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A New Method for Spectrophotometric Assay of Activity of Cross-Linked Penicillin Acylase Aggregates

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Abstract—A new method for monitoring reactions catalyzed by an immobilized enzyme, cross-linked penicillin acylase aggregates (PA CLEA), is suggested. Appropriate chromogenic substrates for spectrophotometric assay of catalytic activity of immobilized enzyme were chosen and their kinetic parameters determined. Active sites in PA CLEA preparations were titrated by the suggested method; it is shown that almost all active sites are retained during immobilization. This method is characterized as highly expressive, simple, and precise, and may be used for control of PA immobilization efficiency as well as for study of operational, thermal, and pH stability immobilized enzyme preparations.

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Key words: cross-linked enzyme aggregates, penicillin acylase, activity assay method, chromogenic substrates

Penicillin acylases (PA) are one of the few enzymes used in large-scale biocatalyst industry. Using penicillin acylases, the nuclei of β-lactam antibiotics [1-3] and new penicillins and cephalosporins [4-6] are now obtained in industry; enzymatic synthesis of optically active amines is also very perspective [7, 8]. It is natural that for efficient industrial use, there is a need for preparation of immobilized biocatalyst with high specific activity, stability within a wide range of the reaction conditions, and retained catalytic properties of the native enzyme on immobilization. In the past ten years, many new immobilized PA preparations have been developed and studied. It is worth mentioning studies on enzyme covalent immobilization [9-11], its inclusion in carrier pores [12, 13], and especially on development of immobilized PA preparations free of ballast carrier—cross-linked crystals [14, 15] or cross-linked enzyme aggregates (CLEA) [16-18].

Quantitative characterization of catalytic properties of the obtained biocatalyst is one of the key stages of any of the abovementioned immobilization methods; based on this characteristic, perspectives of further development of a certain preparation are evaluated. The absence of convenient and express method for characterization of properties of the studied immobilized enzyme often hinders development of preparations. To study the properties of immobilized PA, pH statting and HPLC are now mainly used; each of these methods has significant drawbacks. Thus, pH statting is suitable only for determination of carbonic acids and ammonia released during hydrolysis of a corresponding substrate, and HPLC, although being universal, is rather time-consuming and laborious for quantitative characterization of catalytic properties of an enzyme.

However, there exists a precise, rapid, and simple route [19]: the spectrophotometric method most popular for assay of activity of native PA has still not been adapted for study of immobilized enzyme preparations. In this study, we suggest a procedure for spectrophotometric assay of catalytic activity of PA CLEA.

MATERIALS AND METHODS

Reagents. In this study, we used phenylmethylsulfonyl fluoride (PMSF) from Merck (Germany); *p*-nitro-*o*-carboxyanilide of phenylacetic acid (NCAPA) was synthesized as described earlier [19]; *p*-nitro-*m*-car-

Abbreviations: CLEA) cross-linked enzyme aggregates; NCAPA) *p*-nitro-*o*-carboxyanilide of phenylacetic acid; NCAPG) *p*-nitro-*m*-carboxyanilide of D-(–)-phenylglycine; PA) penicillin acylase; PEG) polyethylene glycol; PMSF) phenylmethylsulfonyl fluoride.

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boxyanilide of D-(−)-phenylglycine (NCAPG) was kindly donated by Dr. E. de Vries (The Netherlands); penicillin acylase (PA) from *Escherichia coli* was purified as described earlier [19]. Other reagents and buffer components were from Merck.

Assay of activity of native PA and cross-linked enzyme aggregates. Activity of the native PA was assayed by monitoring accumulation of a colored product of NCAPA hydrolysis (<5% substrate conversion) at 400 nm in 0.01 M KH_2PO_4, 0.1 M KCl, pH 7.5 (cuvette thermostatted at 25°C). The reaction was initiated by addition of 50 μl of enzyme solution to 500 μl of NCAPA solution in the cuvette of a UV-1601 spectrophotometer from Shimadzu (Japan). Monitoring of the product accumulation was started after intensive stirring. Activity of PA CLEA was assayed analogously to that of the native enzyme. The absence of absorption fluctuations on incubation of PA CLEA preparations in the absence of the substrate was proved in control experiments.

