PROCEEDINGS OF THE YEREVAN STATE UNIVERSITY

Chemistry and Biology 2013, № 1, p. 32–35

Biology

IMMOBILIZATION OF RECOMBINANT L-AMINOACYLASE FROM *GEOBACILLUS STEAROTERMOPHILUS* AND CHARACTERISTICS OF OBTAINED PREPARATIONS

H. A. AGANYANTS[1 *], Ye. A. HOVHANNISYAN[2**], H. O. KOLOYAN[1], A. S. HOVSEPYAN[1], A. A. HAMBARDZUMYAN [1]

[1] *SPC "Armbiotechnology" NAS RA*
[2] *Chair of Biochemistry YSU, Armenia*

Thermophilic L-aminoacylase *Geobacillus stearotermophilus* was immobilized on silochrome C-80 with glutaraldehyde. Immobilization process does not affect the temperature optima of derived preparations, but increase in the thermal stability of the immobilized aminoacylase was observed. Michaelis constants (K_m) were calculated for N-acetyl-L-methionine, N-acetyl-L-valine and N-acetyl-L-alanine. It was shown that as a result of immobilization K_m for N-acetyl-L-methionine increased more than 2-fold.

Keywords: *Geobacillus stearotermophilus*, L-aminoacylase, immobilization, thermal stability, Michaelis constant.

Introduction. Aminoacylases (N-acetylamino acid amidohydrolases, EC 3.5.1.14) catalyze the hydrolysis of N-acetyl-L-amino acids to form the corresponding L-amino acids and play a crucial role in the metabolism of various organisms. The enzyme was isolated from a number of organisms [1–2] and has been actively studied. Proceeding from the industrial significance, aminoacylases are among the ten enzymes mostly used in biotechnology [3]. Enzyme immobilization is a promising direction in the development of industrial biotechnology [4]. For many years immobilized on solid support aminoacylases were actively studied in order to find the best options for immobilization and preparation of enantiomerically pure amino acids [5–8]. The major objective of the enzyme immobilization is to obtain a biocatalyst with high stability and favorable production characteristics. Multiple-use, quick stopping of the process, simple product isolation procedures are the main advantages of immobilized catalysts. The stability of the enzyme-carrier system is determining the selection of appropriate method for immobilization. For this reason, one of the most accepted methods is covalent immobilization by forming irreversible covalent bonds in the enzyme-carrier system [9]. Silochrome C-80 is inorganic, rigid, inexpensive, nontoxic macroporous silica and is widely used in the process of immobilization [10–12].

* E-mail: hovrau@mail.ru ** E-mail: eranuhihovhannisyan@yahoo.com

The purpose of this paper is immobilization of *Geobacillus stearothermophilus* L-aminoacylase, isolated from a recombinant strain of *E. coli* BL21Ω (DE3)/pETama on silochrome C-80, the study of the characteristics of the immobilized biocatalyst and comparison with the characteristics of free L-aminoacylase.

Materials and Methods. Glutaraldehyde, phenylmethylsulfonyl fluoride (PMSF), 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), γ-aminopropyl-3-ethoxy silane of "Sigma" Company USA, silochrome C-80 (pore diameter 400–600 *Å*, particle size of 0.35–0.5 *mm*, the specific area of 60–120 m^2/g) and other reagents of CIS countries production were used in this study. The purified L-aminoacylase isolated from a recombinant strain of *E. coli* BL21Ω(DE3)/pETama was used for the immobilization [13].

The enzyme activity was determined at 52^0C in the reaction medium of 200 μl final volume, containing 100 *mM* HEPES pH 7.2, 0.2 *mM* $CoCl_2$, 40 *mM* N-acetyl--DL-methionine and the appropriate amount of free or immobilized enzyme. The reaction was stopped by adding trichloroacetic acid (TCA) to 3%. The quantity of the produced L-methionine was determined by the reaction of amino acid with orthophthalaldehyde and measuring optical density of the solutions obtained at a wavelength of 340 *nm*. One unit of activity was assumed by the amount of enzyme catalyzing the formation of 1 *mmol* L-methionine for 1 *min* under the reaction conditions. The protein concentration was determined by the method of Groves and Davis [14].

