

PEGylated Recombinant L-Asparaginase from *Erwinia carotovora*: Production, Properties, and Potential Applications

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Abstract—N-hydroxysuccinimide ester of monomethoxy polyethylene glycol hemisuccinate was synthesized. It acylated amino groups in a molecule of recombinant L-asparaginase from *Erwinia carotovora*. A method of L-asparaginase modification by the obtained activated polyethylene glycol derivative was developed. The best results were produced by modification of the enzyme with a 25-fold excess of reagent relative to the enzyme tetramer. The modified L-asparaginase was isolated from the reaction mixture by gel filtration on Sepharose CL-6B. The purified bioconjugate did not contain PEG unbound to the protein, demonstrated high catalytic activity, and exhibited antiproliferative action on cell cultures.

Keywords: recombinant L-asparaginase from *Erwinia carotovora*, PEG-asparaginase, polyethylene glycol, antitumor therapeutics

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INTRODUCTION

L-Asparaginase or L-asparagine-amidohydrolase (EC 3.5.1.1.) catalyzes hydrolysis of L-asparagine with the formation of L-aspartic acid and ammonia. For almost 40 years, bacterial L-asparaginases (from *Escherichia coli* and *Erwinia chrysanthemi*) have been used as a main drug for the combined chemotherapy of lymphoproliferative disorders and lymphomas, in particular for acute childhood lymphoblastic leukemia (ALL) [1–3]. Possibilities for the use of L-asparaginase for treatment of solid tumors also have been reported [4, 5].

The L-asparaginase chemotherapy schemes currently used for ALL treatment ensure remission in 80% of cases. However, preparations of native L-asparaginases demonstrate relatively low stability under physiological conditions, as well as immunological incompatibility and allergenicity [6], which lead to the search for more efficient pharmaceutical preparations of this enzyme. Soluble preparations of stabilized forms of L-asparaginase produced by their chemical modification or by immobilization on water-soluble supports are examples of such forms [7, 8]. Site-directed mutagenesis also has been used to produce stabilized forms of L-asparaginase with improved therapeutic properties [9–11].

However, the majority of these derivatives of L-asparaginase are characterized by rapid clearance from blood plasma and are bioincompatible.

Modification of the surface amino groups in proteins by short polyethylene glycol chains (PEG, pegylation) is one of the most promising methods for the improvement of physicochemical and biomedical characteristics of various pharmaceutically valid proteins (increase of biostability, better tolerance, decrease of immunogenicity, and others). Chemical modification of a protein molecule causes changes in its structure, which increases its hydrophilicity and decreases the electrokinetic potential. These properties, in turn, slow protein capture by the cells of mononuclear phagocytic system (which, as a rule, causes slower clearance), and the bioavailability of the enzyme preparation increases.

PEGylation was first conducted in 1977 to modify albumin and catalase [12, 13]. The advantages and some drawbacks of PEG-derivatives of therapeutic proteins were reviewed in [14]. The following therapeutically significant proteins were produced: some forms of L-asparaginase [15], adenosine deaminase [16], interferon $\alpha 2a$ [17], colony stimulating factor (PEG-filgrastim) [18], and others.

In our previous work [19] a laboratory procedure for conjugation of the enzyme with PEG was developed with the objective to improve therapeutic properties of the recombinant L-asparaginase from *Erwinia carotovora*. Methoxypolyethylene glycol *p*-nitrophenyl carbonate (mPEG-NPh) with a molecular mass of 5000 Da was used as a modification agent.

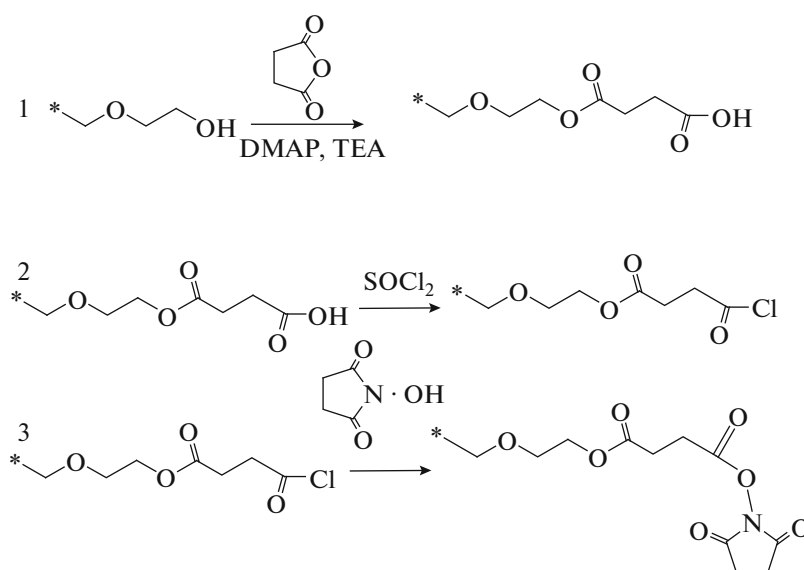


Fig. 1. Scheme for synthesis of activated PEG derivative.

