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Quantitative characteristic of the catalytic properties and microstructure of cross-linked enzyme aggregates of penicillin acylase

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ABSTRACT

The microstructure and the catalytic properties of cross-linked enzyme aggregates (CLEA) of penicillin acylase (PA) obtained under different conditions were investigated. The period of time left between the enzyme precipitation and the cross-linking step was found to influence the structural organization of the resulting enzyme preparation. Confocal fluorescent microscopy of the so-called "fresh" and "mature" CLEAs PA allowed to estimate the "characteristic" diameter of CLEA PA particles, which appeared to be about 1.6 μm, and revealed that the "mature" type was composed of relatively big particles as compared to the "fresh" type. Complementary kinetic studies showed that the "mature" CLEA PA were more effective in both hydrolytic and synthetic reactions. It was suggested that the aggregate size might regulate the extent of covalent modification of PA and thereby influence the catalytic properties of CLEA.
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1. Introduction

Presently, penicillin acylase (PA) catalyzed regio- and enantio-selective conversions are exploited for the production of beta-lactam antibiotics nuclei (6-aminopenicillanic and 7-aminodesacetoxycephalosporanic acids) [1,2], preparation of semi-synthetic penicillins, cephalosporins [3,4], optically active amino compounds [5–7], etc. Quite often these chemicals are synthesized in very acidic or alkaline conditions where PA readily inactivates. Therefore, the development of immobilized PA preparations which are highly stable and active under extreme pH values is of great practical importance. Indeed, during recent decades a number of papers have reported the immobilization of PAs. Generally, efforts have focused on improving traditional immobilization techniques such as covalent [8–10] or physical [11,12] binding of the enzyme to an appropriate supporting material. Obvious progress has been achieved in terms of enzyme operational stability. However, catalytic activity of the carrier-bound PA relative to its weight is usually marked lower as compared to the native enzyme [13], which is an obvious drawback for high output applications. Among the most common reasons for this is enzyme inactivation during the immobilization procedure and the high impact of the carrier bulk weight. Another important issue is that the synthetic efficiency and stereoselectivity of immobilized PAs in most cases cannot compete with those of the native enzyme.

In this context, special attention should be paid to the methods of enzyme cross-linking resulting in cross-linked enzymes (CLEs) [14], cross-linked enzyme crystals (CLECs) [15,16], and cross-linked enzyme aggregates (CLEAs) [17,18]. The latter approach is especially interesting because the recent studies demonstrated that CLEA PA are not only highly stable but also possess catalytic activity and synthetic efficiency comparable to those of the native enzyme [19].

In the present study the catalytic and physicochemical properties of CLEA PA have been quantitatively characterized. Special attention was dedicated to the analysis and comparison of the microstructures of the enzyme preparations obtained under different conditions.

2. Materials and methods

2.1. Chemicals

6-Aminopenicillanic acid (6-APA) and D-phenylglycine amide (D-PGA) were supplied by DSM (The Netherlands). 5-Nitro-3-[(phenylacetyl)amino]benzoic acid (*iso*-NIPAB) was synthesized in our laboratory according to the previously published procedure [20]. The preparation of native PA was obtained as described earlier [21]. Organic solvents (extra high purity) were bought from

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Kriokhrom (Russia). Other reagents including buffer components were purchased from Merck (Germany).

2.2. Preparation of CLEA PA

PA CLEA were obtained according to the modified procedure originally described by Cao et al. [17]: PA was dissolved in 100 mM phosphate buffer pH 7.0 to the final concentration of 7×10^{-5} M. Several equal portions of PEG-6000 (200 mg each) were sequentially added to 10 mL of this solution and dissolved via intensive mixing. Addition of PEG-6000 was carried out until the residual PA activity in the supernatant dropped below to 3% of its initial value and the majority of PA was precipitated in a form of white curd-like aggregates. This was usually achieved after 10 portions of PEG-6000 added within 1 h at 0 °C.