Immobilization of the enzyme on silochrome C-80 was carried out by the method developed in [10]. To activate the resin was subjected to heat treatment (5 *h*, 500^0C). Further, for the introduction of amino groups in the silica matrix the carrier was incubated for 48 *h* at 45^0C in 2% acetone solution of γ-aminopropyl-3--ethoxy silane stirring on an orbital shaker (100 *rpm*), after which the carrier was washed with acetone (2 times) and dried at 45^0C. Aminated silica was activated with glutaraldehyde. For this purpose, 100 *mg* of aminated carrier was added to 1 *ml* of 5% glutaraldehyde solution and incubated for 30 *min* at 20^0C. Incubation was carried out on an orbital shaker (100 *rpm*). An activated carrier was washed with water (7–10 times) and then mixed with 1 *ml* of buffer solution A (0.2 *mM* PMSF, 0.2 *mM* $CoCl_2$, 100 *mM* HEPES, pH 7.2), containing 1 *mg* of purified enzyme and was incubated for 20 *h* at 20^0C under the above-mentioned conditions. The resulting biocatalyst was washed five times with 100 *mM* Tris HCl buffer (pH 8.3) to block free aldehyde groups and then two times with distilled water.

To determine the temperature optimum, the activities of the free and immobilized enzyme preparations were determined in the temperature range of 25–80^0C according to the above method.

To determine the thermal stability, the free and immobilized enzyme preparations were incubated at different temperatures (25–75^0C) for 20 *min* and rapidly cooled in an ice bath. The specific activity was determined by the above method.

For determining Michaelis constants (K_m) the activity of enzyme preparations was determined at concentrations of N-acetyl-L-amino acids of 0.1–5.0 *mM*. K_m was calculated using multivariate linear regression analysis according to our programs written in Gauss 4.0.

Results and Discussion.

Immobilization conditions. We first held immobilization of bacterial L-aminoacylase on porous silica. Carrier was activated with glutaraldehyde by

standard methods as described above. In the process of binding enzyme to an activated carrier to increase the yield of aminoacylase activity in the reaction medium, stabilizers were added in different ratios. Results of the study are shown in Fig. 1. As follows from figure, buffer solution A containing 20 *mM* N-acetyl-DL-methionine shows the best stabilizing effect during immobilization. The pointed conditions were used in further immobilizations of the study.

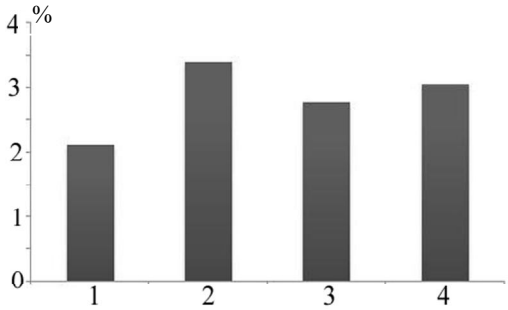

Fig. 1. Immobilization of recombinant L-aminoacylase on silochrome C-80:
1 – Buffer solution A;
2 – Buffer solution A containing 20 *mM* N-acetyl--DL-methionine;
3 – Buffer solution A without $CoCl_2$;
4 – Buffer solution A containing 20% glycerol.

Determination of the Optimum Temperature. The results of the study of the temperature optimum of the free and immobilized preparations of L-aminoacylase are presented in Fig. 2, A. These results showed that the immobilization process did not affect the optimum temperature of obtained preparations. Both enzyme preparations showed the highest activity at 65^0C.

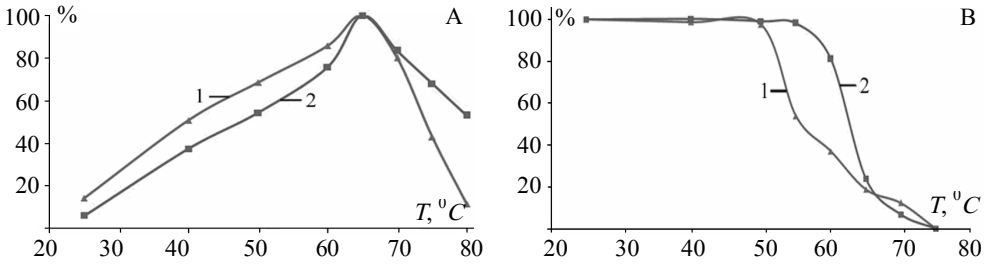

Fig. 2. The temperature dependence of the relative activity of free and immobilized aminoacylase preparations (A); the same after 20 *min* incubation (B): 1 – free enzyme (100% corresponds to 10 *u/mg* specific activity); 2 – immobilized enzyme (100% corresponds to 5.6 u/g_{silica} specific activity).

To Determine the Thermal Stability, free and immobilized L-aminoacylase preparations were incubated for 20 *min* at temperatures of 25 to 75^0C and the residual activity was determined. The results are shown in Fig. 2, B.

As follows from figure, immobilization resulted increases the thermal stability of the enzyme. If a free enzyme preparation retains 50% of the initial activity when incubated for 20 *min* at 55^0C, then the immobilized enzyme retains 50% of the activity at 62^0C.

