Immobilization-Stabilization of Penicillin G Acylase from *Escherichia Coli*

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ABSTRACT

We have developed a strategy for immobilization-stabilization of penicillin G acylase from *E. coli*, PGA, by multipoint covalent attachment to agarose (aldehyde) gels. We have studied the role of three main variables that control the intensity of these enzyme-support multiinteraction processes:

1. surface density of aldehyde groups in the activated support;
2. temperature; and
3. contact-time between the immobilized enzyme and the activated support prior to borohydride reduction of the derivatives.

Different combinations of these three variables have been tested to prepare a number of PGA-agarose derivatives. All these derivatives preserve 100% of catalytic activity corresponding to the soluble enzyme that has been immobilized but they show very different stability. The less stable derivative has exactly the same thermal stability of soluble penicillin G acylase and the most stable one is approximately 1,400 fold more stable. A similar increase in the stability of the enzyme against the deleterious effect of organic solvents was also observed. On the other hand, the agarose aldehyde gels present a very great capacity to immobilize enzymes through multipoint covalent attachment. In this way, we have been able to prepare very active and very stable PGA derivatives containing up to 200 International Units of catalytic activity per mL. of derivative with 100% yields in the overall immobilization procedure.

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Index Entries: Immobilization of penicillin G acylase; thermal stabilization of penicillin G acylase; Enzyme(amine)-agarose(aldehyde) multiinteraction; penicillin G acylase and organic cosolvents; capacity of aldehyde-agarose gels to bind enzymes; enzymatic hydrolysis of penicillin G.

INTRODUCTION

Penicillin G acylase, *PGA*, (E.C. 3.5.1.11.) exhibits very interesting catalytic properties and therefore it is potentially useful indeed as catalyst of a number of biotechnological processes:

1. nowadays it is industrially used to hydrolyze penicillin G, *Pen G*, to phenylacetic acid, *PAA*, and 6-aminopenicillanic acid, *6APA*,
2. it also catalyzes the subsequent step of N acylation of 6APA (and other antibiotic "nuclei") with PAA analogues to yield the corresponding semisynthetic antibiotics,
3. this enzyme exhibit a very important stereospecificity towards a number of nucleophiles and so it is potentially useful for the resolution of phenylacetyl derivatives of compounds of industrial interest (e.g., carnithine (*1*), aspartame (*2*),
4. the possible use of PGA in protection/deprotection of amine or hydroxyl groups in sugars (*3*), peptides (*4*) or penicillin G (*5*) chemistry has also been reported recently.

This acylase is produced by a wide variety of microorganisms (bacteria as well as fungi), but nowadays the enzyme from *E. coli* is the most widely used. The preparation of very active and very stable (concerning heat, organic cosolvents. . .) derivatives of this enzyme may improve its actual industrial application and it might also facilitate the industrial implantation of the other potential uses pointed out above.

In the last years we have developed a strategy for immobilization-stabilization of enzymes by multipoint covalent attachment to preexisting supports (*6–9*). We have proposed the attachment of enzymes, through their amine groups, to monolayers of identical aldehyde groups moderately separated from the surfaces of agarose gels as a very adequate "immobilization-stabilization system". Because of the main characteristics of this "system" (absence of steric hindrance for the amine-aldehyde reaction, stability of aldehyde groups, reversibility of each one-point amine-aldehyde attachment. . .), it offers very great possibilities for an intense, but nondistorting, enzyme-support multiinteraction process (*6*).

In this communication, we present a study on the application of our stabilization strategy to penicillin G acylase from E. coli, PGA. We have prepared a number of PGA-agarose derivatives using different experimental conditions. We have tested the role of different variables that control the intensity of the enzyme(amine)-agarose(aldehyde) multiinteraction process:

1. surface density of aldehyde groups in the activated gels;
2. contact-time between the immobilized enzyme and the activated support prior to borohydride reduction of the derivatives; and
3. temperature.