The suspension of PA aggregates was thoroughly mixed and immediately divided in two equal (5 mL) parts. One part was cross-linked by 1 mmol of glutaraldehyde at 0 °C: 10 aliquots (42.5 μL) of 25% glutaraldehyde were injected into intensively stirred suspension during 2 h over equal periods of time. Residual enzymatic activity in the supernatant after the cross-linking step was less than 1% of its initial value. In order to wash off the excess of glutaraldehyde, the reaction mixture with the synthesized CLEA PA was carefully centrifuged at 1500 rpm and the supernatant was substituted with the pH 7.0 phosphate buffer followed by thorough re-suspension. The washing step was repeated twice again. CLEA PA obtained according to this protocol was denoted as “fresh” preparation.

The second part of PA suspension was centrifuged at 13,000 rpm for 20 min and placed in a refrigerator (4 °C). After 7 days of incubation the aggregates were cross-linked with glutaraldehyde as described above. This CLEA PA was denoted as “mature” preparation.

2.3. Study of microstructure of CLEA PA

The microstructures of “fresh” and “mature” CLEAs PA were studied using Zeiss LSM 510 Axiovert confocal fluorescent microscope (Plan-Neofluar 100×/1.3 oil objective, 488 nm excitation argon laser). Experimental data were analyzed using Zeiss LSM Image Examiner software and Image-Pro Plus software: individual particles identified on image were separated into groups according to their average diameter. Groups have been determined from 0.0 up to 3.5 μm in 0.1 μm step.

2.4. Spectrophotometric assay of PA activity

Hydrolytic activity of the native and cross-linked PA was determined spectrophotometrically by registering the initial accumulation rate (at less than 5% substrate conversion) of the colorimetric product of *iso*-NIPAB hydrolysis using Shimadzu UV-1601 Spectrophotometer (Japan) at 400 nm [21]. To determine the kinetic parameters (k_{cat} and K_M) of the *iso*-NIPAB hydrolysis by CLEA PA, the dependence of the initial hydrolysis rate on substrate concentration was analyzed in terms of Michaelis–Menten equation. To characterize the maximum hydrolytic activity (V_{max}) of the enzyme preparations, saturated concentrations of the *iso*-NIPAB (2.5 mM) were used. All reactions were carried out at standard conditions: 25 °C, 0.01 M phosphate buffer, pH 7.5, 0.1 M KCl.

2.5. Titration of PA active sites

Concentration of active sites in the native PA and CLEA PA preparations was determined by titration with phenylmethanesulfonyl fluoride (PMSF) [22]. An aliquot of PA or CLEA PA was adjusted to pH 6.0, vigorously suspended and divided into several equal samples. Then, an appropriate amount of PMSF solution was added to each of these samples and thoroughly mixed for 20 min. The residual enzymatic activity was determined from the hydrolysis rate of 2.5 mM *iso*-NIPAB (saturating concentration) as described in the previous section. Concentration of PA active sites was calculated from the residual activity linearly fitted versus PMSF concentration.

2.6. Catalytic properties of the “fresh” and “mature” CLEAs PA in the ampicillin synthesis

The hydrolysis of D-PGA, an activated acyl donor, and the synthesis of ampicillin were studied and compared to characterize catalytic properties and synthetic potential of different CLEA PA preparations. Enzymatic reactions were carried out at 25 °C under constant stirring. Specific amounts of reagents (from 2 to 100 mM of D-PGA in case of activated acyl donor hydrolysis; 100 mM of D-PGA and 100 mM of 6-APA in case of ampicillin synthesis) were added to 4.9 mL of water and pH was adjusted to 7.0. The volume was increased to 5.0 mL with distilled water. After thermo equilibrating for 5 min a reaction was initiated by injection of 20–100 μL of CLEA PA suspension (enzyme active site concentration 6.5×10^{-5} M). The pH value was maintained by automatic titration (Metrohm Titrino 719 S). Samples (20–50 μL) were withdrawn throughout the course of the reaction and diluted up to 1 mL with a corresponding volume of the eluent (980–950 μL) to terminate the enzymatic process. Samples were analyzed by HPLC as described below.

