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Creation of a Producent, Optimization of Expression, and Purification of Recombinant *Yersinia Pseudotuberculosis* L-Asparaginase

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Recombinant *E. coli* strain producing *Y. pseudotuberculosis* Q66CJ2 (YpA) L-asparaginase II was created. Gene *ansB* homologue encoding *Y. pseudotuberculosis IP 32953* L-asparaginase precursor was synthesized. The gene was cloned in pBad24 expression vector and expressed in *E. coli* BL21 (DE3) strain. Optimal conditions for the producer strain culturing were selected. An effective method for isolation and purification of the enzyme by two-staged column chromatography was developed.

Key Words: *L-asparaginase; antitumor enzyme; Yersinia pseudotuberculosis*

L-Asparaginases (L-asparagine amidohydrolase, EC 3.5.1.1) catalyze cleavage of L-asparagine with the formation of L-asparaginic acid and ammonium. Some of them (*E. coli* (EcA) and *Erwinia chrysantemi* (ErA) are used for the treatment of acute lymphoblastic leukemia. Some other bacterial asparaginases also exhibited antitumor effects, *e.g. Wollinella succinogenes*, *Thermus thermophilus*, *Proteus vulgaris*, *Pseudomonas fluorescens*, *Serratia marcescens*, *Erwinia aroidea*, *Erwinia carotovora*, *Aspergillus terreus*, *Helicobacter pylori*, and *Mycobacterium tuberculosis* [2,4,6,7]. On the other hand, the problems of side effects (allergic reactions, disorders in liver functions, *etc.*) and resistance developing during repeated chemotherapy courses remain unsolved. Hence, the search for and obtaining of L-asparaginases from new sources with more valuable therapeutic characteristics remains a pressing problem.

Creation of recombinant producer strains improves significantly the efficiency of L-asparaginase production. High cytostatic activity of *E. pseudotuberculosis* L. asparaginases (YpA) has been demonstrated for MOLT-4 and Jurkat T-lymphoblastic leukemia cells, MCF-7 mammary adenocarcinoma, rat Gasser's node neurinoma, and LnCap human prostatic carcinoma cell cultures [1].

We tried to obtain a stable recombinant *E. coli* strain producing YpA and to optimize its expression and purification.

Our tasks were as follows: to obtain an *ansB* gene homologue encoding YpA *IP 32953* precursor, to find the most effective expression system for obtaining

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YpA and optimize the expression, and to develop a laboratory method for isolation of YpA.

MATERIALS AND METHODS

Cloning and expression of YpA were carried out using pET23a (Novagen) and pBad24 (Invitrogen) vectors, NdeI, EcoRI, HindIII, and XbaI restrictases, T4 DNA ligase, Taq DNA polymerase, and Pfu DNA polymerase (Fermentas). Standard methods were used in operations with DNA.

The following recipient strains were used: *E. coli* BL21 (DE3) [F-, ompT, hsdSB, (rB-, mB-), dcm, gal, λ(DE3)], *E. coli* BL21 (DE3) plysS [F-, ompT, hsdSB, (rB-, mB-), dcm, gal, λ(DE3), plysS (CmR0)] (Bioengineering Center, the Russian Academy of Sciences); *E. coli* TOP10 [F', mcrA, Δ(mrr, hsdRMS, mcrBC), f80lacZΔM15, DlacX74, deoR, recA1, araD139, D(ara-leu)7697, galU, galK, rpsL(StrR), endA1, nupG], *E. coli* JM83 [F-, f80dlacZΔM15, D(lac-proAB), ara rpsL, recA+, rK+, mK+] (Institute of Genetics). The resultant producer strains were cultured in Erlenmeyer's flasks in 50 or 200 ml LB medium with ampicillin (100 μg/ml) on a GFL 3033 shaker (180 rpm, 37 or 28°C). Optical density of the culture was measured on an Aquarius 7000 spectrophotometer at λ=600 nm and expressed in optical units (OU_{600}).

Arabinose was added at optical density of the culture 0.5-2.3 OU_{600} to the final concentration of 0.02-0.50%, IPTH at the same optical density to the final concentration of 0.1 mM. In order to find the optimal duration of culturing, the samples were collected every 40 min throughout 17 h after addition of the inductor and asparaginase activity in them was measured by direct nesslerization. The quantity of the enzyme catalyzing the release of 1 μmol ammonium per 1 min at 37°C was taken for L-asparaginase activity unit (1 U). The productivity of the culture was expressed in U/OU_{600}.

