Tech Biotechnol , 41, 297
[6] Germain, P , Slagmolen, T and Crichton, R.R (1989) Biotechnol Bioeng., 33, 563
[7] Klibanov, A M (1983) Science, 219, 722
[8] Vandamme, E J and Voets, J P , (1974) Adv in Appl Microbiol., 17, 312
[9] Oliver, G , Valle, F., Rosetti, F , Gomez, M , Santamaria, P , Gosset, G and Bolivar, F (1985) Gene, 40, 9
[10] Klibanov, A M. (1983) Biochem Soc Trans , 11, 19
[11] Lineweaver, H and Burke, D (1934) J Am Chem Soc , 56, 658.
[12] Royer, G P (1980) Catal Rev Eng , 22, 29
[13] Patwardhan, V S. and Karanth, N G (1982) Biotechnol Bioeng , 14, 763