Kinetic parameters of the enzymatic hydrolysis of acyl donor (D-PGA) were determined from the initial hydrolysis rates when plotted versus the corresponding substrate concentrations and fitted by the Michaelis–Menten equation. Synthesis and hydrolysis rates in the ampicillin synthesis were determined from the initial part of the reaction progress curves. In all calculations the conversion of substrates did not exceed 2% of their initial concentrations.

2.7. HPLC analysis

Samples were analyzed by HPLC equipped with Waters M6000 pump, 4.6 mm × 250 mm Nucleosil C-18 “Phenomenex” column and Waters Lambda-Max 481 UV detector at 215 nm. The eluent (acetonitrile:water = 30:70, v/v) contained 0.68 g/L KH_2PO_4 and 0.68 g/L sodium dodecylsulphate; pH of the eluent was adjusted to 3.0 with phosphoric acid.

2.8. The stability and catalytic activity of the “mature” CLEA PA at different pH

pH-inactivation of the immobilized PA was carried out in a thermostated cell (25 °C) under controlled pH and constant stirring. The corresponding pH-stability of the “mature” CLEA PA was estimated from the residual enzymatic activity measured by the rate of hydrolysis of 2.5 mM *iso*-NIPAB at pH 7.5 (saturating concentration).

In order to determine the pH-dependence of the hydrolytic activity, initial reaction rates of *iso*-NIPAB hydrolysis at saturating concentration were measured as described above with the exception for pH value which was varied. The following buffer solutions were used to maintain pH: 0.01 M potassium acetate with 0.1 M KCl ranging from pH 2.7 to 4.0; 0.03 M potassium phosphate with 0.1 M KCl ranging from pH 5.0 to 11.5.

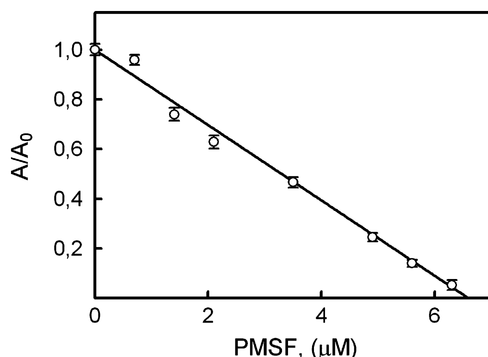

Fig. 1. Titration of cross-linked aggregates of penicillin acylase (PA CLEA) by phenylmethylsulfonyl fluoride (PMSF).

3. Results and discussion

3.1. Kinetic studies and active site titration

Determination of active sites in the CLEA PA suspension was crucial to calculate absolute values of kinetic parameters for the immobilized enzyme. Active sites of CLEA PA were quantified using titration by irreversible inhibitor, PMSF (Fig. 1). Experimental data demonstrated a good linearity and complete inhibition of the immobilized enzyme by PMSF. Moreover, the comparison of these data with the titration of the native PA revealed that only about 10% of enzymatic activity had been lost during the immobilization: PA active site concentration in the stock solution was 7.0×10^{-5} M as compared to 6.5×10^{-5} M in the CLEA PA suspension of the same volume.

As can be seen from Section 2, catalytic activity of the immobilized enzyme was monitored by spectrophotometric assay. Even though the cross-linked PA aggregates are water-insoluble particles they possess a high dispersion degree and form a relatively stable suspension. Sedimentation of this suspension proceeds slowly and therefore does not significantly vary the optical density of the reaction mixture. Thus, in "control" experiments when no chromogenic substrate was added, the optical density change was 20 times lower compared to the values registered in the presence of *iso*-NIPAB. In order to increase the accuracy and reproducibility of kinetic measurements a fairly concentrated suspension of CLEA PA should be used. Therefore, the relatively "slow" colorimetric substrate, *iso*-NIPAB, had been chosen for the assay so even a high concentration of PA active centres would cause a detectable reaction rate. This fast, accurate and reproducible spectrophotometric method for the determination of CLEA PA catalytic activity was described in more details earlier [23].