After incubation the cells were precipitated by centrifugation (15 min, 2500*g*). The biomass was resuspended in buffer A (10 mM KH_2PO_4, 1 mM glycine, 1 mM EDTA, pH 7.5) and subjected to ultrasonic treatment (USDN-2T disintegrator) for 10 min (1 min sonication, 1 min interval). The cell extract was obtained by centrifugation of US-treated suspension (60 min, 15,000*g*) and layered onto a column with Q-Sepharose (1.5×16.0 cm) equilibrated with buffer A. Fractions containing YpA were diluted 10-fold with buffer A, united, and applied onto a column with DEAE-Toyopearl (1.0×12.5 cm) equilibrated with buffer A. Protein was eluted with linear gradient of KCl concentration (0.0-1.0 M). Elution velocity was 78 and 30 ml/h, respectively. At the final stage of isolation the enzyme solution was desalinated and concentrated in an Amicon cell with Millipore filter (NMWL 30.000). All stages of purification were carried out at 4°C.

Protein concentration was measured by modified Lowry's method [5]. Electrophoresis was carried out after Laemmli. Protein (5 μg) was put into a well. Protein reference samples with molecular weight of 14-97 kDa (Pharmacia) served as markers.

RESULTS

The DNA fragment of 1055 b. p. containing the gene homologous to *ansB* gene encoding L-asparaginase II precursor in *Y. pseudotuberculosis IP 32953* strain and carrying Ndel and EcoRI restriction sites, was assembled from DNA fragments obtained by PCR using synthetic overlapping oligodeoxynucleotide primers. The fragment was cloned by Ndel and EcoRI restriction sites in pET23a expression vector, which was transformed into *E. coli* BL21 (DE3) strain. The resultant clones (n=140) were tested for asparaginase activity by the dish test [3]. Plasmid was isolated from the active clone and the correctness of the cloned construction was confirmed by restriction analysis. The cloned DNA fragment was sequenced. It was shown that the sequence of synthetic *ansB* gene analog in it did not differ from *Y. pseudotuberculosis IP* natural *ansB* gene sequence.

The resultant producer had an essential brawback: unregulated unstable expression, its level reducing

TABLE 1. Changes in Activities of L-Asparaginase from Different Producers during Their Culturing

Producer	Activity, U/ml		Optical density, OU_{600}		Productivity, U/OU_{600}	
	reinoculation 1	reinoculation 12*	reinoculation 1	reinoculation 12	reinoculation 1	reinoculation 12
E. coli BL21 (DE3)/pBad/YERS	11.9	12.1	6.15	6.28	1.94	1.93
E. coli JM83/pBad/YERS	5.7	0.4	5.41	5.50	1.05	0.10
E. coli TOP10/pBad/YERS	2.7	0.2	2.51	2.30	1.07	0.12

Note. Parameters were evaluated 17 h after addition of inductor; *culturing was carried out after 12 reinoculations in dishes.

with reinoculations. Variations in the culturing conditions (medium composition, temperature, aeration conditions, IPTH inductor concentration) and transfer of the plasmid into strain BL21 (DE3) plysS under a more stringent control of protein expression was inessential for the level and stability of expression. The need in a producer strain with stable controlled expression necessitated the search for other expression systems better adapted for our purposes. With this aim in view, *ansB* gene was transferred into pBad24 expression vector under the control of promotor from araBAD arabinose operon. In order to realize this transfer, the fragment carrying the target gene was cleaved out by HindIII and Xbal restriction sites from pET 23a (*ansB*) plasmid, purified by electrophoresis in agarose gel, and after subsequent elution was cloned in pBad24 plasmid by the same sites. The resultant pBad24/YERS plasmid was used for transformation of three recipient strains BL21 (DE3), JM 83, and TOP 10.