However, the use of mPEG-NPh for preparation of PEG-asparaginase (PEG-ASP) was limited due to instability of the bond linking carboxyl group and polyethylene oxide. It must be mentioned that this reagent is quite expensive, because none of the activated PEG derivatives are currently manufactured in Russia.

Another approach to the preparation of PEG-ASP was selected in this work, which included synthesis of N-hydroxysuccinimide ester of monomethoxy polyethylene glycol hemisuccinate (mPEG-suc-NHS) and its following attachment to amino groups of L-asparaginases. This compound was shown to have more stable ureidyl ester bond between the succinic aldehyde and PEG. Synthesis mPEG-suc-NHS has been conducted at the Department of Chemistry, Lomonosov Moscow State University, and it can be produced on an industrial scale by chemical companies in Russia.

The objective of this study is to modify the recombinant L-asparaginase from *E. carotovora* by the mPEG-suc-NHS derivative, purify the obtained protein from admixtures, and investigate its catalytic activity, stability, and structure, as well its effect on proliferation of cancer cells in culture.

METHODS

The preparation of activated PEG derivative was performed in three stages. The scheme for PEG modification is presented in Fig. 1. In the first stage, monomethoxy-PEG was acylated by succinic anhydride in anhydrous chloroform in the presence of 4-dimethylaminopyridine and anhydrous triethylamine. The reaction mixture was transferred into an aqueous solution of HCl, and the reaction product PEG-hemisuccinate (mPEG-suc) was extracted by methylene chloride. In the second stage, mPEG-suc

was modified by thionyl chloride, which resulted in the formation of monomethoxy-PEG-hemisuccinate chloroanhydride (mPEG-suc-Cl) used subsequently in the third stage of the synthesis of N-hydroxysuccinimide ester under mild conditions. The reaction was carried out for 2–3 days, followed by chloroform evaporation at 30°C and dissolution of the residue in anhydrous benzene; the product was precipitated from hexane. The final product (mPEG-suc-NHS) was air-dried and kept in a desiccator over alkali without moisture access.

PEG determination. The PEG content in aqueous solutions was determined as described previously [20, 21].

Production of recombinant L-asparaginase from *E. carotovora*. Recombinant L-asparaginase from *E. carotovora* used in this work was isolated following expression in the previously constructed strain *E. coli* BL21(DE3)/pACYC_LANS(KM) [22–24].

Modification of L-asparaginase by mPEG-suc-NHS derivative. An aliquot of enzyme (30–40 mg/mL) was dissolved in a borate buffer (0.1 M, pH 8.6), and a dry powder of mPEG-suc-NHS was introduced into the solution. The reaction mixture was incubated for 4 h at 4°C. The modified enzyme was separated from admixtures by chromatography on a column (0.8 × 30 cm) with sepharose CL-4B.

Determination of PEG-ASP molecular mass. The molecular mass of the modified enzyme was determined by gel-filtration on an TSK 4000SW column (0.1 M Na₂HPO₄, pH 7.0, sample volume of 20 µL, elution rate of 1 mL/min, detection at 280 nm). The calibration curve as constructed by protein standards was described by cubic polynomial

$$\log M = 6.29 - 0.041V + 0.00758V^2 - 0.00041V^3,$$

where V is the sample elution volume.

Determination of enzymatic activity of recombinant L-asparaginase. L-asparaginase was assessed by direct nesslerization [25]. The amount of enzyme catalyzing the release of 1 μ mol of ammonia in 1 min at 37°C was taken as one activity unit. The specific activity was expressed as units of L-asparaginase activity per 1 mg of protein.

SDS-PAGE and determination of the protein concentration. The protein concentration was assessed by the Lowry method [26]. Protein electrophoresis was carried out in a 12.5% PAAG in the presence of SDS according to the Laemmli method [27]. Protein markers (Protein Molecular Weight Marker, Fermentas, Lithuania) were used to determine the protein molecular mass.