Production of cross-linked penicillin acylase aggregates. At the first stage enzyme (concentration of active sites 10^{-5}-10^{-4} M) was precipitated from 0.1 M KH_2PO_4, pH 7.0 (buffer A), cooled to 4°C by addition of small portions of polyethylene glycol (PEG-6000); the final PEG concentration was 25% (w/v), and the residual PA activity in the supernatant was <3% of that of the initial solution. Then the suspension was chemically linked using 25% glutaraldehyde. The reaction was performed at 0°C by addition of small portions of glutaraldehyde (final concentration 1% (w/v)) with intensive stirring. When no hydrolytic activity against NCAPA was detected in the supernatant, the reaction mixture was filtered, and the precipitate was multiply washed with buffer A. The washed precipitate was placed into the same buffer with the volume equal to that of the initial PA solution.

Determination of the active site concentration in the native and immobilized penicillin acylase. The concentration of active sites in solution of the native PA and suspension of cross-linked enzyme aggregates (CLEA) was determined by titration with the specific irreversible inhibitor PMSF [19]. Titration with PMSF was performed as follows: a portion of initial CLEA suspension was tenfold diluted with buffer A and pH of the mixture was brought to 6.0. The resulting suspension was divided into equal parts (1000 μl) with intensive stirring. The required PMSF volume was added to each part, the maximal volume of titrant being not more than 50 μl. After addition of PMSF, the mixtures were intensively stirred for 20 min at room temperature, and the residual enzymatic activity was assayed. The concentration of active sites in the studied suspension was determined via the portion of the *x* axis intercepted by linear regression plotted in "residual activity–PMSF concentration" coordinates.

Evaluation of kinetic parameters of hydrolysis of chromogenic substrates. Apparent kinetic parameters (k_{cat} and K_m) of hydrolysis of chromogenic substrates catalyzed by the native and immobilized PA were determined via dependence of the initial reaction rates on the substrate concentration. For this, experimental data were approximated by nonlinear regression using the Michaelis–Menten equation and Sigma Plot 2001 program (SPSS, USA). Enzymatic hydrolysis was performed in a thermostatted cuvette of the UV-1601 spectrophotometer as described above. For cross-linked PA aggregates, each experiment was repeated not less than three times.

RESULTS AND DISCUSSION

Spectrophotometric assay of CLEA activity. A specific feature of PA CLEA is that this form of immobilized catalyst is obtained without any ballast carrier but via chemical linkage of preliminarily precipitated enzyme. In this case, the preparation of immobilized biocatalyst is a finely dispersed suspension.

The microstructure of the preparation particles obtained by fluorescence confocal microscopy is shown in Fig. 1. As shown, cross-linked PA aggregates are spherical particles with diameter 0.5-2.0 μm. The data suggest that such suspensions will be stable against sedimentation in aqueous solutions during a time period sufficient for assay of catalytic activity of the immobilized enzyme. To check this suggestion, we performed a series of experiments monitoring the rates of change in absorption of solutions containing various volumes of PA CLEA suspension. Some typical curves of time-dependent change in absorption of such solutions are presented in Fig. 2.

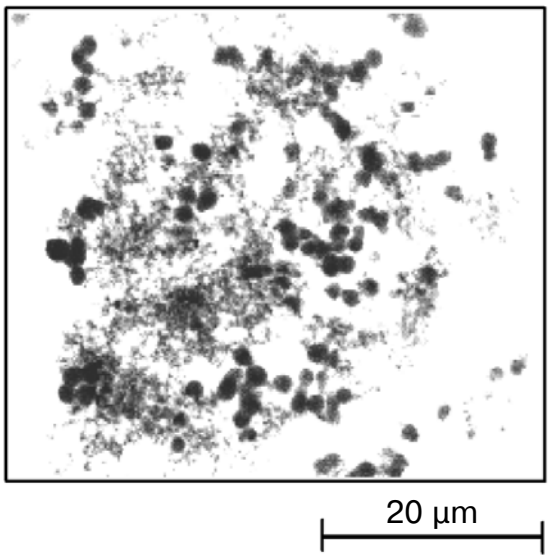


Fig. 1. Structure of cross-linked penicillin acylase aggregates obtained using an LSM 510 Axiovert fluorescent confocal microscope from Zeiss (Germany), magnification 100×/1.3.