We have studied the activity and stability of the different derivatives, and we have also tested the maximal capacity of Sepharose CL 6B gels to bind PGA. In this way, we have been able to prepare derivatives that present excellent perspectives for industrial application (*10*).

MATERIALS AND METHODS

Materials

Sepharose CL 6B was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Semipurified extracts of penicillin G acylase from *E. coli* (330 International Units per mL. and 31 mg. of protein per mL.) and penicillin G sulfoxide were generously donated by Antibioticos S.A., Madrid, Spain. Penicillin G and glycidol were purchased from Sigma Chem. Co., St. Louis, Mo.

Activation of Agarose Gels

Glyoxyl-Sepharose CL gels (Ag-O-CH_2-CHO) were prepared by etherification of agarose gels with glycidol (2,3 epoxypropanol) and further oxidation of the resulting glyceryl-Sepharose (Ag-O-CH_2-CHOH-CH_2OH) with periodate. Total amount of glyceryl groups in the intermediate Sepharose derivative is the sum of the ones formed through etherification with glycidol plus the ones previously present in Sepharose Cl as a side product of the cross-linking reaction. A detailed description of this overall activation method has been given in a previous paper (*6*).

Preparation of Penicillin G Acylase-Agarose Derivatives

Reaction mixture: 0,30 mL. of semipurified extract (100 International Units) were added to 105 mL. of 0.05 M bicarbonate buffer pH 10.0 containing 9mM (3 mg/mL) of penicillin G sulfoxide and 5 mL of this solution were separated as a blank. Then 7 g of wet activated gel (approx. 10 mL.) were added to the solution and the suspension, **immobilization suspension,** was very gently stirred inside a High-Low Temperature Incubator. At the same time, 0.35 g of wet inactivated gel (glyoxyl-agarose reduced with sodium borohydride) were added to the blank and stirred in the same conditions as the immobilization suspension. At different times, aliquots of supernatant and whole suspension of both immobilization suspension and blank were withdrawn and assayed as described below.

End-point of the enzyme support multiinteraction process: After a given con-
tact-time between the immobilized enzyme and the activated support,
derivatives were reduced with borohydride in the same "optimal condi-
tions" previously found for reduction of trypsin-agarose derivatives *(8).*
In this way, Schiff's bases formed between the enzyme and the support
are reduced into very stable secondary amine bonds and remaining alde-
hyde groups in the gel are converted into inert hydroxyl ones.

Capacity of Aldehyde-Sepharose CL 6B
to Bind PGA

Experiments were carried out as described above as general immobili-
zation method and the concrete experimental conditions (temperature,
surface density of aldehyde gels...) were the same described in Results
for the preparation of P4 derivative. The amount of enzyme offered to the
activated support was ranged between 10 and 400 IU of acylase per mL.
of support. Since soluble PGA in the blank did not adsorb on the gel and
remained fully active, we have been able to calculate the amount of en-
zyme immobilized on the activated support by measuring the decrease of
activity of the supernatant of the immobilization suspension.

Enzymatic Assays

Soluble and immobilized enzyme was assayed with penicillin G as
substrate, titrating the liberated PAA with 0.01 M NaOH 0.1 M C1Na in a
pH-stat (model TTT80 Radiometer, Denmark). Experiments were carried
out at pH 8.0 at 25°C and using 20 mL. of 30 mM of penicillin G in 0.1 M
C1Na, 50 mM phosphate as assay mixture. 0.1–0.4 I.U. of enzyme were
used in each experiment. The presence of 9mM of penicillin G sulfoxide in
the immobilization suspension did not affect the observed reaction rates.
Only very small aliquots of these suspensions were added to the assay
mixture (usually 0.1 mL.) and then [substrate]/[inhibitor] ratio was very
high (approx. 600). In these conditions, the inhibitory effect of penicillin G
sulfoxide was negligible. On the other hand, high concentration of phos-
phate buffer was used in this assay in order to avoid the generation of
gradients of pH inside the porous structure of these catalyst mainly when
very active derivatives were assayed by using this hydrolytic reaction.