The strains selected by the dish test [5] were cultured under standard conditions: the inductor (arabinose) was added at optical density of 0.80-1.98 OU_{600} to a final concentration of 0.2%. Asparaginase activity of strains JM83/pBad/YERS and TOP10/pBad/YERS without inductor was just trace, that of strain BL21 (DE3)/pBad/YERS did not exceed 2 U/ml. After addition of arabinose the level of *ansB* expression in BL21 (DE3)/pBad/YERS strain was significantly higher than in two other strains: almost 2-fold higher by productivity and 2-fold higher than that of JM83/pBad/YERS and 4.4 times higher than that of strain TOP10/pBad/YERS by activity (Table 1). Basal asparaginase activity of acceptor strains under conditions of induction was at the trace level. In addition, this strain was characterized by higher accumulation of biomass; the level

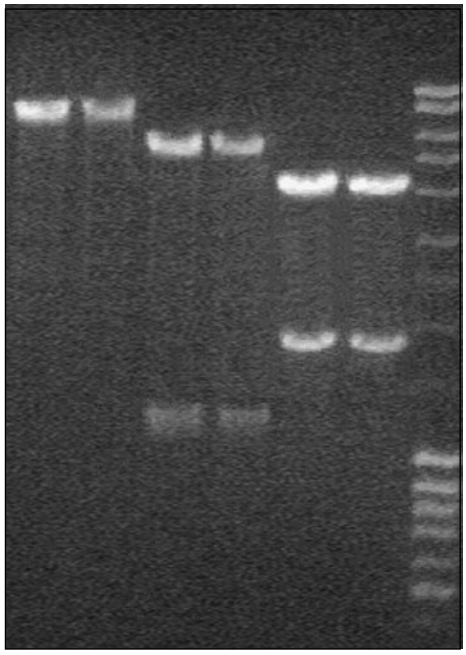

Fig. 1. Restriction analysis of plasmid DNA isolated from BL21(DE3)/pBad/YERS cultures after reinoculations 1 and 12. Electrophoresis of pBad24/YERS DNA (tracks *1*, *3*, *5*) and pBad24 DNA without insertion (tracks *2*, *4*, *6*) in 0.8% agarose gel. *1*, *3*, *5*: reinoculation 1; *2*, *4*, *6*: reinoculation 12. *1*, *2*) SalGI, fragment, 5620 b. p.; *3*, *4*) EcoRI, fragments, 1109 and 4511 b. p.; *5*, *6*) EcoRV fragments, 1664 and 3956 b. p.; *7*) MassRuler DNA Ladder Mix ready-to-use (Fermentas No. SM0403).

TABLE 2. Relationship between *E. coli* BL21 (DE3)/pBad/YERS Productivity and Inductor Concentration and Time of Its Addition into the Medium

Optical density during induction, OU_{600}	Inductor concentration, %	Activity*, U/ml	Optical density, OU_{600}	Productivity, U/OU_{600}
0.5	0.02	14	4.70	3.0
	0.10	16	5.10	3.2
1.45-1.6	0.02	15	4.85	3.0
	0.05	17	4.95	3.4
	0.10	17	4.94	3.4
	0.20	16	4.99	3.2
	0.50	16	5.36	2.9
2.3	0.02	15	5.10	2.9
	0.20	20	4.93	4.1
3.5	0.02	8	4.80	1.7
	0.20	6	5.10	1.2

Note. *Parameters were evaluated 17 h after addition of inductor.

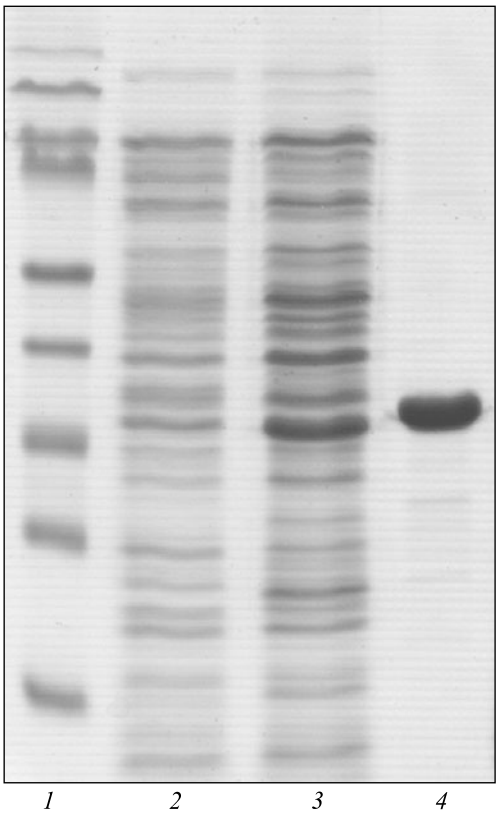

Fig. 2. Electrophoresis of purified YpA in 12% PAAG-SDS. *1*) molecular weight reference samples (Fermentas, No. SM0671: 170, 130, 95, 72, 55, 43, 34, 26, 17 kDa); *2*) cell extract (*E. coli* BL21(DE3) strain); *3*) cell extract after induction (BL21(DE3)/pBad/YERS strain); *4*) YpA from DEAE-Toyopearl column (active fraction).