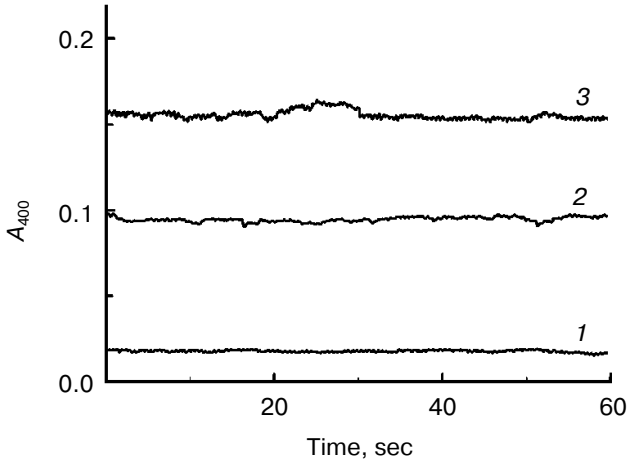


Fig. 2. Changes in absorption in the experimental cell on addition of 25 (*1*), 50 (*2*), and 100 µl (*3*) PA CLEA to 500 µl of buffer A at 400 nm and 25°C. The rates of change in absorption (ΔA/min): *1*) $(6.1 \pm 0.8)\cdot10^{-4}$; *2*) $(2.2 \pm 0.2)\cdot10^{-3}$; *3*) $(3.2 \pm 0.4)\cdot10^{-3}$.

The data indicate that during the time needed for monitoring the rate of enzymatic reaction, any significant changes in absorption due to sedimentation of enzymatic suspension do not occur (the rate of change in absorption due to sedimentation of CLEA suspension is at least 20 times lower than the rate of enzymatic conversion as monitored). Absorption fluctuations arising from the presence of heterogeneous particles in experimental cell have statistical origin and even when using enzyme with twofold excess (curve *3*) do not exceed ±0.0026 ΔA/min, this being almost two orders of magnitude lower than the monitored changes in absorption (in this study, not less than 0.1 ΔA/min) caused by enzymatic hydrolysis of chromogenic substrate.

Thus, sedimentation of particles and absorption fluctuations arising from heterogeneity of the system do not have statistically significant effect on monitoring of absorption in the course of enzymatic conversion.

Choice of substrates for spectrophotometric assay of activity of PA CLEA. To obtain reproducible spectrophotometric results, significant aliquots (25-50 µl) of PA CLEA allowing minimization of errors during sampling of enzymatic suspension should be used. This requirement limits choose of substrates suitable for assay of enzymatic activity. In particular, good candidates are substrates with a low k_{cat} value allowing use of relatively large enzyme concentrations in the experimental cell. According to [19], for the native PA from *E. coli*, such substrates are NCAPA and NCAPG. In fact, study of hydrolysis of these substrates catalyzed by PA CLEA showed (Figs. 3 and 4) that the spectrophotometric method could be efficiently used for a wide variety of substrate and immobilized enzyme concentrations. As shown in Fig. 3, the initial rate of enzymatic conversion linearly increases on increase in the immobilized PA concentration in the cell from 0.03 to 1.2 µM, that is, more than one order of magnitude. This allows the use of this method not only for assay of the immobilized enzyme activity, but also for evaluation of kinetic parameters.

Titration of the active sites of cross-linked PA aggregates. To determine the absolute values of catalytic constants for the studied conversions, concentration of the active sites of the enzyme should be known. For this, PA active sites were titrated in the native enzyme solution as well as in suspension of cross-linked aggregates obtained from an equal quantity of the native enzyme. Such titra-

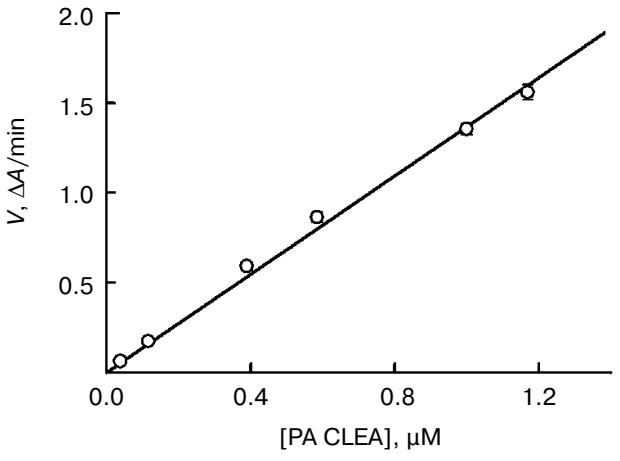


Fig. 3. Initial rate of NCAPA hydrolysis catalyzed by PA CLEA versus the immobilized enzyme concentration. Experimental data (points) are the average value of three independent experiments (± standard deviations). Reaction conditions: 2 mM NCAPA, 0.01 M KH_2PO_4/0.1 M KCl, pH 7.5, 25°C.