Irreversible Thermal Inactivation

Samples of derivatives or soluble PGA were suspended in 0.1 M phos-
phate buffer pH 8.0 and incubated at 45°C. At different times aliquots
were withdrawn and assayed at pH 8.0 and 25°C as described above.

Table 1
Preparation of Penicillin G Acylase-Agarose Derivatives. Activity.

Derivative	Surf. Dens.[a]	Time[b]	Temp.[c]	Imm. Yield[d]	Activity[e]
P1	1	3	25	40	100
P2	4	3	25	100	100
P3	10	3	25	100	100
P4	17	3	25	100	100
P5	17	0.3	25	100	100
P6	17	1.5	25	100	100
P7	17	24	25	100	100
P8	17	3	4	100	100
P9	17	3	15	100	100
P10	17	3	30	100	100

[a]Surface density of aldehyde groups in the activated gels (aldehyde residues per 1000 A^2 of gel surface).
[b]Contact-time of immobilization-multiinteraction (hours).
[c]Temperature of immobilization-multiinteraction (°C).
[d]Immobilization Yield (% of soluble enzyme offered to the support that has been immobilized).
[e]Activity of the derivative (% corresponding to soluble enzyme that has been immobilized).
[f]Overall Immobilization Yield (% of activity corresponding to soluble enzyme offered to the support that has been recovered in the final derivative).

Stability in the Presence of Organic Cosolvents

Samples of derivatives or soluble PGA were suspended in different water-methanol mixtures (different pH and different concentration of cosolvent) and incubated at 25°C. At different times, aliquots were withdrawn and assayed in aqueous medium at pH 8.0 and 25°C as described above. Water-methanol mixtures were prepared by mixing aqueous phosphate or acetate buffers and cosolvent (final concentration of buffer was 50 mM in all cases). The pH of the mixtures was adjusted after addition of the cosolvent and it is given as pH_{app}.

RESULTS AND DISCUSSION

Preparation of PGA-Agarose Derivatives

We have prepared very different PGA-agarose derivatives by using quite different combinations of the three variables that we have commented in Introduction (see Table 1). We have always used bicarbonate buffer with pH 10.0 as immobilization medium because we have previously found the most of proteins tested (e.g., a very similar penicillin G

acylase from Kluyvera citrophila) only immobilize on these type of alde-
hyde support at this pH (6). In addition to that, this pH was found the
most adequate for this type of enzyme support multiinteractions when
we used the model enzyme trypsin (7). In the standard procedure, we
have always offered a small amount of enzyme, 10 I.U. per mL to the sup-
port. In all cases, the enzyme immobilizes very quickly (in less than 20
min) and completely on the activated support, except in the case of P1
derivative (see Table 1). In this latter case, we have used a very poorly
activated gel containing only 1 aldehyde residue per 1000 A^2 of gel surface
and so, by steric reasons as we have commented in a previous paper (6),
each enzyme molecule can only form a unique covalent attachment with
this activated support. Therefore, this poor immobilization yield, 40%,
obtained in the case of P1, seems to reflect the reversibility of the one-
point amine-aldehyde reaction. However, when we use more activated
gels the enzyme can already form two-point or more intense multipoint
attachments and hence it is able to become irreversibly attached to the
support even before reduction with $NaBH_4$ as we have clearly demon-
strated in a previous paper (6). In fact, we can observe in Table 1 that the
small amount of enzyme offered to all other more activated supports (10
I.U. of PGA per mL of support) immobilizes completely.