Determination of kinetic parameter of L-asparaginase. K_m values were determined from the rate of ammonia formation during L-asparagine hydrolysis. Enzyme activity was determined at different pH levels. The following buffers were used for this purpose: 0.1 M citrate buffer (pH 3–7), 0.1 M sodium phosphate buffer (pH 6–8), 0.05 M tris-HCl buffer (pH 7–9), and 0.0125 M borate buffer (pH 9–11). The reaction mixture (1 mL) contained L-asparaginase (0.030–0.070 units) and L-asparagine (20–250 μ M). The plotting, data processing, and calculations of K_m and V_{max} was conducted with Microsoft Excel program with the double reciprocal plot according to the Lineweaver–Burk method.

Determination of activity pH optimum. The effect of pH on the enzymatic activity of L-asparaginase was investigated at 37°C in standard buffer systems: 0.01 M sodium acetate (pH 3.0–6.0), 0.01 M sodium phosphate (pH 6.0–8.0), 0.01 M tris-HCl (pH 7.0–9.0), and 0.0125 M sodium borate (pH 9.0–11.0) buffers [28].

Temperature optimum of L-asparaginase activity. The effect of the temperature of enzymatic activity was determined in the abovementioned standard buffers in the temperature range from 30 to 80°C.

Determination of endotoxin presence. Endotoxin contaminants were assayed with the LAL-test [29].

Determination of the number of free amino groups in proteins was carried out as described in [30].

MALDI-TOF-MS-analysis on the surface of AFM chip. Mass spectroscopy was performed on Bruker Microflex and Autoflex III (Bruker Daltonics, Germany) time-of-flight mass-spectrometers in the electrostatic reflection mode with a power of 17 kV and pulse delay time of 200 ns. The mass spectra components were identified with the flexAnalysis version 2.0 (Bruker Daltonics, Germany) and MASCOT Daemon (Matrix Science, United Kingdom) software with the NCBI or MSDB databases. The search parameters included no more than two missed hydrolysis sites, and the measurement accuracy for monoisotopic masses was less than 100 ppm.

Determination of PEG-ASP isoelectric point. Isoelectric focusing (IEF) in an immobilized pH gra-

dient (IPG) was conducted on an Offgel Fractionator 3100 (Agilent, United States) instrument at 250 V for 1 h, and then at 3500 V, until the minimal current in each gel (on average 35 kVh) was reached. Gels were stained with Coomassie R-250 and scanned.

Investigation of antiproliferative activity in vitro. The following human leukemic cells lines were used in this work: K-562 (chronic myeloid leukemia, Russian Scientific Oncology Center, Russia), Jurkat (acute lymphoblastic leukemia, Orekhovich Scientific Research Institute of Biomedical Chemistry (IBMKh), Russia), HL-60 (promyelocytic leukemia, IBMKh, Russia), and MCF-7 (breast adenocarcinoma, American Type Culture Collection, United States). L-asparaginase EcA *E. coli* Medac, Germany) served as a control in the determination of antitumor activity of the native EwA and PEG-ASP. PEG-ASP was produced with a PEG/protein molar ratio of 1 : 25 and purified on a Toyopearl HW 55 column (1.8 $\times$ 70 cm). The specific activity of the PEG-ASP was 314 IU/mg of protein; the protein concentration was 1.38 mg/mL. The L-asparaginases activity was assayed prior to each experiment. Cells were cultivated in a humid environment (5% CO₂) at 37°C as described in [5, 6]. A DMEM medium was used to cultivate MCF-7 cells, and RPMI 1640 was used for K-562, Jurkat, and HL-60 cell lines. All media contained 10% of fetal calf serum (FCS), as well as 2 mM of glutamine, 10 units/mL of penicillin, and 10 μ g/mL of streptomycin (Paneko, Russia). The number of viable metabolically active cells was determined by the MTT-test as described in [31, 32].

Reagents. L-Asparagine, L-glutamine, D-asparagine, acryl amide, N,N,N',N'-tetramethylethylenediamine, K₂HPO₄, Na₂HPO₄, KCL, DSD, Folin's reagent, Coomassie R-250 (Serva, Germany), succinic anhydride, chloroform, N,N'-dicyclohexylcarbodiimide, 4- dimethylaminopyridine, triethylamine, methylene chloride, thionyl chloride, N-hydroxysuccinimide, benzene, hexane, Tris, Sepharose CL-6B, DEAE-Sepharose FF, TSK 4000 SW, Toyopearl HW 55 (Sigma-Aldrich, United States), diasfere CT-150 (Biokhimmak CT, Russia), Nessler reagent, TCA, PEG 5000, methoxypolyethylene glycol (Fluka, Switzerland), NaOH, Na₂B₄O₇ · 10H₂O (Merck, Germany), HCl, CH₃COOH, CH₃COONa (Reakhim, Russia), media DMEM, RPMI 1640, FCS, (Gibco, United States) were used.

RESULTS AND DISCUSSION

Modification of L-asparaginase by mPEG-suc-NHS.