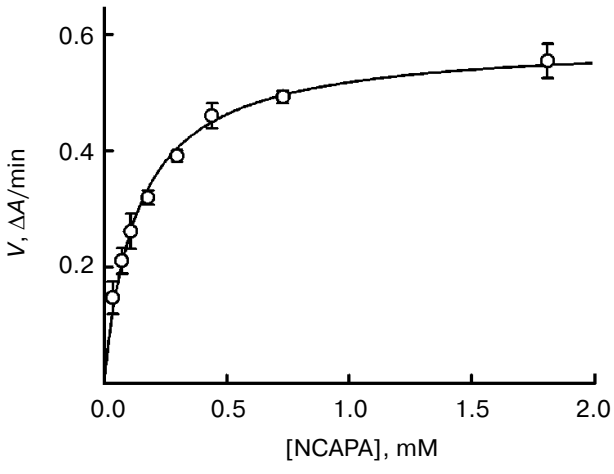
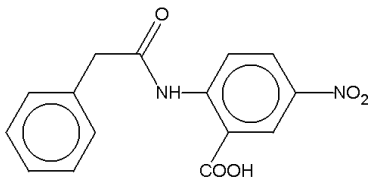
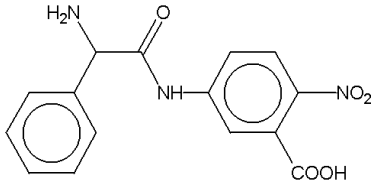


Fig. 4. Initial rate of NCAPA hydrolysis catalyzed by PA CLEA versus substrate concentration. Experimental data (points) are the average values of three independent experiments (± standard deviations). Reaction conditions: 1 µM PA CLEA, 0.01 M KH_2PO_4/0.1 M KCl, pH 7.5, 25°C.

Kinetic parameters of hydrolysis of chromogenic substrates catalyzed by cross-linked enzyme aggregates and native PA

Substrate	Structural formulae	PA CLEA		Native PA	
		K_m, mM	k_{cat}, sec^{-1}	K_m, mM	k_{cat}, sec^{-1}
NCAPA		0.14 ± 0.01	1.0 ± 0.1	0.075 ± 0.008	1.5 ± 0.1
NCAPG		6.0 ± 0.08	10.0 ± 0.7	2.0 ± 0.3	12.0 ± 0.5

tion demonstrates immobilization efficiency, that is, number of active sites transferred from solution to CLEA (Fig. 5).

The data demonstrate that after immobilization, the concentration of the active sites in CLEA preparations is not less than 90% of that for the initial enzyme; taking filtration and multiple washing of the immobilized preparation into account, this indicates that during immobilization, inactivation of the enzyme by the action of glutarate aldehyde does not occur. After assay of the concentration of PA active sites, it is possible to use the values of catalytic constants for free and immobilized enzyme instead of the values of maximal rate (Fig. 4). Corresponding kinetic parameters and the structures of substrates are presented in the table; as can be seen, the native and immobilized PA have k_{cat} values close to each other, whereas in case of PA CLEA K_m values for NCAPA and NCAPG are respectively two and three times higher than those for the soluble enzyme form.

So, a simple, precise, and very rapid procedure of spectrophotometric monitoring of catalytic activity of PA CLEA has been developed. This method can be used to monitor the efficiency of the PA immobilization process as well as for study of operational, thermal, and pH stability of immobilized enzyme preparations.