For the native PA kinetic parameters of *iso*-NIPAB at pH 7.5 were found to be $k_{cat} = 1.2\ s^{-1}$ and $K_M = 70\ \mu M$. Immobilized PA was characterized by the values of $k_{cat} = 1.0\ s^{-1}$, $K_M = 230\ \mu M$ for the "fresh" CLEA PA and $k_{cat} = 0.9\ s^{-1}$, $K_M = 140\ \mu M$ for the "mature" CLEA PA (Fig. 2). The values of k_{cat} were very similar for all the enzyme preparations. These results mainly confirm the observations of other authors [24] that cross-linking of enzyme aggregates provides a very "mild" and effective way for enzyme immobilization. At the same time, the apparent Michaelis constants of the CLEAs PA were substantially higher as compared to the native enzyme indicating that diffusion limitations take place or/and certain distortion of the substrate binding have happened. Moreover, K_M of the "fresh" and the "mature" preparations were different. In order to understand the difference, the microstructures of "fresh" and "mature" CLEA PA have been studied.

3.2. Structural investigations

Microstructure of the "fresh" and "mature" CLEA PA was investigated using confocal fluorescent microscopy, at 100×/1.3 optical magnification (Fig. 3A and B). Green-colored areas represent the cross-linked aggregates of PA, where the tyrosine, tryptophan and phenylalanine residues provide the fluorescence at 505–530 nm. Even from the unprocessed data it could be noticed that the "mature" preparation is characterized by more solid and structured packing of the spherical particles, whereas in the "fresh" preparation the fine dispersed and even amorphous zones are more pronounced. In order to characterize the preliminary observations quantitatively the images were analyzed by Zeiss LSM Image Examiner and Image-Pro Plus software. The green area was separated into objects representing individual particles containing enzyme as shown by yellow frames on the Fig. 3. These objects were consequently characterized by their area and average diameter, then sorted and counted. As a result, the distribution of particles was generated for the "fresh" and "mature" PA preparations (see Fig. 4). The characteristic particle size was found to be $1.6 \pm 0.2\ \mu m$ for the both types of immobilized PA which is in a good agreement with the data for Candida Antarctica lipase CLEA [25] where particles were characterized by a diameter of 1 μm.

Although the "fresh" and the "mature" CLEA PA were very similar in terms of characteristic particle size, the "mature" preparation had a bigger fraction of large particles as compared to the "fresh" CLEA PA. This is especially important because the quantity of enzyme molecules contained inside a particle is proportional to the particle volume, not diameter. Thus, a 2-μm sized particle will contain eight times more enzyme molecules than a particle of 1 μm diameter. To illustrate this effect more clearly the integral volume distribution of particles was plotted (Fig. 5). As can be seen from the plot, the curve of "fresh" CLEA PA exceeds the corresponding curve for the "mature" CLEA PA. It means that the majority of PA in the "fresh" CLEA is distributed among many small particles while in the "mature" CLEA it is concentrated in relatively large particles. For example, particles with a diameter less than 1.9 μm contained nearly 50% of total PA in the case of "fresh" CLEA PA and only 35% in the case of "mature" CLEA PA.