The reaction of protein modification was initiated by addition to the protein of the dry mPEG-suc-NHS powder at pH 8.8, which allowed minimization of the side reaction of N-hydroxysuccinimide ester hydrolysis. The optimal ratio of the rates of enzyme modification and N-hydroxysuccinimide ester hydrolysis was achieved in the process. The activity of the enzyme did not decrease as a result of modification, and the con-

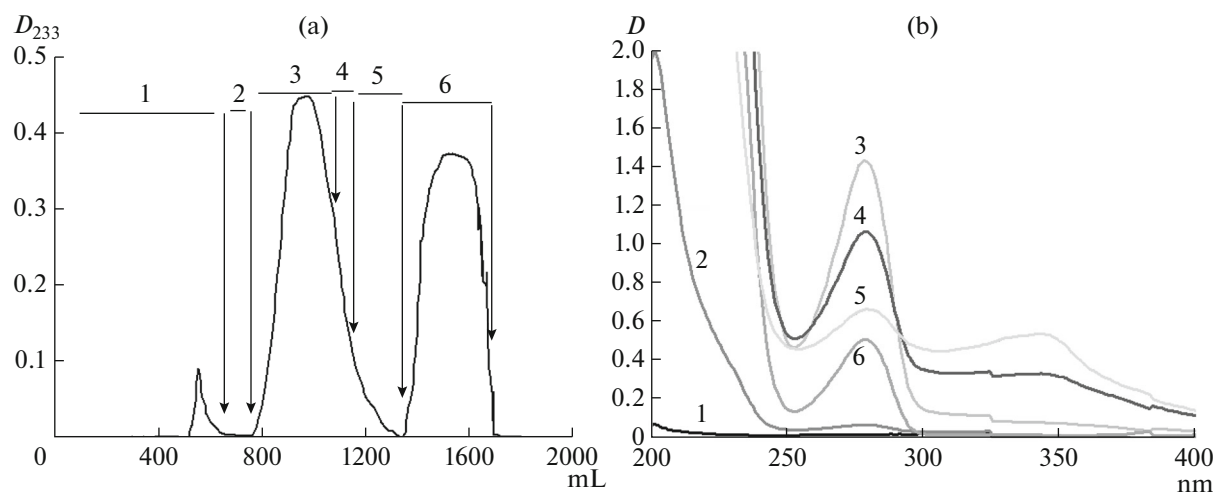


Fig. 2. Chromatogram of PEG-ASP on Sepharose CL-6B (a) and UV spectra of the six obtained components (b); 1–6 see explanations in text.

tent of unmodified monomeric subunits and residual amino groups was approximately 10 and 30%, respectively. Investigation of the degree of modification of L-asparaginase and the change of its activity under different reaction conditions showed that a 25-fold excess of mPEG-suc-NHS over the enzyme tetramer was optimal and that the optimal pH was 8.6.

PEG-ASP purification. Dialysis through membranes with a molecular weight cut-off of 50 kDa and two-step chromatography on DEAE-Sepharose FF and Diasfere CT-150 proved to be ineffective in the removal of the excess of mPEG-suc-NHS from the reaction mixture due to the low (less than 50%) yield of the target product. The enzyme purified from free PEG was produced by gel filtration on Sepharose CL-6B using 20 mM KH_2PO_4 , pH 7.2 as an eluent. The yield of the target product was about 70% in the process, and it contained no more than 10% of nonmodified subunits. This method was used for large-scale production of PEG-ASP.

The chromatogram of PEG-ASP on the sepharose CL-6B is presented in Fig. 2a, and the UV spectra of components eluted from the gel by 20 mM KH_2PO_4 , pH 7.2, with an elution rate of 50 mL/h (sample volume of 17 mL, gel volume of 1.8 L) are presented in Fig. 2b. The obtained components were identified. As can be seen in Fig. 2b, component 3 has an absorption maximum at 278 nm, and component 4 displays a wide shoulder in the range 300–370 nm, which is characteristic for mPEG-suc-NHS. Two broad absorption bands at 280 and 330 nm were observed in the UV spectrum of component 5, which indicated not only high protein content but mPEG-suc as well. Hence, component 3 (Fig. 2, fractions 3 and 4) eluted in the volume from 780 to 1100 mL was a modified enzyme (PEG-ASP). Components 4 and 5 eluted in the volume 1050–1250 mL contain, in addition to the PEG-ASP exhibiting absorption at 280 nm, unbound mPEG-suc, which was manifested by the increased

absorption at 340 nm characteristic for mPEG-suc. Component 6 eluted in volume 1250–1800 mL displayed a UV spectrum identical to that of N-hydroxy-succinimide.

