ISOLATION, PURIFICATION, AND SOME PHYSICOCHEMICAL PROPERTIES OF GLUTAMINE ASPARAGINASE FROM Pseudomonas boreopolis 526

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UDC 579.841.11.577.152.351

KEY WORDS: gel filtration; molecular weight; chromatography; subunit; ultrafiltration.

A new enzyme preparation glutamine asparaginase (ED 3.5.1.38), mainly isolated from microorganisms of the genus Pseudomonas [3, 8, 12], has recently been used in the treatment of cancer together with preparations of L-asparaginases. Clincial trials have shown that the preparation has a broader spectrum of antitumor action, for it has a marked antitumor effect on many tumors resistant to L-asparaginases and, in addition, unlike the latter, it also inhibits the growth of some solid tumors [9, 13]. This explains the increased interest of research workers in the enzyme glutamine asparaginase.

This paper gives data relating to the isolation, purification, and study of some physicochemical properties of glutamine asparaginase from Pseudomonas boreopolis 526, in which high deamidase activity was demonstrated in the writers' laboratory as long ago as in 1970 [2]. Much later, in combined studies with colleagues from the All-Union Oncologic Scientific Center, Academy of Medical Sciences of the USSR, this enzyme was found to have a marked cytotoxic action in certain lymphatic leukemias [4].

EXPERIMENTAL METHOD

The improved method of isolation and purification of the enzyme, developed by the writers, included the following stages: extraction, heat treatment, treatment with protamine sulfate, chromatography on DEAE-Sephacel, chromatography on CM-Sephadex, and gel-filtration on Sephacryl S-200.

An acetone powder of bacterial cells (30 g) was suspended in 800 ml of 0.05 M Tris-HCl buffer, pH 7.5, containing EDTA and 10^{-5} M di-isopropylfluorophosphate for 14 h at 4°C. The resulting protein extract was centrifuged for 30 min at 15,000 rpm. The cell residue was re-extracted in the original buffer (300 ml) for 5 h under the same conditions, and then centrifuged. The supernatant was collected, the residue discarded, and the extracts pooled.

The crude extract was subjected to heat treatment at 55°C for 40 min in the presence of 0.05 M sodium glutamate, after which the protein solution was quickly cooled to 4°C. Denatured proteins were removed by centrifugation. Protamine sulfate was added drop by drop to the protein solution to a final concentration of 0.2%, and kept at 4°C for 1 h. Nucleic acids which were precipitated were removed by centrifugation.

The protein solution, containing 2.45 mg protein/ml, was mixed with 400 ml of DEAE-Sephacel, previously equilibrated with 0.05 M Tris-HCl buffer, pH 7.5, for 40 min and filtered on a Büchner funnel. The resin was washed with buffer until no more active protein appeared in the eluate. The active protein was concentrated by ultrafiltration on an "Amicon" cell (The Netherlands), using a Pm-30 membrane. Loss of protein when a membrane of this type is used is 8.5-10%, and loss of enzyme activity is 4-6%. The resulting protein concentrate was dialyzed against 50 volumes of 0.025 M citrate-phosphate buffer, pH 5.0. The buffer was changed twice in the course of 12 h.

The dialyzed enzyme was applied to a column (2.2 × 12 cm) with CM-Sephadex A-50 at the rate of 60 ml/h. Glutamine asparaginase was eluted in a linear gradient pH 5.0-7.0 of 0.025 M citrate-phosphate buffer. The rate of elution was 30 ml/h. Protein which contained activity appeared within the range pH 5.8-6.7. Fractions containing active protein were pooled, concentrated to 5 ml, and dialyzed against 0.1 M Tris-HCl buffer, pH 7.5, containing 0.2 M NaCl. Dialysis was carried out for 8 h with two changes of buffer.

Patrice Lumumba Peoples' Friendship University, Moscow. Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 99, No. 5, pp. 557-560, May, 1985. Original article submitted July 27, 1984.