Capacity of Aldehyde-Sepharose CL 6B
to Bind PGA

When we have offered 10–200 IU of PA per mL. of activated support,
in the concrete experimental conditions reported in Table 1 for the prepara-
tion of P4, the offered enzyme immobilizes very quickly and completely.
With higher amounts of offered enzyme, no more than 200 IU per mL. are
immobilized. Even when 400 IU were offered, a slight decrease in the
capacity of the support to bind PA was observed (only 170 IU per mL.
become immobilized) (Fig. 1). That highest amount of 200 IU per mL cor-
responds to 25 mg of protein/mL. of activated support. Since aldehyde
groups are very stable (half-life at pH 10 and 25°C is 10 d (6)) and immo-
bilization (through a two-point or more intense multipoint attachment) is
quite irreversible, it seems that a monolayer of fully protein-covered gel
surface can be very easily achieved. However, when a large excess of a
semipurified extract is used, a competence among the excess of PGA and
the excess of the other proteins of the extract to immobilize on the acti-
vated support must be established. The slight decrease in the binding of
PGA observed in our case seems to indicate that reactivity of PGA with
these activated gels in our experimental conditions is slightly lower than
one of the other proteins of the extract.

Activity of PGA-Ararose Derivatives

Every PGA-agarose derivative (prepared in the presence of the com-
petitive inhibitor penicillin G sulfoxide) preserves fully active as compared
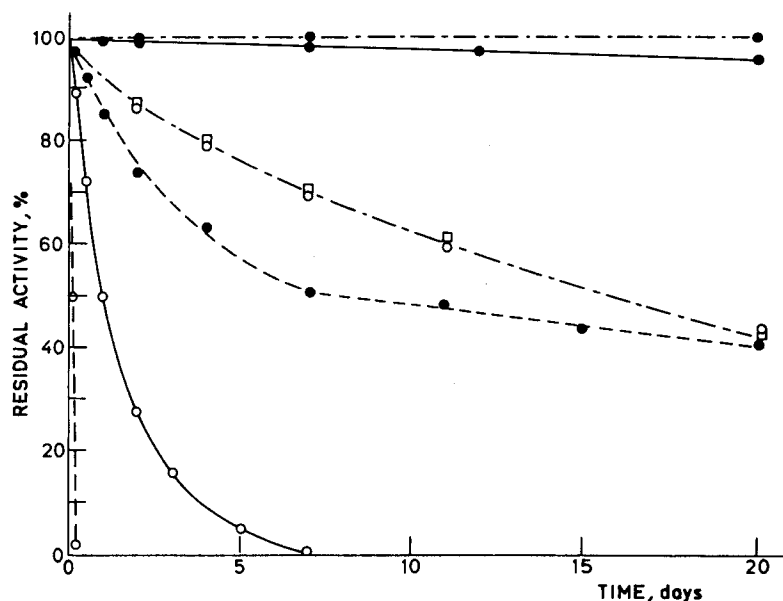
Fig. 1. Time courses of Irreversible Inactivation of PGA and PGA-agarose in the presence of methanol. Experimental procedure is described in Materials and Methods: □: soluble PGA, ○: one-point attached P1 derivative, ●: multi-point attached P4 derivative. - - -: incubation of enzyme in 50% methanol, pH 5.0, 25°C, ————: incubation in 50% methanol, pH 7.5, 25°C, - · - · - ·: incubation in 40% methanol, pH 7.5, 25°C.

with the soluble enzyme that has been immobilized (see Table 1). That is, the activity of P1 is 4 IU/mL and the other derivatives exhibit a catalytic activity of 10 IU/mL. In these latter cases, the activity of derivatives is also exactly the same one corresponding to the soluble enzyme offered to the activated supports. As we shall observe below, in the case of P2–P10 derivatives the preservation of full catalytic activity occurs in spite of the fact that the stability of the enzyme has been greatly increased. Similar results were previously found in the case of trypsin (amine)-agarose (aldehyde) derivatives (7). When this stabilization strategy was planned (6), we had assumed that the reversibility of each one-point amine-aldehyde attachment may prevent the enzyme from important distortions as consequence of the formation of a new additional enzyme-support attachments. Results obtained now with PGA and previously with trypsin seem to confirm our initial assumption. In addition to that, the presence of the competitive inhibitor of the enzyme during these intense enzyme-support multiinteraction processes may also protect the enzyme from some slight distorting interactions (9). A study on inhibition of PGA by penicillin G sulfoxide and on the protecting effect of this inhibitor during

the PGA(amine)-agarose(aldehyde) multiinteraction will be the matter of a forthcoming paper.