These results were corroborated by the SDS-PAGE results. The yield of purified PEG-ASP was 75% (400 mg from 535 mg L-asparaginase), and the specific activity was 340 IU/mg.

Assessment of the pyrogenic contaminations in the produced PEG-ASP by the LAL-test showed that the preparation contained 1.4 units of endotoxins per mL, and the elution buffer was 0.4 units of endotoxins per mL.

Hence, the PEG-ASP was purified from admixtures of the free mPEG-suc-NHS by chromatography on Sepharose CL-6B. Moreover, the purified PEG-ASP demonstrated sufficiently high activity (92% of the initial one) and was practically free of endotoxin contamination.

Degree of PEG-ASP purification. The degree of purification was determined by SDS-PAGE, UV spectroscopy, and HPLC. As can be seen in Fig. 3b, the PEG-ASP preparation contained a small amount of native enzyme subunits. Analysis of the electropherogram with SigmaGel 1.0 software (Fig. 3) revealed that they comprised 10%; proteins with molecular masses of approximately 52 (~42%), 60.8 (~17%), and 70 kDa (~15%), as well as proteins with higher molecular masses (16%) were present.

The average amount of PEG attached to the tetramer of the modified enzyme was calculated based on these data according to equation

$$N = \frac{\sum_{i=0}^4 4N_i P_i^4}{\sum_{i=0}^4 P_i^4},$$

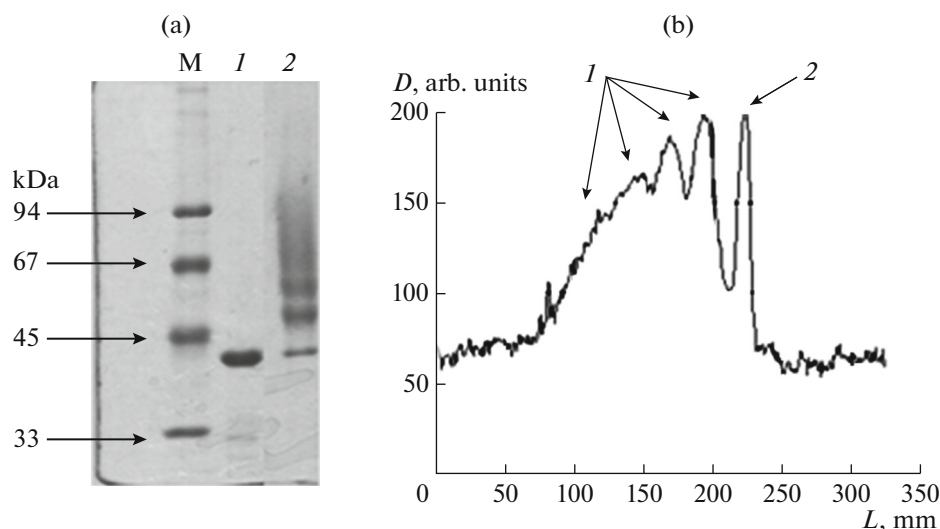


Fig. 3. (a) Electrophoresis in 12.5% PAAG in the presence of SDS: 1—native enzyme (2 μ g), 2—PEG-ASP (5 μ g) following modification by a 30-fold excess of mPEG-suc-NHS; (b) densitogram of the obtained gel containing PEG-ASP (1) and native L-asparaginase (2).

where N_i is the number of PEG chains in i th fraction of the enzyme and P_i is the content of this fraction in the protein preparation. It was found that, on average, 4.5 PEG chains were attached to the PEG-ASP tetramer, and the native tetramers were practically absent in the preparation of the modified enzyme. It can be suggested that the observed presence of native subunits as contaminants of the PEG-ASP is related to the shielding of amino group on the neighboring subunits by the PEG attached in the vicinity of the contact site of subunits, thus preventing new mPEG-suc-NHS molecules from attaching.

The UV spectra of the purified PEG-ASP exhibited an absorption maximum at 280 nm characteristic for proteins, while the shoulder in the region of 300–370 nm characteristic for free PEG was almost absent.

The PEG-ASP was eluted from the TSK 4000 SW HPLC column (Beckman, Germany) at a volume of around 17 mL, which was 3 mL less than for the native L-asparaginase (Fig. 4a). This corresponded to molecular mass of the modified enzyme of 580 kDa (Fig. 4b).