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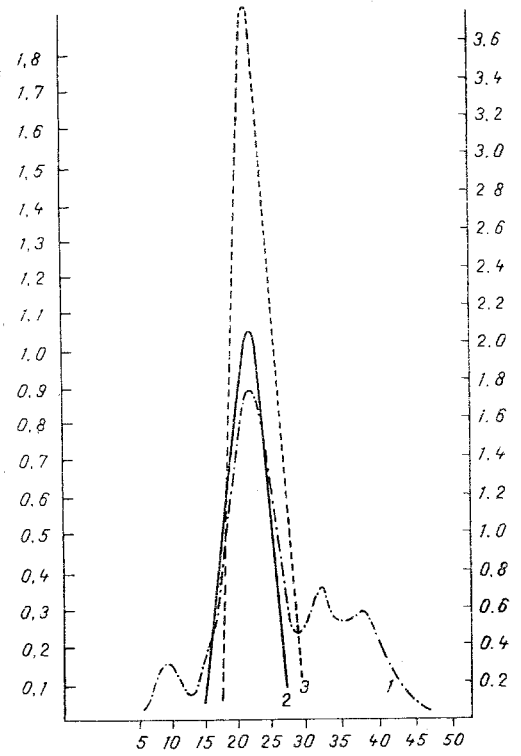

Fig. 1. Chromatography of glutamine asparaginase from Pseudomonas boreopolis 526 on Sephacryl S-200. Abscissa, number of fractions; ordinate: on left – optical density at 280 nm (in relative units), on right – rate of hydrolysis of substrate (in μmoles/min). 1) Protein; 2) asparaginase activity; 3) glutaminase activity.

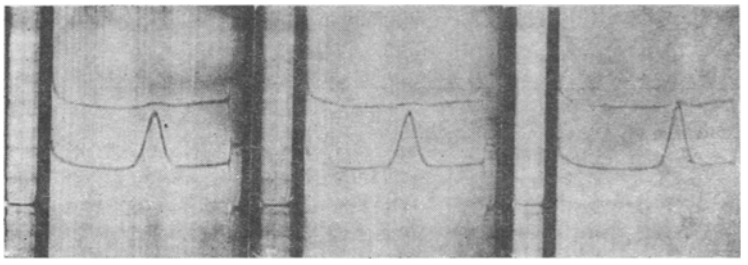

Fig. 2. Sedimentation diagram of glutamine asparaginase at the homogeneous preparation stage. Rotor speed 56,100 rpm, photographed 70 min after reaching full speed. Protein concentration in solution 3.7 mg/ml. 0.05 M Tris-HCl buffer, pH 7.5.

The dialyzed protein (6 ml) was applied to a column with Sephacryl S-200 (2.6 × 100 cm) previously equilibrated with 0.1 M Tris-HCl buffer, pH 7.5, containing 0.2 M NaCl. Protein was eluted from the column at the rate of 30 ml/h. The solution profile of glutamine asparaginase is illustrated in Fig. 1. Fractions with specific activity of 100 units as asparagine and 140 units as glutamine were pooled and lyophilized. The resulting preparation was kept in sealed ampules at 4°C.

To determine the degree of homogeneity of the enzyme preparation two methods were used: sedimentation analysis and electrophoresis in polyacrylamide gel. The sedimentation diagram of the preparation, obtained on a Spinco (model E) ultracentrifuge, is shown in Fig. 2. Electrophoresis was carried out in tubes [10]. The gels were fixed for 1 h in 20% TCA solution and stained in 0.2% Coomassie Blue R-250 solution for 1 h.

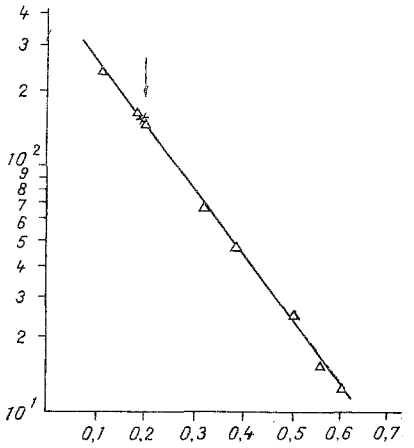

Fig. 3. Determination of molecular weight of glutamine asparaginase from Pseudomonas boreopolis 526 by gel-filtration. Abscissa, log of molecular weights (in daltons); ordinate, $V_e - V_0/V_t$.