When we have increased the amount of enzyme immobilized on the support, we have observed that activity of derivatives containing up to 50 IU/mL continued to be exactly the same of the soluble enzyme that had been immobilized. Derivatives containing higher amounts of PA showed slight diffusional problems in the conditions of our standard assay. However, increasing the concentration of penicillin G up to 140 mM, they showed the activity corresponding to the soluble enzyme that had been immobilized.

Thermal Stability of PGA-Agarose Derivatives

The kinetics of irreversible thermal inactivation of stabilized PGA-agarose derivatives are complex and cannot be described by a simple first-order model; they are clearly multiphasic ones. Pseudo half-life times, $pt_{1/2}$, (time necessary to reach 50% of residual activity) were drawn directly from experimental time-courses of inactivation corresponding to each derivative. These parameters can be considered as representative values of the stability of the derivatives for comparative purposes. We define stabilization of each derivative as the ratio between its $pt_{1/2}$ and the one corresponding to soluble enzyme. This latter value being approx. 2 h in the experimental conditions we have used for thermal deactivation.

In tables 2, 3 and 4, we represent the stabilizations achieved for the different PGA-agarose derivatives. P1 derivative (one-point attached one during whose preparation the enzyme is attached to the support reversibly) exhibits exactly the same stability as the soluble enzyme (Table 2). It seems that this first PGA-agarose attachment preserves the overall structure of this dimeric enzyme (threedimensional structure of each subunit and assembly between subunits) fully intact. On the contrary, the other P derivatives resulted greatly stabilized. Stabilizations oscillate among 40 for P2 to 1,400–1,500 for P4, P7, and P10 derivatives. Stabilization increases as surface density of aldehyde groups (Table 2), temperature (Table 3), and contact-time enzyme-activated support (Table 4) do. Surface density of aldehyde groups in the activated support seems to be the variable that plays the most important role in enzyme stabilization. So, P5 and P8 derivatives are clearly more stable than P1 and P2 ones. The former derivatives were prepared by using the most activated gel but in very unfavorable conditions (20 min of contact-time or 4°C respectively). On the contrary, P1 and P2 were prepared in optimal conditions but using poorly activated gels.

Optimal conditions for the preparation of PA-agarose derivatives seem to be the ones corresponding to P4. Higher temperatures or higher contact-times do not exert important effect on stabilization (see Tables 3

Table 2
Effect of Surface Density of Aldehyde Groups
in the Activated Gels on the Stability of PGA-agarose derivatives

Derivative[a]	Surface Density[b]	Stabilization[c]
P1	1	1
P2	4	40
P3	10	600
P4	17	1400

[a] Conditions for the preparation of the derivatives are given in Table 1.
[b] Surface density of aldehyde groups is expressed as in Table 1.
[c] Stabilization is defined in Results and Discussion ($pt_{1/2}$ derivative/$pt_{1/2}$ soluble penicillin G acylase).

Table 3
Effect of Contact-Time Between the Immobilized Enzyme
and the Activated Support on Thermal Stabilization of PGA-agarose Derivatives

Derivative[a]	Contact-Time[b]	Stabilization[c]
P5	0.3	150
P6	1.5	700
P4	3.0	1400
P7	24.0	1500

[a] Conditions for the preparation of the derivatives are given in Table 1.
[b] Contact-Time is expressed in hours and it is defined in Materials and Methods.
[c] Stabilization is defined in the text (Results and Discussion) and in Table 2.