Mass spectrometric analysis of PEG-ASP. The modified product was reliably identified as L-asparaginase from *E. carotovora* (mowse score > 200) by mass spectrometric analysis of tryptic hydrolysates of PEG-ASP with the MASCOT system. In order to confirm the formation of complexes with PEG, the mass spectra of the peptides with molecular mass up to 7.2 kDa were obtained (Fig. 5). It was found in the region of 6.3 kDa that the peptide group was present, demonstrating low-intensity signals differing by 44 Da. This group most likely corresponded to the K.GIVYAGM-GAGSVSK.R peptide (m/z 1296.6) localized between the amino acid residues 244–257, which had an average molecular mass of 5 kDa and was conjugated to PEG-5000. The low intensity of the recorded signals can be explained by the fact that a group of peptides

with close molecular masses differing by 44 Da was formed during conjugation of ASP with PEG from a large number of peptide molecules with this molecular mass; the number of these molecules decreased in the process. Hence, it can be concluded that mPEG-suc-NHS was attached to the enzyme predominantly via the Lys257 residue localized in the sixth subterminal alpha helix, not in the active site structure.

Determination of isoelectric point of PEG-ASP. It is known that modification of the amino groups in proteins results in an acidic shift of their isoelectric points. As can be seen in Fig. 6, the single subunit of native enzyme (track 1) demonstrated $pI = 8.85 \pm 0.1$. A small amount of protein component was observed at this pH in the PEG-ASP with 50% of amino groups modified; however, the majority of the protein was characterized with lower pI values (track 2). No protein component was observed at pH 8.8 for the PEG-ASP with 90% of amino groups modified (tracks 3 and 4), while a significant amount of protein was found with a pI in the range pH 5–6. Such changes of acid–base properties is likely indicative of modification of the amino groups in the enzyme molecule.

Comparison of enzymatic activity of PEG-ASP with its non-PEGylated form. Modification of L-asparaginase by a 25-fold molar excess of mPEG-suc-NHS did not result in significant changes in the enzyme activity. An increase of K_m values for L- and D-asparagine, and L-glutamine by 23, 16, and 24%, respectively, in the reaction catalyzed by the PEG-ASP in comparison with the ones for the native enzyme was observed. The V_{max} value of the reaction catalyzed by PEG-ASP was slightly lower than for the native enzyme (by 15% for asparagine). It can be suggested that the attachment of several bulky PEG 5000 polymeric chains to ASP limits the conformational mobil-

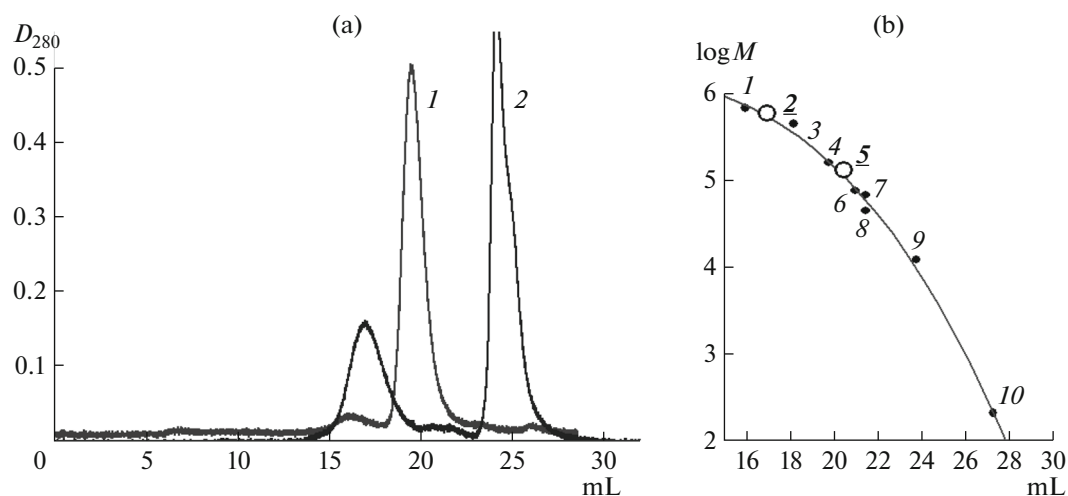


Fig. 4. HPLC on TSK 4000 SW of PEG-ASP (a) and of standard marker proteins (b): thyroglobulin (1), ferritin (3), aldolase (4), asparaginase (5), conalbumin (6), BSA (7), ovalbumin (8), cytochrom C (9), and tryptophan (10).