TABLE 1. Purification of Glutamine Asparaginase from Pseudomonas boreopolis 526 (enzyme activity given relative to asparagine)

Stage of purification	Volume, ml	Protein concentration, mg/ml	Total quantity of protein, mg	Specific activity, IU/mg	Total activity, IU	Yield, %
Extraction	1100	4,5	4985	3.3	16 583	
Treatment with protamine sulfate	1070	3,4	3638	4.2	15 279	92
Heat treatment	1050	2,45	2572	5,8	14 917	90
DEAE-Sephacel	800	1,0	800	12,4	9 920	59
CM-Sephadex	50	3.1	155	40	6 200	37
Sephacryl S-200	12	2,2	26	100	2 630	16

The molecular weight was determined by two methods. First, the sedimentation equilibrium method (the "full speed" method) on a Beckman (model E) ultracentrifuge with adsorption scanning system and with 12 mm multiplexor and 2° single-sector cells, under split beam conditions, in an AGIi rotor at 280 nm (20°C). The experiment was carried out with a rotor speed of 11,000 rpm for 16 h, preceded by spinning of the rotor for 3.5 h at 14,000 rpm. Sedimentation diagrams, obtained at intervals of 2 h, showed when equilibrium had been reached [5]. The specific partial volume was calculated from the amino acid composition [14]. Second, molecular weight was determined by gel-filtration on a column (1 × 100 cm) with Sephacryl S-200, equilibrated with 0.1 M Tris-HCl buffer, pH 7.5. Blue dextran (2000 kilodaltons) was used to determine the dead volume. To construct a calibration curve, standard protein solutions (2 mg in 0.3 ml), obtained from Serva (West Germany), were passed through the column. The rate of elution of the proteins was 10 ml/h. Molecular weight was determined by means of a standard curve (Fig. 3).

The sedimentation density constant was determined on a Beckman (model E) ultracentrifuge with adsorption scanning system and multiplexor in 12° two-sector cells at 280 nm (20°C) at 60,000 rpm for 2 h.

The total number of SH-groups of the enzyme was determined by titration with Ellman's reagent in 10 M urea. The Ellman's reagent was used in a 50-100-fold excess. The extinction coefficient of Ellman's reagent was taken to be 14,100 [7].

The glutamine and asparaginase activity of the enzyme was determined at 37°C over the range of pH 3.0-10.0 and with substrates in a concentration of 0.02 M. The following buffer solutions were used: 0.05 M citrate-phosphate buffer, pH 3-6; 0.05 M phosphate buffer, pH 5-7.4; 0.5 M Tris-HCl buffer, pH 7.5-9.0; 0.05 M Veronal buffer, pH 9.0-10.0. To determine the thermostability of the enzyme it was incubated in 0.05 M Tris-HCl buffer, pH 7.5, at different temperatures. Aliquots of 20 μl, containing 8 μg of enzyme, were taken at definite time intervals. Enzyme activity was judged from the quantity of ammonia formed, which was determined

TABLE 2. Substrate Specificity of Glutamine Asparaginase from Pseudomonas boreopolis 526

Name of substrate	% of relative activity of enzyme
L-Asparagine	100
D-Asparagine	64
L-Glutamine	156
D-Glutamine	108
Isoglutamine	0
Glycyl-DL-Asparagine	4,6
Glycyl-D-asparagine	0
L-Alanyl-DL-asparagine	1,4
L-Glutamyl-L-asparagine	0
Aspartic acid diamide	6
α-Ethyl-asparagine-NH_2	2,5
α-Propyl-asparagine-NH_2	0

by the method of direct nesslerization. Thermostability also was determined in the presence of 0.05 M sodium glutamate.

The activation energy was determined at a temperatore of over 35°C. Its value was calculated by the equation $E_a = -4.576 \cdot 10^3 \tan \alpha$. The value of tan α was found by plotting the reaction velocity of hydrolysis of the substrate against reciprocals of absolute temperatures.

Substrate specificity was determined in 0.05 M Tris-HCl buffer, pH 7.5, with substrates in a concentration of 0.02 M. Hydrolysis of asparagine was taken as 100%, and all other values were extrapolated to this value.

Electrophoresis in 0.1% sodium dodecylsulfate was carried out in Laemmli's system [10]. Gels were fixed in 20% TCA for 8 h and stained with Coomassie Blue R-250. Protein was determined by Lowry's method [11].