3.3. Influence of the structural deviations on the catalytic properties of CLEA PA

To clarify the influence of the structural organization of "fresh" and "mature" CLEA PA on their catalytic properties, two model enzymatic reactions, D-PGA hydrolysis and ampicillin synthesis, have been studied using both enzyme preparations (Table 1). In

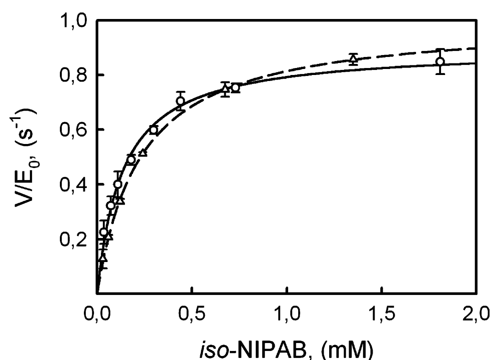

Fig. 2. Determination of the kinetic parameters, k_{cat} and K_M, of the *iso*-NIPAB hydrolysis catalyzed by fresh (△) and mature (○) CLEA PA. Lines show the fitting of the experimental data by the Michaelis–Menten equation.

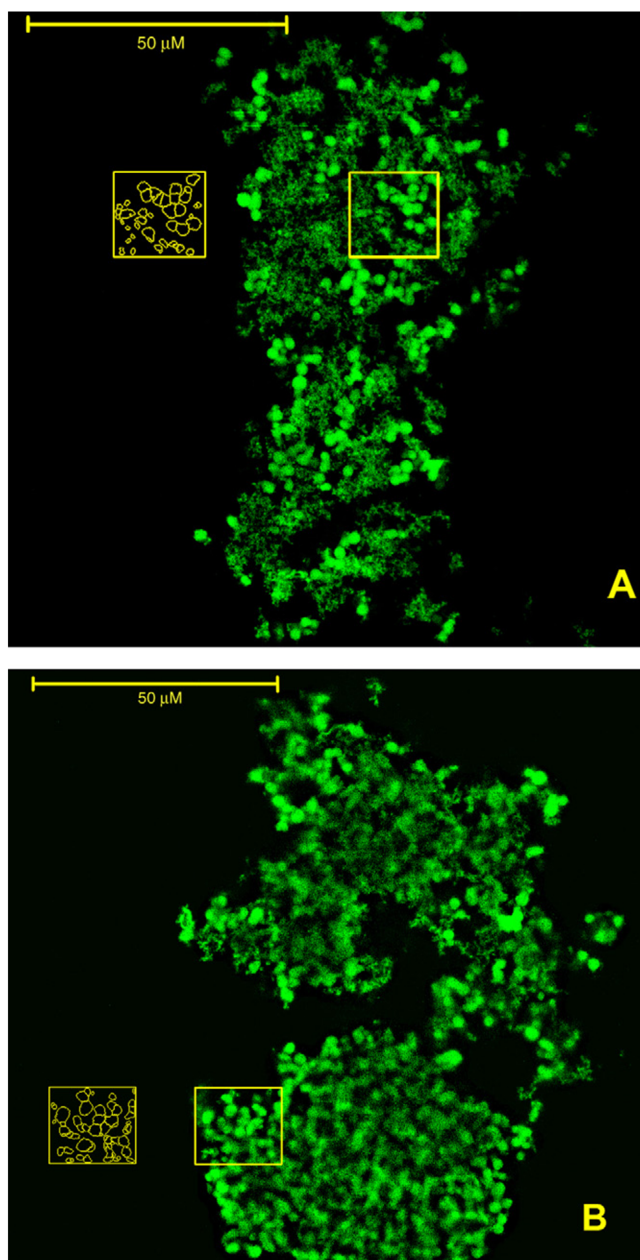

Fig. 3. Microstructures of the “fresh” (image A) and the “mature” (image B) CLEA PA, obtained by the confocal fluorescent microscopy (100×/1.3 magnification). The right frames confine the areas, selected for the quantification of the obtained data, the left frames demonstrate the results of image processing and outline of the “digital” CLEA particles. (For interpretation of the references to color in the text, the reader is referred to the web version of the article.)