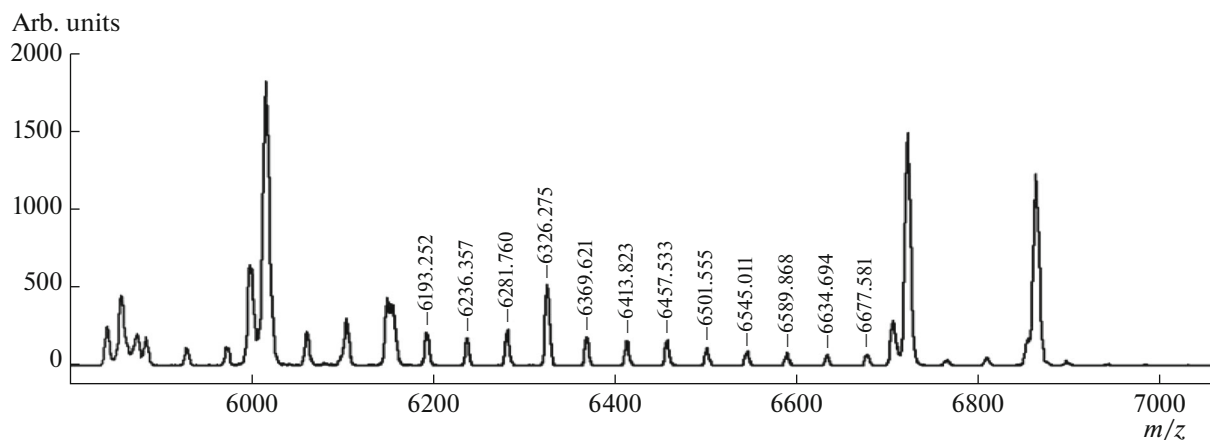


Fig 5. Mass spectra of peptides from tryptic hydrolysate of PEG-ASP conjugated with PEG.

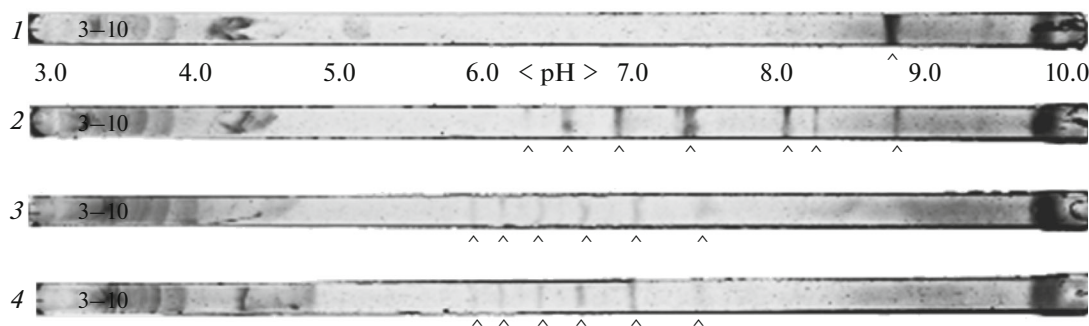


Fig. 6. IEF in immobilized pH gradient of native L-asparaginase (1) and PEG-ASP (2–4).

ity of the enzyme and the accessibility of its active center to substrates due to steric hindrances.

Effect of pH and temperature on the activity of native and pegylated ASP. The temperature optimum of native and PEG-modified forms of L-asparaginase was 50–55°C with a PEG-ASP specific activity higher

by 30–40% than the activity of the native enzyme. This is likely related to the improved thermal stability of the PEG-ASP due to the decreased activity of water molecules in the vicinity of the protein macromolecule surrounded by the chains of hydrophilic polymer [32]. At the same time, the pH dependencies of the