Table 4
Effect of Temperature at Which Immobilization-Multiinteraction
Is Carried Out on the Thermal Stabilization of PGA-agarose Derivatives

Derivative[a]	Temperature[b]	Stabilization[c]
P8	4	600
P9	15	1000
P4	25	1400
P10	30	1400

[a] Conditions for the preparation of the derivatives are given in Table 1.
[b] Temperature is given in °C.
[c] Stabilization is defined in the text (Results and Discussion) and in Table 2.

and 4). On the other hand, activation degrees higher than 17 aldehyde residues per 1000 A^2 of gel surface could not be achieved because this amount seems to be the one corresponding to the total amount of activable surface hydroxyl groups in these agarose gels (6). This highest surface density of 17 aldehyde residues/1000 A^2 corresponds to a concentration of 70 μequivalents aldehyde per mL. of wet Sepharose CL 6B gel (6).

The increase of the values of these three variables tested must increase the possibilities of enzyme-support multiinteraction as we have discussed in a previous paper (7). For example, surface density of active groups controls the possibilities of the support to multiinteract with the enzyme, and these possibilities must increase as surface density of active groups does (Table 2). At the same time, the increase in enzyme stability, observed when we left the enzyme, already irreversibly immobilized to continue interacting with the activated support (before borohydride reduction of the derivatives) (Table 3), is quite similar to the one previously found when we have stabilized trypsin (7). Our former "logical conclusion", discussed in the case of trypsin, seems to be now confirmed again: additional enzyme-support multipoint attachments is kinetically quite slower than the first enzyme-support attachment (immobilization). Therefore, establishment of new correct alignments between new amine groups of the enzyme and new aldehyde groups of the activated support may be the kinetic controlling step for the additional multipoint attachment. From this same point of view, the increase of temperature during the enzyme-support multiinteraction must also promote an increase in the stability of the derivatives (Table 4) because the flexibility of the immobilized protein must increase with temperature and parallels the possibilities of formation of those new amine-aldehyde correct alignments commented above.

This same pattern stability-variables tested was observed previously in the case of the enzyme trypsin. In that former case, by working with pure trypsin, we have been able to establish a clear experimental correlation between stabilization and multipoint covalent attachment (7). Now, since we have used semipurified extracts of penicillin G acylase, we have been unable to perform those experimental studies used with trypsin-agarose to probe the real extension of the multipoint covalent attachment (e.g., amino acid analysis of hydrolysates). However, some experimental evidences of multipoint covalent attachment can also be established in the case of these P2–P10 derivatives:

1. irreversibility of the immobilization process in all derivatives and
2. no desorption of any subunit of this dimeric enzyme when every derivative was incubated in very strong dissociating conditions with SDS (results not shown, (11)).

For all these reasons, it seems that stabilization of penicillin G acylase may be a direct consequence of PGA-Agarose multipoint covalent attachment and stabilization increases when the intensity of multipoint covalent attachment does. This fact would be very simply explained of PGA were a monomeric enzyme: multipoint covalent attachment increases rigidity of the threedimensional structure of the enzyme and so increase stability.

However, the mechanism of inactivation of dimeric PGA seems to be more complex (e.g., it has been proven that dissociation of subunits is involved in PGA inactivation and the isolated monomers are fully inactive (*14*)), and it is not well documented. So, first we may assume that multipoint covalent attachment promotes the preservation of the overall conformation of this complex enzyme (e.g.,

1. prevention of subunit dissociation;
2. preservation of correct assembly between subunits and
3. "freezing" of the threedimensional structure of each subunit).

The establishment, which is the main role, is quite difficult. However, it seems clear that the first point (prevention of enzyme from dissociation) is not the only, nor even the main, stabilizing effect of multipoint covalent attachment. In fact, this prevention should be quite similar for P2-P10 derivatives because they have all the two subunits simultaneously attached to the support (*11*) and preserved full catalytic activity after immobilization. Hence, dissociation constant of the two subunits must have been dramatically decreased in all these cases. However, the observed stabilizing effects are fully different (P2 derivative has only stabilized 40 fold and P4 has been 1,400 fold). Moreover, we have reported the preparation of a similar PGA derivative (the two subunits simultaneously attached to the support) which was even only five fold more stable than one-point attached PGA (*14*). So, it seems that multipoint covalent attachment of PGA may also prevent the enzyme from incorrect assembly between subunits and from distortions of three dimensional structure of each subunit.