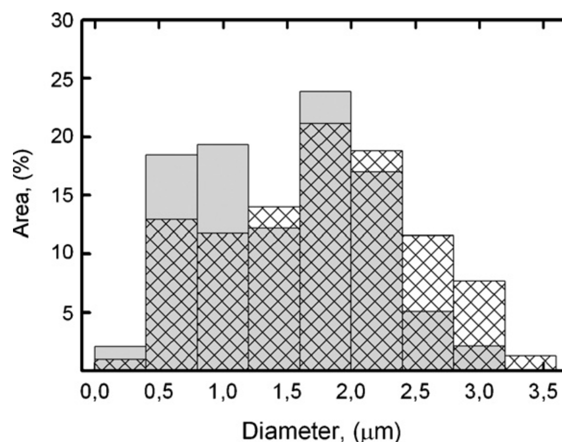

Fig. 4. Differential distribution of the CLEA PA particles in “fresh” (filled) and “mature” (hatched) enzyme preparations.

the synthetic reaction both CLEA PA preparations demonstrated the synthesis/hydrolysis ratio (S/H) which was as about 15–30% lower than that for the native PA. Similar effect was also recently described for the lipase activity [25]. Nevertheless, it should be noted that the “mature” CLEA PA demonstrated better results as compared to the “fresh” preparation.

The data for the hydrolysis of D-PGA were remarkably similar to those for the hydrolysis of *iso*-NIPAB described in the first section. Again, the “mature” CLEA PA possessed a maximum catalytic activity which was higher than that of the native enzyme or the “fresh” preparation. The apparent Michaelis constant was the lowest in case of the native enzyme; the “mature” and the “fresh” CLEA PA demonstrated 1.5 and 1.8 times higher values correspondingly. One of the obvious reasons for the increased binding constants of CLEAs PA is diffusion limitations because relatively big particles of CLEA should contain at least 10^7 molecules of PA. Therefore, the

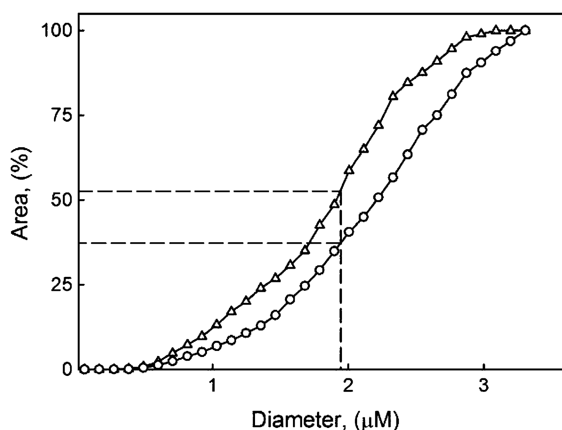

Fig. 5. Integral distribution of the CLEA PA particles in “fresh” (△) and the “mature” (◯) CLEA PA.

Table 1
Comparison of the catalytic properties of the “fresh” and the “mature” CLEA PA

Enzyme preparation	D-PGA hydrolysis		Ampicillin synthesis[a]		
	K_M (mM)	V_{max} (mM/min)	V_{synt} (mM/min)	V_{hydr} (mM/min)	S/H
“Fresh” CLEA PA	33	0.48	0.23	0.1	2.3
“Mature” CLEA PA	28	0.56	0.29	0.11	2.6
Native PA	18	0.47	–	–	3.2

[a] D-PGA 100 mM, 6-APA 100 mM. Reaction conditions: pH 7.0, 25 °C, enzyme active site concentration 6.5×10^{-7} M.

