Improved Method of Purification and Properties of Glutaminase-Asparaginase from *Pseudomonas aurantiaca* 548

Z. I. Lebedeva and T. T. Berezov

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An improved method of purifying the antitumor enzyme glutaminase-asparaginase from *P. aurantiaca* 548 is developed. The method includes extraction, treatment with protamine sulfate, heating in the presence of sodium glutamate as the stabilizer, ion-exchange chromatography on DEAE-sephacel, and gel-filtration AcA-44 ultragel. This method allows one to boost the yield of *P. aurantiaca* 548 glutaminase-asparaginase with lysine N-terminal residue.

Key Words: *glutaminase-asparaginase; amidohydrolase; purification; properties*

Glutaminase-asparaginase (GA, EC 3.5.1.38) belongs to the amidohydrolase family. Unlike L-asparaginase (EC 3.5.1.1), GA with the same rates catalyzes hydrolysis of L-glutamine and L-asparagine yielding the corresponding dicarboxylic acid and ammonia. At present, about 10 GA have been isolated as homogenous preparations from different microorganisms; these data have been reviewed [2,8]. Great progress has been recently made in the study of spatial structure of GA [10]. This enzyme attracts great attention, because of its possible use in enzymotherapy of human tumors [11].

We have previously undertaken a search for a domestic GA producent [1] and developed a method for isolation of GA from nonpathogenic *P. aurantiaca* 548, which allows one to obtain homogenous enzyme with a 10% yield [4]. Comprehensive studies of the molecular and catalytic properties of GA have proved that this enzyme meets the majority of requirements for primary screening of antitumor antigens [3-5,9]. However, further investigation of the antitumor properties of GA is limited due to its restricted availability.

Institute of Biomedical Chemistry, Russian Academy of Medical Sciences, Moscow

In the present study we modified the method of purification of the enzyme in order to boost its yield and identified N-terminal amino acid residue of GA.

MATERIALS AND METHODS

Biomass of *P. aurantiaca* (Russian Bank of Microorganisms, strain 548) was grown in 100-liter fermenters by the method developed by us. The microbial cells were separated, treated with acetone, and used as the primary source of the enzyme [3]. All stages of purification except for heating were performed at 0-4°C, centrifugation was carried out at 18,000 rpm and 4°C for 30 min. In the course of GA isolation its activity was assessed by hydrolysis of L-glutamine and L-asparagine.

Ammonium formed in the reaction was determined using Nessler reagent as described previously [4]. The amount of GA hydrolyzing 1 μmol substrate for 1 min at 30°C was taken as a unit of enzyme activity. Specific activity of GA was expressed in units (U) per mg protein; GA-specific staining in gel by its enzyme activity was performed as described elsewhere [6].

The N-terminal residue of GA was identified by dansylation according to the Erdmann's method

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TABLE 1. Purification of Glutaminase-Asparaginase from *P. aurantiaca* 548

Stage	Protein, mg	Total activity, U		Specific activity, U/mg protein		Activity yield, %	
		L-glutamine	L-asparagine	L-glutamine	L-asparagine	L-glutamine	L-asparagine
1. Extraction	3800	13680	12920	3.6	3.4	100	100
2. Protamine sulfate extraction	2620	12838	12052	4.9	4.6	93.8	93.3
3. Heating in the presence of 0.05 M sodium L-glutamate	1750	12275	8750	7.3	5.0	93.4	67.7
4. DEAE-sephacel chromatography	269	7102	5272	26.4	19.6	51.9	40.8
5. Gel filtration through AcA-44 ultragel	54	5940	4320	110.0	80.0	43.4	33.4

adapted for proteins [7]. The procedure of cleavage with phenyl isothiocyanate (PITC) included solubilization of GA in water followed by consecutive addition of triethylamine, pyridine, acetonitrile, and 5% PITC in acetonitrile, extraction with chlorobutane, and cleavage of phenylthiohydantoin derivatives (PTH) by adding trifluoroacetic acid to the resultant pellet (48°C, 20 min). Formed PTH were identified by reverse-phase high-performance liquid chromatography.