We have compared the thermal inactivation of soluble PGA and different derivatives at pH 8.0 and high temperatures. pH is the one at which industrial hydrolysis of penicillin G is performed but temperatures were considerably higher (industrial hydrolysis of penicillin G is performed in the range 28–35°C). We have increased the temperatures in order to reduce the duration of the stability assays. However, we assume that thermal stabilizations achieved at moderately high temperatures may correlate quite well with thermal stabilizations at low temperatures. In both cases, the first steps of enzyme inactivation seem to be the same: small conformation changes on the overall enzyme structure.

So, these dramatic increases in thermal stability of our derivatives seem to indicate a very good perspective of these derivatives to be used in industrial processes of hydrolysis of penicillin G. Evidently, a more complete perspective about the possibilities of our derivatives to be used in that important industrial process should additionally include studies of time-courses of complete reaction, generation of gradients of pH inside these catalysts, microbial contamination, resistance to anticontaminant treatments, operational stability and so on. These matters will be the subject of a forthcoming paper (manuscript in preparation).

Stability of PGA-Agarose Derivatives
in the Presence of Organic Cosolvent

We have tested the stability of P1 and P4 derivatives in different water-methanol mixtures (Fig. 1). In all cases, we observe that P4 derivative is much more stable than P1 one. Stability of P4 in 50% methanol (pH_{app} 7.5), 25°C, is approximately 2,000 fold higher than one corresponding to P1 ($pt_{1/2}$ of P4 is approx. 10 d and the one of P1 is 7 min). We have also compared the stability of P1 and soluble PGA in 40% methanol (pH_{app} 7.5), 25°C, and we observe that one-point attached derivative has exactly the same stability as soluble PGA and the half-life times obtained (15 d) are also quite similar to those previously reported by Kasche for soluble PGA and a commercial PGA derivative (PGA-Eupergit from Römh-Pharma), (12).

The exact mechanism of the action of organic cosolvents on enzyme structure is quite different to the one of the action of heat. In addition to the modification of the medium with which the surface of the protein is interacting, now the presence of organic cosolvent in the range of pH we have studied (5.0–7.5) modifies also the overall surface charge of the protein (by modifying the pKs of the surface carboxylic groups of the protein). So, interaction surface-medium is completely different when the enzyme is being inactivating by heat or by organic cosolvents. However, the main overall effect of these two inactivating agents must be similar in the two cases. That is, the induction of conformational changes on the overall structure of this complex enzyme (unfolding of threedimensional structure of each subunit, dissociation of subunits, modification of the assembly of these two subunits...). If we assume, as commented above, that multipoint covalent attachment of PGA (with the two subunits simultaneously attached to the support) prevents the enzyme from these types of conformational changes, the good experimental correlation that we have found between thermal stabilization and stabilization in the presence of methanol seems to be quite logical.

The time-courses of inactivation of stabilized PGA-agarose derivatives seem to be also clearly multiphasic (see Fig. 1), following a quite similar pattern to the one commented above in the case of thermal inactivation. On the contrary, inactivation of soluble and one-point attached enzyme seems to be fairly monophasic (Fig. 1). This fact could be the result of the presence of different enzyme subpopulations in the case of multipoint attached immobilized derivatives.