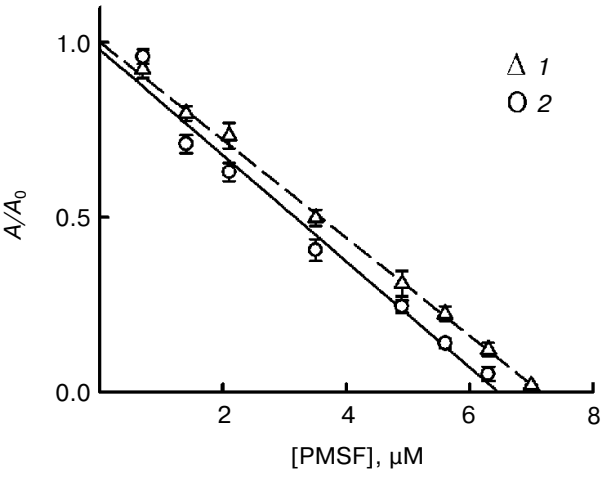


Fig. 5. Titration with PMSF of the active sites of the native PA used for immobilization (*1*) and cross-linked PA aggregates (*2*). Experimental data (points) are the average values of three independent experiments (± standard deviations). Reaction conditions: 0.01 M KH_2PO_4/0.1 M KCl, pH 7.5, 25°C.

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REFERENCES

1. Matsumoto, K. (1993) *Bioprocess Technol.*, **16**, 67-88.
2. Shewale, J. G., and Sivaraman, H. (1989) *Proc. Biochem.*, **24**, 146-154.
3. Bruggink, A., Roos, E. C., and de Vroom, E. (1998) *Org. Proc. Res. Dev.*, **2**, 128-133.
4. Youshko, M. I., Moody, H. M., Bukhanov, A. L., Boosten, W. H. J., and Švedas, V. K. (2004) *Biotechnol. Bioeng.*, **85**, 323-329.
5. Van Langen, L. M., de Vroom, E., van Rantwijk, F., and Sheldon, R. (1999) *FEBS Lett.*, **456**, 89-92.
6. Schroen, C. G., Nierstrasz, V. A., Bosma, R., Kroon, P. J., Tjeerdsma, P. S., de Vroom, E., VanderLaan, J. M., Moody, H. M., Beeftink, H. H., Janssen, A. E., and Tramper, J. (2002) *Biotechnol. Bioeng.*, **80**, 144-155.

7. Guranda, D. T., van Langen, L. M., van Rantwijk, F., Sheldon, R. A., and Švedas, V. K. (2001) *Tetrahedron: Asymmetry*, **12**, 1645-1650.
8. Guranda, D. T., Khimiuk, A. I., van Langen, L. M., van Rantwijk, F., Sheldon, R. A., and Švedas, V. K. (2004) *Tetrahedron: Asymmetry*, **15**, 2901-2906.
9. Mateo, C., Abian, O., Fernandez-Lorente, G., Pedroche, J., Fernandez-Lafuente, R., Guisan, J. M., Tam, A., and Daminati, M. (2002) *Biotechnol. Progr.*, **18**, 629-634.
10. Bianchi, D., Golini, P., Bortolo, R., and Cesti, P. (1996) *Enzyme Microb. Technol.*, **18**, 592-596.
11. Jianguo, L., Wei, C., Shuai, W., and Fan, O. (2001) *Reactive*, *Functional Polymers*, **48**, 75-84.
12. He, J., Li, X., Evans, D. G., Duan, X., and Li, C. (2000) *J. Mol. Catalysis B: Enzymatic*, **11**, 45-53.
13. Prabhune, A., and Raman, S. H. (1991) *Appl. Biochem. Biotechnol.*, **30**, 265-272.
14. St. Clair, N. L., and Navia, M. A. (1992) *J. Am. Chem. Soc.*, **114**, 7314-7316.
15. Margolin, A. L. (1996) *Trends Biotechnol.*, **14**, 223-230.
16. Cao, L. Q., van Rantwijk, F., and Sheldon, R. A. (2000) *Org. Lett.*, **2**, 1361-1364.
17. Cao, L. Q., van Langen, L. M., van Rantwijk, F., and Sheldon, R. A. (2001) *J. Mol. Catalysis B: Enzymatic*, **11**, 665-670.
18. Mateo, C., Palomo, J. M., van Langen, L. M., van Rantwijk, F., and Sheldon, R. A. (2004) *Biotechnol. Bioeng.*, **86**, 273-276.
19. Youshko, M. I., Shamolina, T. A., Guranda, D. F., Synev, A. V., and Švedas, V. K. (1998) *Biochemistry (Moscow)*, **63**, 1104-1109.