An aliquot of 1-5 nmol of native protein preparation were dansylated (1 h, 37°C). Dansylated amino acids were identified by two-dimensional chromatography in polyamide films, using H_2O:CH_3COOH (20:3) system for the first direction and benzene: CH_3COOH (9:1) system for the second direction. An ethyl acetate:methanol:CH_3COOH (20:1:1) system was used in the second direction to improve resolution.

RESULTS

The improved procedure of purification of GA and the data of a representative experiment are presented in Table 1.

Stage 1. *Preparation of crude extract.* Acetonic powder (25 g) was dissolved in 400 ml 0.05 M potassium-phosphate buffer, pH 7.0; the suspension was shacked for 2 h at 4°C and centrifuged.

Stage 2. *Protamine sulfate treatment.* Protein content in the supernatant was measured by the method of Lowry, 1.5% protamine sulfate solution in the same buffer was added dropwise to the supernatant (1 ml per 100 mg protein, here 38 ml) and after a 20-min shacking the sediment was removed by centrifugation.

Stage 3. *Heating in the presence of a stabilizer.* Sodium L-glutamate was added to the supernatant (300 ml) to a final concentration of 0.05 M (here 2.6 g). The mixture was placed in a thermostat at 55°C, heated to 50°C and incubated at this temperature for 20 min. The resultant suspension was then cooled in a water bath to 4°C and 30 min later the sediment was removed by centrifugation. $(NH_4)_2SO_4$ was added to the supernatant (308 ml) to 90% saturation. The mixture was centrifuged and the GA-containing pellet underwent further purification.

Stage 4. *Ion-exchange chromatography on DEAE-sephacel.* The pellet was suspended in 5 ml 0.05 M potassium-phosphate buffer (pH 7.0) and dialyzed against this buffer (1 liter) to complete dissolving. The solution (13 ml) was desalted on a Sephadex G-50 column (3.5×50 cm, 5 ml/min). Protein-containing fractions were pooled (150 ml) and applied onto a DEAE-sephacel column (5×60 cm) equilibrated with 0.01 M potassium-phosphate buffer, pH 7.0. The preparation was eluted with the same buffer at a rate of 30 ml/h and collected as 8-ml fractions. Under these conditions GA did not bind with the sorbent and was eluted immediately after the front of the solvent. Elution was stopped after disappearance of GA activity in the eluate, GA-containing fractions were pooled and $(NH_4)_2SO_4$ was added to 90% saturation.

The fact that unlike other proteins GA practically does not bind to the ion exchanger at pH 7.0 allows one to perform DEAE-sephacel chromatography in a free volume (without column). We used this opportunity in some cases in order to accelerate the isolation procedure when some GA losses can be neglected.

Stage 5. *Gel filtration through AcA-44 ultragel.* The pellet was dialyzed against 0.01 M phosphate buffer (pH 7.0) to complete dissolving and applied onto the column (2.5×100 cm) equilibrated with the same buffer. The preparation was eluted at a rate of 16 ml/h and collected as 4-ml fractions. The enzyme was eluted from the column as a symmetrical peak; the GA-containing fractions were pooled (27 ml) and lyophilized for long-term storage.

Polyacrylamide gel disk electrophoresis (pH 7.5 or pH 7.0 in the presence of SDS) showed that the obtained GA is a homogenous preparation. Enzyme assay (hydrolysis of L-glutamine) in gel demonstrated GA activity coincides with the protein band.

The proposed method provides a 30% increase in the yield of pure GA.

The N-terminal residue was identified by the Erdmann's method and by dansyl derivatization. The first method yielded a chromatographic peak identified as PTH-lysine. The dansyl method revealed the presence of (N-dansyl)lysine in dansyl-protein hydrolyzate. Thus, lysine is identified by two independent methods as the only N-terminal amino acid of GA from *P. aurantiaca* 548. This is an additional evidence of homogeneity of the isolated GA.

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