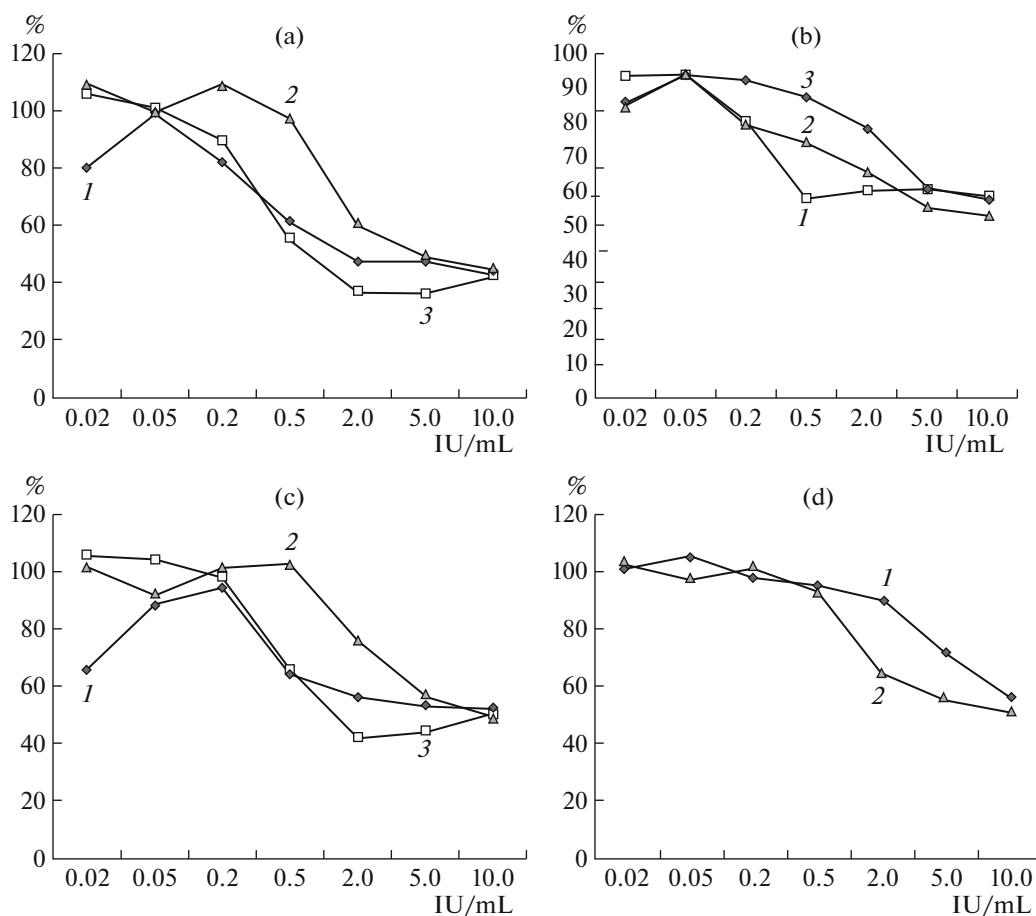


Fig. 7. Antitumor cytotoxic activity (% of viable cells) of native EwA (1), PEG-ASP (2) and L-asparaginase EcA from *E. coli* (3) towards Jurkat (a), HL-60 cells (b), K-562 (c), and MCF-7 (d) cells.

PEG-ASP and native enzyme activity were almost identical, which indicated that the charged groups close to the enzyme active center were not affected during modification by PEG.

Antiproliferative activity. We have established in our previous work [5] that the native recombinant L-asparaginase from *E. carotovora* exhibits an antiproliferative effect on animal and human tumor cell lines sensitive to this enzyme, and promising results have been obtained in preclinical studies of this enzyme [33]. As can be seen in Fig. 7a and 7c, the number of viable K-562 and Jurkat cells decreased significantly at the PEG-ASP concentration of 2.0 IU/mL, which was higher than for the native EwA and EcA (0.5 IU/mL). The PEG-ASP at concentrations of 2.5 and 7.5 IU/mg suppressed the growth of MCF-7 solid tumor more effectively than the EcA (Fig. 7d). The antitumor activity of the PEG-ASP towards HL-60 cells was also higher than that of the EwA and was comparable with the EcA (Fig. 7b). In particular, the decrease of viable HL-60 cells by 50% in comparison with the control was observed at the PEG-ASP and EcA concentration of 5.0 IU/mL. Hence, the PEG-ASP from *E. carotovora* demonstrated clearly pronounced antiproliferative activity towards the cell cultures. It can be suggested that the slowdown in

enzyme capture by the reticuloendothelial system caused by the introduction of PEG may increase the efficiency of L-asparaginase action in in vivo experiments.

Hence, a method was developed for modification of L-asparaginase by the modified derivative mPEG-suc-NHS and for the isolation of pure PEG-ASP via chromatography on sepharose CL-6B. The PEG-ASP was free of PEG unbound to the protein and from endotoxins. The activity of L-asparaginase modified by a 25-fold excess of mPEG-suc-NHS decreased by less than 20%, and its specific activity was approximately 300 IU/mg of protein. Modification of L-asparaginase with PEG resulted in an acidic shift of the PEG-ASP pI and an increase of the enzyme thermal stability. The PEG-ASP demonstrated more pronounced antitumor action towards the HL-60 cells in comparison with the native enzyme, which was comparable with the effect of L-asparaginase from *E. coli*. It was suggested that the accessibility of active center to substrates was limited as a result of attachment of PEG polymeric chains to the enzyme (K_m values for L-asparagine, L-glutamine, and D-asparagine

increased), as well as its conformational mobility ($V_{\max}$ values decreased).

The described method for modification of L-asparaginase with the activated derivative mPEG-suc-NHS offers promise for the development of domestic therapeutic preparation PEG-ASP. This is a hot topic currently, because the non-PEGylated ASP is not produced in Russia, and the imported preparation Oncaspar (Medac, Germany) containing PEG-ASP from *E. coli* is used in clinical practice.

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