Dedicated to Prof. P.T. Frangopol on the occasion of his 75th anniversary

Enzymatic Synthesis of ^{15}N-L-aspartic Acid Using Recombinant Aspartase from Escherichia Coli K12

IULIA LUPAN[1], SERGIU CHIRA[1], MARIA CHIRIAC[2], NICOLAE PALIBRODA[2], OCTAVIAN POPESCU[1]*

[1] Babeº-Bolyai University, Faculty of Biology and Geology, Molecular Biology Center, Institute for Interdisciplinary Experimental Research, 44 Gh. Bilascu Str., 400015, Cluj-Napoca, Romania

[2]National Institute for Research and Development of Isotopic and Molecular Technologies, 65-103 Donath Str., 400293, Cluj-Napoca, Romania

Amino acids are obtained by bacterial fermentation, extraction from natural protein or enzymatic synthesis from specific substrates. With the introduction of recombinant DNA technology, it has become possible to apply more rational approaches to enzymatic synthesis of amino acids. Aspartase (L-aspartate ammonia-lyase) catalyzes the reversible deamination of L-aspartic acid to yield fumaric acid and ammonia. It is one of the most important industrial enzymes used to produce L-aspartic acid on a large scale. Here we described a novel method for [^{15}N] L-aspartic synthesis from fumarate and ammonia ($^{15}NH_4Cl$) using a recombinant aspartase.

Keywords: aspartase, L-aspartic acid, ^{15}N, enzymatic synthesis, mass spectrometry

Aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) catalyzes the reversible conversion of L-aspartate to fumarate and NH_4^+ and plays an important role in the bacterial nitrogen metabolism. Aspartase is found in various organisms and has been purified by conventional biochemical methods from *Escherichia coli* [1, 2]. The *E. coli* enzyme has a molecular weight of about 200,000 Da and is composed of four identical subunits. The enzyme is activated in the presence of Mg^{2+} at alkaline *p*H [3]. The L-aspartic acid (L-Asp) is an ingredient in the chemical synthesis of the artificial sweetener aspartame (*N*-L-α-aspartyl-L-phenylalanine-1-methyl ester) [4]. We described here a novel method for enzymatic synthesis of [^{15}N] L-aspartic acid using recombinant aspartase from *E. coli* K12.

Materials and methods

Molecular cloning of aspartase from Escherichia coli

Gene for aspartase from *E. coli* (1437 bp) was cloned by polymerase chain reaction (PCR) using pET21b expression vector (Novagen). The amplified DNA and pET21b vector were digested with *Bam*HI and *Xho*I restriction enzymes. The ligation reaction was performed at 16ºC for 12-16 h using T4 DNA ligase. The resulting recombinant molecules were introduced by electroporation into *E. coli* DH5α cells. The selection of transformed bacterial cells was made in the presence of ampicillin. The screening of recombinant molecules was made by restriction mapping and sequencing (ABI Prism 310 Genetic Analyzer, Applied Biosystems). The nucleotide sequence of our recombinant aspartase shows some differences when compared to other two aspartases from NCBI data base. However, these differences do not change the amino acid sequence of the recombinant protein.

Expression of recombinant aspartase

In order to express the aspartase gene, the purified recombinant DNA molecule was transferred in competent *E. coli* BL21(DE3) cells. The cells carrying recombinant molecules were cultured at 37ºC in Luria-Bertani medium supplemented with 100 mg/L ampicillin. The expression of protein was induced with 1 mM IPTG and the cultures were further incubated at 37°C for three hours, before being harvested by centrifugation [5]. The bacterial pellet was stored at -80°C.

Assay of aspartase activity

Bacterial cells were defrosted in 50 mM Tris-HCl (*p*H 7.4), sonicated at 4°C and centrifuged at 10.000 g for 30 min. The supernatant was tested for enzyme activity. Aspartase activity was assayed at 30°C measuring the production of fumarate at 240 nm using a Jasco V-530 UV/VIS spectrophotometer. The standard assay mixture (1 mL) contained: 0.1 M L-Asp, 6 mM $MgCl_2$, 50 mM Tris-HCl pH 8.5. The reaction was started by addition of enzyme. One unit of aspartase was defined as the amount of the enzyme that produced 1 μmol of fumarate in 1 min at 30°C. A molar coefficient of $2.53 \cdot 10^3$ M^{-1} cm^{-1} at 240 nm was used.

Biosynthesis of [^{15}N] L-aspartic acid

A typical synthesis of [^{15}N] L-Asp was performed as follows: 18 mmol fumaric acid, 18 mmol [^{15}N] ammonium chloride (~99 atom% ^{15}N) and 11,4 mmol $MgCl_2$ (6 mM final) were dissolved in 20 mL of bidistilled water with stirring at 25°C, and *p*H adjusted to 8.5 with 1N NaOH. Finally, the recombinant aspartase was added (205U). When the amino acid concentration became constant, the reaction was stopped by heating the reaction mixture at 85°C for 15 min. After cooling, the denaturated proteins were removed by filtration or centrifugation, and the labeled L-Asp was isolated from reaction mixture. The structure, purity and isotopic content of [^{15}N] L-Asp were determined by GC-MS analysis following a standard procedure [6].