EXPERIMENTAL RESULTS

A highly purified preparation of glutamine asparaginase from Pseudomonas boreopolis 526 was obtained with a yield of 16% of its content in the original extract, with a ratio of activities of 1:1.5 (Table 1). Determination of the molecular weight of the enzyme by gel filtration gave a value of 145 kilodaltons. Calculation of molecular weight from the ultracentrifugation data gave a value of 134 kilodaltons. These values are close to the corresponding molecular weight obtained for glutamine asparaginases from other microorganisms. The enzyme also differed little from other deamidases in its sedimentation coefficient, which was 7.3. Gel electrophoresis in the presence of sodium dodecylsulfate gave one band with mol. wt. of 34 kilodaltons, evidence that the enzyme consists of four identical subunits.

Many workers have observed that deamidases of microbial origin are resistant to freezing and thawing [6, 12]. However, the enzyme which we obtained is very labile during such treatment. A single freezing and thawing leads to loss of about 50% of its activity. By this property the enzyme is similar to glutamine asparaginase from Pseudomonas acidovorans [8]. The change in enzyme activity is perhaps explained on the grounds that during exposure to certain procedures, including freezing, the enzyme molecules aggregate into supramolecular complexes, with activity below the original level. Like other glutamine asparaginases, the enzyme which we isolated contains no free SH groups. Thus in its physicochemical parameters the enzyme now obtained is very similar to a number of deamidases of this group. Meanwhile the enzyme causes only slight hydrolysis of L-asparagine derivatives at the α-NH_2 group. The catalytic action of the enzyme in this case depends on the length of the carbon chain of the modifying agent (Table 2).

Considering the marked cytotoxic action of the enzyme against a number of lymphatic leukemias [4], its comparatively high stability, its quite good yield during purification, and also the simplicity of culture of the microorganism on semisynthetic medium, it can be concluded that the enzyme is very promising as a subject for further study in clinical oncology.

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AGGREGATION AND SWELLING OF RAT BRAIN SYNAPTIC VESICLES

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UDC 612.8.015

KEY WORDS: synaptic vesicles; rat brain; aggregation; swelling; Ca^{++} ions.

Secretion of transmitters by nerve endings and of hormones by gland cells takes place by exocytosis, i.e., Ca-dependent interaction between secretory granules and complementary sites on the inner surface of the presynaptic membranes or cell membranes of the gland cells. One result of this heterologous membrane–membrane interaction is the secretion of transmitters or hormones into the extracellular medium [1, 2]. It is claimed [5] that the trigger stage of exocytosis may be facilitation of adhesion of a fixed secretory granule (adherent to the cell membrane during excitation of the cell) to another "transit" secretory granule. Under these circumstances the two granules fuse together into one, from which the transmitters or hormones are later secreted into the extracellular medium. This hypothesis is supported by data of electron microscopy, showing that during depolarization of nerve ending membranes there is some decrease in the number of synaptic vesicles (SV) in the terminals [2]. This hypothesis, besides heterologous membrane–membrane interaction, also postulates a homologous type of interaction, i.e., fusion (aggregation) of secretory granules with one another. The views examined above have stimulated the study of Ca-induced aggregation (fusion) of different secretory granules, in most cases of chromaffin granules of the adrenals and liposomes [2, 3, 5, 6].

In the investigation described below some characteristics of aggregation and swelling of isolated SV from rat brain were studied.

EXPERIMENTAL METHOD

The SV fraction was isolated from whole brain of rats weighing 150-200 g [10]. For this purpose, unpurified synaptosomes were obtained from a 10% brain homogenate (0.32 M sucrose, 20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA) after removal of the nuclei (10,000g, 10 min), subjected to osmotic shock, after which the residue was suspended in distilled water (4 ml of water to residue obtained from 1 g of brain tissue). After freezing at −20°C and thawing the suspension was centrifuged at 18,000g for 30 min and the supernatant was then centrifuged at 120,000g for 40 min. The residue thus obtained was the SV fraction. The fraction was suspended in 0.25 M sucrose, containing 20 mM Tris-HCl, pH 7.4, and kept for 1 week at −10°C. The preparation, frozen once only, was used in the work. Protein was determined by Lowry's method.

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(Presented by Academician of the Academy of Medical Sciences of the USSR G. N. Kryzhanovskii.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 99, No. 5, pp. 560-563, May, 1985. Original article submitted June 27, 1984.