Finally, the different water-methanol mixtures we have tested have special practical interest:

1. Kasche has found that the presence of high concentrations of methanol (e.g., 20–40%) at pH 7.5 improves significantly the kinetically controlled yield of cephalexin and ampicillin synthesis catalyzed by PGA, (12);
2. the presence of high concentrations of organic cosolvent at acidic pHs (e.g., pH 5.0) improves the thermodynamically

Table 5
"Activity/Stability" Properties of Several Commercial PGA Derivatives Compared with the Ones of Our Best PGA-agarose Derivative

Derivative	Vol. Activity[1]	Immob. Yield[2]	Thermal Stabil.[3]	Solv. Stabil.[4]	Mean Util. Time[5]
SP[a]	20	ND	1	ND	2–3
H[b]	70–100	ND	1	ND	ND
RP[c]	80–120	70	ND	1[d]	2–3
P4[e]	200	100	1,400	2,000	ND

[a]Snam Progetti Fiber Entrapped PGA. Data obtained in our Laboratory.
[b]Hoescht AG. Data obtained in our Laboratory.
[c]Röhm-Pharma. Data from Technical Sheet.
[d]Reference.
[e]Our best PGA-agarose Derivative. P4 (see Table 1) prepared by offering 200 I.U. of soluble PGA per ml. of activated gel.
[1]Volume activity, expressed as International Units of PGA (see Materials and Methods) per ml. of packed catalyst.
[2]Immobilization yield as given in Table 1.
[3]Thermal Stabilization as defined in text (Results and Discussion).
[4]Stabilization of the derivative in the presence of 40% methanol, pH_{app} 7,5, 25°C (stabilization is defined as commented in thermal inactivation = $pt_{1/2}$ derivative/$pt_{1/2}$ soluble enzyme).
[5]Mean utilization times recommended by the commercial suppliers, expressed in months.
ND: not available or not determined data.

controlled yield for the synthesis of semisynthetic antibiotics catalyzed by PGA as it has been experimentally proven by McDougall et al. by using PEG as cosolvent (13);

3. in general, the use of water-organic solvent mixtures may improve the solubility of different interesting substrates for these enzyme (e.g., phenylacetyl derivatives of racemic mixtures that this enzyme is able to resolve)....

Hence, stabilizations achieved in the presence of methanol for P4 derivative seem to have a very interesting practical relevance concerning with the use of this derivative for the development of new industrial applications of PGA.

CONCLUDING REMARKS

By using a suitable immobilizaton-stabilization strategy, we have been able to prepare penicillin G acylase-agarose derivatives that present important properties of Activity/Stability. The main characteristics of our best PGA-agarose derivative (P4 one) are remarked in Table 5 in comparison with the ones corresponding to a number of commercial PGA catalysts.

From data of Table 5, we can observe that commercial PGA derivatives having a low volume activity, ranging between 10 and 100 International units per mL., and exhibiting a stability closely similar to the one of soluble enzyme are already being used at industrial scale for hydrolysis of penicillin G with a mean utilization time of 2-3 mo. Since the activity/stability characteristics of our best PGA-agarose derivatives, seems to be clearly superior to that ones corresponding to most of the commercial PGA catalysts, it seems that this industrial process of hydrolysis of penicillin G catalyzed by PGA could be greatly improved by using our very stabilized derivatives. Evidently, a more complete comparison of commercial PGA catalysts should also include homemade PGA derivatives (that is derivatives prepared by Bayer, Beechamm...which are only used inside these factories). Unfortunately, we have been unable either to get data from these derivatives or to get samples of them to test in our laboratory.

On the other hand, the very good stability of our PGA-agarose derivatives in the presence of high concentrations of methanol makes these derivatives very useful to explore and develop new industrial applications of PGA: mainly some possible applications of this enzyme that requires the use of quite drastic experimental conditions (synthetic reactions (13), hydrolysis of very hydrophobic substrates (3)...) as we have commented above.

Finally, we would like to remark that stabilization of penicillin G acylase has been achieved through a "rational" strategy and not by random experiments, as we have remarked in Introduction. This stabilization method and its fundamentals has been formerly developed using trypsin as the model enzyme (7). In addition, it also has been successful when applied to a set of enzymes: penicillin G acylase from *E.coli* as reported here, penicillin G acylase from *K.citrophila* (14), lipase from *C. cilindracea* (15), and α-chymotrypsin (16). So, this strategy seems to have a quite general application whatever be the protein to be immobilized.

ACKNOWLEDGMENTS

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