of YpA production remained unchanged throughout 12 reinoculations. Enzyme activity of the two other strains was undetectable after 12 reinoculations, this indicating instability of the expression of the cloned gene. Hence, based on these data, strain BL21(DE3)/pBad/YERS was chosen for further studies and enzyme production. The stability of the resultant genetic construction in this strain throughout 12 reinoculations was confirmed by restriction analysis (Fig. 1).

The optimal gene expression was evaluated by the following parameters: arabinose inductor concentration; producer growth phase during which the inductor was to be added; culturing temperature; duration of culturing after induction. The main criterion was output of biomass with high YpA activity. The cells exhibited the highest productivity after addition of the inductor in the middle of the logarithmic growth phase (1.45-2.3 OU_{600}). Maximum activity (20 U/ml) was attained in cultures with induction at 2.3 OU_{600} 17 h after the inductor addition, the productivity was 4.1 U/OU_{600}. Differences in the inductor concentrations for the range of 0.05-0.2% were inessential for the productivity; decrease of its concentration to 0.02% and its increase to 0.5% led to reduction of productivity (Table 2).

Activity of L-asparaginase per volume unit was the same during culturing at 37 and 28°C, but cell productivity was significantly higher at 28°C (Table 3). This indicated that culturing at 28°C was preferable.

Hence, the optimal conditions of induction were selected in these experiments: inductor concentration of 0.1-0.2%, its addition during the middle of the log-phase at 1.45-2.3 OU_{600}, and culturing temperature of 28°C. The optimal duration of culturing after induction was 7-16 h. The producer strains were cultured under these conditions in subsequent experiments.

TABLE 3. Relationship between *E. coli* BL21(DE3)/pBad/YERS Productivity and Incubation Temperature

Incubation temperature, °C	Inductor concentration, %	Activity, U/ml	Optical density, OU_{600}	Productivity, U/OU_{600}
37	0.02	14	4.7	3.0
	0.20	16	5.1	3.2
28	0.02	14	3.4	4.2
	0.20	16	3.6	4.5

Note. Parameters were evaluated 17 h after addition of inductor.

TABLE 4. Results of YpA Purification

Purification stage	Volume, ml	Total protein, mg	Activity, U/ml	Specific activity, U/mg	Yield, %
Cell extract	54.0	2256.0	363.5	8.7	100
Q-Sepharose	28.5	336.3	564.0	47.8	85
DEAE-Toyopearl	26.0	257.4	619.4	62.7	82

A simple and effective method for enzyme isolation from producer strain cells and its purification was developed. The method included ultrasonic treatment of cell suspension, removal of the debris by centrifugation, and two stages of ion exchange chromatography on columns with a strong and then weak anion exchangers (Q-Sepharose and DEAE-Toyopearl, respectively).

Asparaginase activity in cell extract was 8.7 U/mg, which corresponded to 14% of total protein in the cell (Table 4). Subsequent centrifugation resulted in elimination of almost 50% ballast proteins, though this presumably led to loss of 10-20% enzyme activity because of L-asparaginase adsorption by destroyed cell precipitate. During chromatography, no loss of enzyme activity was observed, while specific activity of the enzyme increased to 62.0 U/mg, *i.e.* more than 7-fold compared to initial level. YpA was eluted from the columns in a single peak at KCl concentration of 0.3 M. Electrophoresis of purified YpA in 12% PAAG-SDS detected one component with a molecular weight of about 34 kDa (Fig. 2), which corresponded to theoretically calculated molecular weight of mature protein – 33.9 kDa. Detection of only mature L-asparaginase indicated correct processing of the synthetic recombinant precursor in the producer strain cells. Electrophoresis data were processed by Gel-Pro analyzer 3.1.00.00 (Media Cybernetics). Estimations showed 93% purification of the target enzyme in the product.

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