Results and discussions

The enzymatic activity of our recombinant aspartase is comparable with data presented by other authors [7, 8]. The aspartase-producing *E. coli* strain obtained by us can be used also to enhance the co-production of other recombinant proteins [9]. The synthesis of L-Asp is a reversible reaction. In this case the substrate is not 100% consumed which leads up to low efficiencies. For this reason, it is very important to set up the optimal reaction conditions. The initial reactant concentrations, the reactant concentrations of reactants at equilibrium, and the values

* *email: opopescu@biolog.ubbcluj.ro*

Table 1

VALUES OF EQUILIBRIUM CONSTANT (K_{eq}) OF THE REACTION CATALYZED BY RECOMBINANT ASPARTASE from *E. coli*

Initial reactant concentrations (mM)			Reactant concentrations at equilibrium (mM)			K_{eq}
L-Asp	NH_4Cl	Fumarate	L-Asp	NH_4Cl	Fumarate	(mM)
1.0	10.0	0	0.749	10.251	0.251	3.43
2.0	10.0	0	1.523	10.477	0.477	3.28
2.0	20.0	0	1.648	20.352	0.352	4.35
4.0	40.0	0	3.566	40.434	0.434	3.70

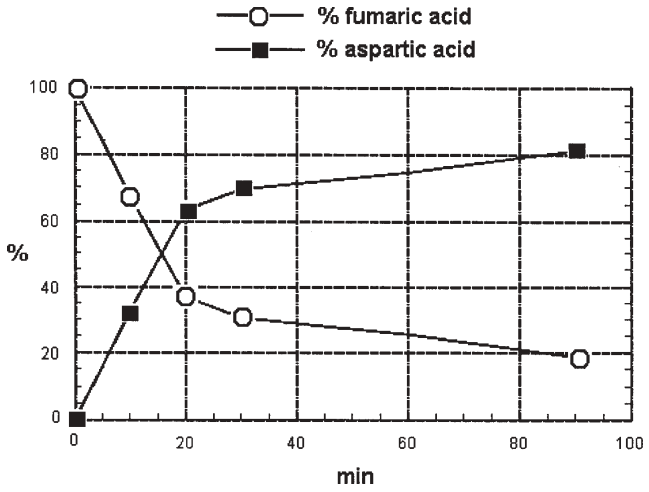

Fig. 1. The fumarate consumption in L-Asn synthesis

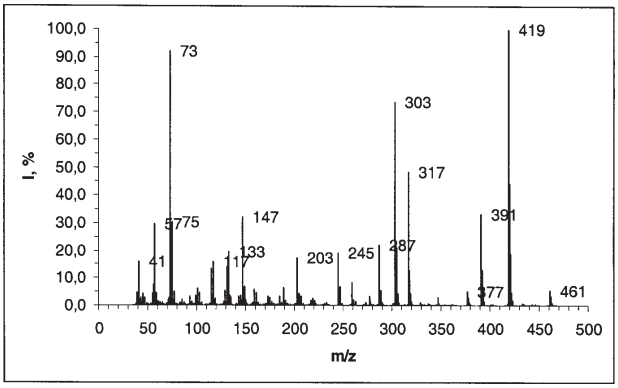

Fig. 2. The mass spectrum of derivatizated [^{15}N] L-Asp ($C_{22}H_{49}{}^{15}NO_4Si_3$; Mw = 476.3).

of equilibrium constant are presented in table 1. The mean value of K_{eq} is 3.69 mM.

In figure 1 are presented the consumption of fumaric acid and the formation of L-Asp function of time. It was obtained 10.45 mmol [^{15}N] L-Asp (yield: 60%). The mass spectrum of labeled L-Asp is presented in figure 2. As usual in amino acid derivatization, molecular ions are not present in the spectra, but the amino acids may be identified by three abundant fragment ions.

The ion fragments (m/z) characteristic for [^{15}N] L-Asp are: 303, 317, 391, 419 and 461. The average values of the isotope labeling found for three distinctive ions were in good agreement with the ^{15}N label of the ammonium chloride used in biosynthesis.

Conclusions

The enzymatic preparation of [^{15}N] L-Asp by the described procedure presents the following advantages:

- good yield of [^{15}N] L-Asp under simple reaction conditions;
- the [^{15}N] L-Asp is easy to isolate in a pure state;
- the atom % [^{15}N] in labeled L-Asp is equal to that of the [^{15}N] ammonium chloride used;
- this method can be easily adapted for the large-scale preparative purposes increasing only the reaction volume and keeping constant the reagent concentrations.

References

1. SUZUKI, S., YAMAGUCHI, J., TOKUSHIGE, M., Biochim. Biophys. Acta**,** **321**, 1973, p.369
2. TAKAGI, J.S., IDA, N., TOKUSHIGE, M., SAKAMOTO, H., SHIMURA, Y., Nucleic Acids Res., **13**, 1985, p.2063
3. SUZUKI, S., YAMAGUCHI, J., TOKUSHIGE, M., Biochim. Biophys. Acta**,** **321**, 1973, p.369
4. VAN DER WERF, M.J., VAN DEN TWEEL, W.J., KAMPHUIS, S., HARTMAN S., DE BONT, J.A., Trends Biotechnol., **12**, 1994, p.95
5. CLEMENT, J.-M., POPESCU, O., B. I. Pasteur, **89**, 1991, p.243
6. CHAVES DAS NEVES, H.J., VASCONCELOS, A.M., J. Chromatogr., **392**, 1987, p.249
7. PAPIERZ, M., GADOMSKA, G., SOBIERAJSKI, B., CHMIEL, A., Pol. J. Microbiol., **56**, 2007, p.71
8. GADOMSKA, G., PLUCIENNICZAK, A., CHMIEL, A., Pol. J. Microbiol., **56**, 2007, p.77
9. WANG, Z.W., CHEN, Y., CHAO, Y.P., J. Biotechnol., **124**, 2006, p.403

Manuscript received: 20.08.2008