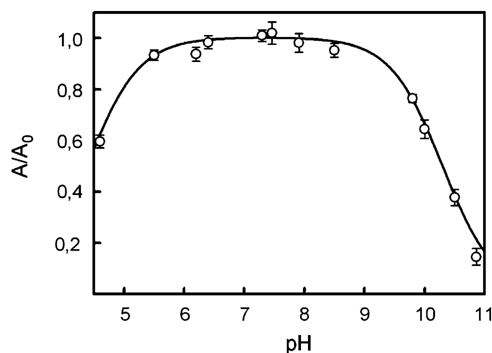

Fig. 6. pH-dependence of the catalytic activity of the "mature" CLEA PA (○, experimental data; solid line, calculated curve). Curve is calculated according to the equation: $A = A_0/(1 + [H^+]/K_a + K_b/[H^+])$.

"mature" CLEA PA would be expected to have higher K_M value as compared to the "fresh" preparation because the latter is composed of smaller particles and hence has a higher surface area. However, the same reason should cause very intensive covalent modification of PA inside the "fresh" particles during the synthesis of CLEA (aggregates cross-linking step). At the same time, PA deep inside big particles, which are common for the "mature" CLEA, will be less modified. As it was shown before, covalent cross-linking of PA with glutaraldehyde is very likely to cause distortion of the native enzyme conformation [26,24] that might result in a growth of the binding constant. If the impact of the K_M growth due to this process will overcome the impact of diffusion limitations then the "fresh" CLEA PA will possess a higher apparent K_M as compared to the "mature" CLEA PA.

3.4. pH-dependencies of CLEA PA activity and stability

The catalytic activity of the "mature" CLEA PA was investigated in a broad pH range. The immobilized PA demonstrated a typical bell-shaped pH dependence of the catalytic activity (Fig. 6), similar to that for the native enzyme but extended in alkaline region (pK for the native PA and "mature" CLEA PA are 9.9 and 10.5, correspondingly).

Study of the pH-stability revealed quite interesting, different from the native enzyme, behavior of the cross-linked biocatalyst. Particularly, in both acidic and alkaline regions the decay of the catalytic activity of CLEA PA with inactivation time could not be described in terms of the first order kinetics (Fig. 7A and B) which is typical for the native enzyme, when

$$A = A_0\,e^{-kt}, \quad (1)$$

however, if the "mature" preparation were to consist from two different fractions of PA characterized different stability, and then inactivation course could be described according to following equations:

$$A = A_0\ e^{-k_1 t} + A_{01}\,e^{-k_2 t}, \quad (2)$$

which is a superposition of two first-order processes. Fitting of experimental data with this equation gives the excellent correlation for all tested pH values (Fig. 7, solid lines).

Comparison of the pH-stabilities for the native and immobilized PA (Fig. 8) showed that stability of the first, less stable fraction in CLEA PA is very close to the stability of the native enzyme. The second, more stable fraction had remarkably lower inactivation constant in both acidic and alkaline regions.

Interestingly, that the slopes of the left and right branches obtained from $\log k_{in}$—pH plots are very similar for both inactivation constants of CLEA PA (−2.5/+1 for "fast" k_1 and −2.46/+1.3 for

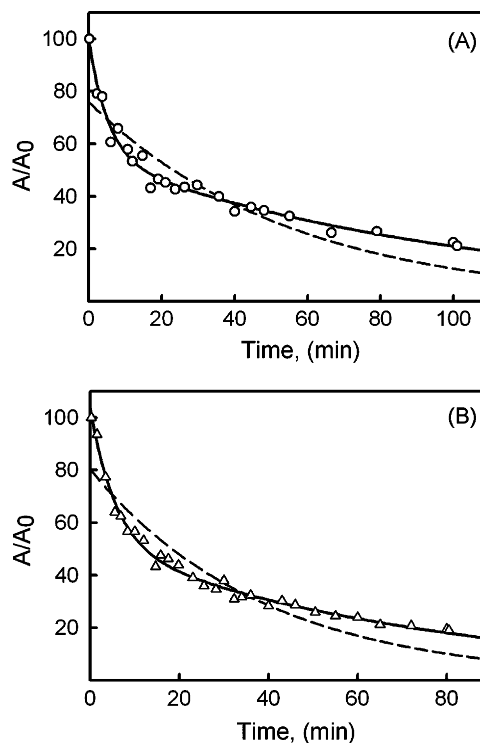

Fig. 7. Inactivation of PA in the "mature" CLEA PA preparation: (A) pH 3.0 and (B) pH 11.0. Dashed lines represent fitting of the experimental data by the first-order kinetics: $A = A_0 \cdot e^{-kt}$, solid lines by the second-order exponential decay: $A = A_0\ e^{-k_1 t} + A_{01}\,e^{-k_2 t}$.