The affinities of free and immobilized aminoacylases to some substrates are shown in the Table. It is shown that as a result of immobilization the Michaelis constant (K_m) for N-acetyl-L-methionine increases from 1.29 (for the free enzyme) to 2.96 *mM*. For comparison, K_m for N-acetyl-L-methionine, N-acetyl-L-valine and N-acetyl-L-alanine were made up 7.7, 2.5 and 1.2 correspondingly for lactococcal aminoacylase [1], and K_m for N-acetyl-L-methionine was 6.6 *mM* for *Pyrococcus furiosus* aminoacylase [15].

K_m values for different N-acetyl derivatives of L-amino acids

Substrate	K_m, *mM*	
	Free Enzyme	Immobilized Enzyme
N-acetyl-L-methionine	1.43 ± 0.64	3.44 ± 2.14
N- acetyl-L-valine	1.31 ± 0.80	–
N- acetyl-L-alanine	0.56 ± 0.08	–

Conclusion. Immobilization of L-aminoacylase of *G. stearothermophilus* on silochrome C-80 was carried out. It is shown that as a result of immobilization the thermal stability of the enzyme increases and affinity of N-acetyl-L-methionine reduces. These qualities are good prerequisites for using the obtained catalysts in the production of enantiometrically pure amino acids.

Received 14.01.2013

REFERENCES

1. **Curley P., van der Does C., Driessen A.J., Kok J., van Sinderen D.** Purification and Characterisation of a Lactococcal Aminoacylase. // Arch. Microbiol., 2003, v. 179, № 6, p. 402–408.
2. **Koreishi M., Asayama F., Imanaka H., Imamura K., Kadota M., Tsuno T., Nakanishi K.** Purification and Characterization of a Novel Aminoacylase from *Streptomyces Mobaraensis*. // Biosci. Biotechnol. Biochem., 2005, v. 69, № 10, p. 1914–1922.
3. **Tewari Y.B.** Thermodynamics of Industrially-Important, Enzyme-Catalyzed Reactions. // Appl. Biochem. Biotechnol., 1990, v. 23, p. 187–203.
4. **Aggarwal S., Sahni S.** The Commercial Exploitation of Immobilized Enzymes. International Conference on Environmental, Biomedical and Biotechnology IPCBEE, 2012, v. 41, p. 18–22.
5. **Chibata I., Tosa T., Sato T., Mori T., Matsuo Y.** Preparation and Industrial Application of Immobilized Aminoacylases. In Proc. IV IFS: Ferment Technol. Today, 1972, p. 383–389.
6. **Mattiasson B.** In Immobilized Cells and Organelles. Boca Raton, FL: CRC Press, 1983, v. 1, p. 3–25.
7. **He B., Li M., Wang D.** Studies on Aminoacylase from *Aspergillus Oryzae* Immobilized on Polyacrylate Copolymer. // Reactive Polymers, 1992, v. 17, p. 341–346.
8. **German A.B., Neklyudov A.D.** Obtaining and Properties of Immobilized Aminoacylase from *Streptoverticillium olivoreticuli*. // Appl. Biochem. Microbiol., 2001, v. 37, № 1, p. 63–66.
9. **Yamaguchi R., Arai Y., Kaneko T., Itoh T.** Utilization of Partially N-Succinated Derivatives of Chitosan and Glycolchitosan As Supports for the Immobilization of Enzymes. // Biotechnol. Bioeng., 1982, v. 24, p. 1081–1091.
10. **Dyukova K.G., Ambartsumyan A.A., Alebyan G.P.** Covalent Immobilisation of Purified *Citrobacter Freundii* Tyrosine Phenol-Lyase on Inorganic Carriers. // Biotechnology, 2011, v. 2, p. 58–65 (in Russian).
11. **Babich O.O., Soldatova L.S.** Study and Properties of Free and Immobilised L-Phenylalanine Ammonia-Lyase of *Rhodosporidium Toruloides* on Silochrom C-80. // Proceedings of Samara Scientific Center RAS. Questions of General Biology, 2012, v. 14, № 1, p. 279–282.
12. **Paloyan A.M.** Covalent Immobilization of Enzymes of *Erwinia Carotovora* which Transaminate Phenylpyruvate and Their Characterisation. // Biolog. J. Armenia, 2012, v. 3, № 64, p. 34–39.
13. **Aganyants H.A.** Purification and Study of L-Aminoacylase Characteristics of *Escherichia Coli* Recombinant Strain. International Symposium: "Perspectives for Development of Molecular and Cellular Biology 3", September 26–29.2012, p. 19–23.
14. **Peterson G.L.** Determination of Total Protein. // Meth. Enzymol., 1983, v. 91, p. 95–119.
15. **Story S.V., Grunden A.M., Adams M.W.W.** Characterization of an Aminoacylase from the Hyperthermophilic Archaeon *Pyrococcus Furiosus*. // J. Bacteriol., 2001, v. 183, № 14, p. 4259–4268.