"slow" k_2). Additionally they are close to the values, obtained for the native enzyme (−3/+1.1, results from [27]). Hence, it could be suggested that immobilization does not change the mechanism of pH-inactivation of PA; the same ionogenic groups maintain the catalytically active protein conformation in the native enzyme and in CLEA PA.

3.5. Peculiarities of the CLEA PA structural organization

Summarizing the data on the pH-stability, synthetic and hydrolytic activity of CLEA PA it appears that there are at least two different types of PA molecules making up the CLEA particle. The

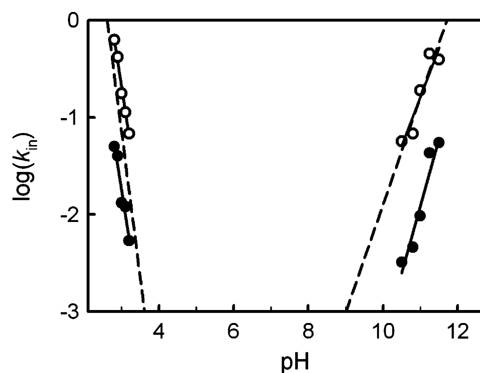

Fig. 8. Stability of two PA fractions in the "mature" CLEA PA: ●, the more stable fraction; ○, the less stable fraction; solid lines, linear fitting according to the equations: $\log(k_{1in}) = -2.55 \times pH + 6.99$, $\log(k_{2in}) = -2.46 \times pH + 5.64$ in acidic region and $\log(k_{1in}) = 1.0 \times pH - 11.9$, $\log(k_{2in}) = 1.3 \times pH - 17.2$ in alkaline region. Dashed lines show the pH-dependence of the first order inactivation rate constants for the native PA calculated according to the equations $\log(k_{in}) = -3.0 \times pH + 7.85$ and $\log(k_{in}) = 1.11 \times pH - 13$ [27].

first type is presented by the molecules presumably located near the particles surface and in less structured/amorphous regions. PA from these regions should be intensively cross-linked by glutaraldehyde. The molecules of the second type are located deeper inside the CLEA globule. Therefore, these PA molecules are much less distorted by the chemical cross-linking and possess catalytic properties that are close to those of the native enzyme. Thus, the enlargement of the average particle size, which happens over time with precipitated PA aggregates (the so-called "maturation" process), leads to the increase of the second, less distorted by the following chemical cross-linking, fraction of CLEA PA molecules. This, in turn, should preserve high catalytic activity of the immobilized enzyme which indeed could be observed from the comparison of the catalytic properties of the "fresh" and "mature" CLEA PA in the hydrolysis of D-PGA and synthesis of ampicillin (Table 1). On the other hand, the increase of the particle's size might cause the diffusion limitations and growth of the apparent K_M-value. Therefore, in each specific case the optimal particle size, which would combine high catalytic activity of an enzyme after immobilization and good substrate access, i.e. low diffusion limitations, should be determined.

4. Conclusions

Immobilization of PA in a form of CLEAs was shown to be a very effective and mild procedure leading to a highly disperse, water-insoluble material in which presumably two fractions of the enzyme do exists. The ageing effect has been demonstrated to influence the structural organization and the catalytical properties of CLEA PA. This fact enables one to tune the properties of the immobilized enzyme by modifying procedure of CLEA synthesis.

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