IDENTIFICATION, ISOLATION, AND PHYSICOCHEMICAL PROPERTIES OF NEUROSPECIFIC α_1-GLOBULIN

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UDC 612.822.1:547.962.4].08

KEY WORDS: cytoplasmic antigen; identification; properties; neurospecific proteins

The aim of this investigation was to seek new cytoplasmic proteins specific for the nervous system. In the modern view, neurospecific proteins (NSP) are structural components by means of which the unique functions of the nervous system are carried out [3, 13]. Relatively few such proteins have yet been described [1, 6], and for that reason the study of new NSP is a very urgent problem. Methods of their identification, based on immunization of animals by extracts of mammalian brain tissue [3, 14, 15], which were used previously, have now completely exhausted their usefulness and are hardly likely to lead to the discovery of new NSP. In order to identify new cytoplasmic brain antigens we therefore used semipurified and concentrated (by 500-600 times) α_1-globulin fractions of a human brain tissue extract for immunization [5].

EXPERIMENTAL METHOD

The first series of polyspecific antisera were prepared in the following way: human brain tissue (taken during the first 6 h after death) was washed free from blood, homogenized, and extracted with 0.05 M Na-phosphate buffer, pH 7.6, with the addition of Triton X-100 and Tween-80, in a concentration of 1 g/liter. After three cycles of freezing and thawing the homogenate was centrifuged at 15,000*g* for 25 min. Ammonium sulfate was added to the resulting supernatant (extract) up to 30% saturation, and the mixture was incubated for 12 h at 4°C and centrifuged for 30 min at 15,000*g*. The residue was dissolved in the minimal volume of 0.05 M carbonate buffer, pH 9.2, and subjected to gel-filtration in a column (1.6 × 85 cm) with Sephadex G-200 ("Pharmacia Fine Chemicals," Sweden) at the rate of 5 ml/h. The column was calibrated against standards obtained from "Serva" (Germany). During gel-filtration protein fractions with molecular weights of between 40 and 120 kD were isolated. These fractions were concentrated and subjected to PAG disk electrophoresis. Areas corresponding to zones of α_1-globulins were then cut out of the gel. Proteins were extracted from them with three portions of 0.1 M veronal-medinal buffer, pH 8.6, for 24 h. After accumulation of the necessary amounts of proteins of the test fraction for immunization, it was concentrated a further 15-20 times and used to immunize rabbits weighing 3-4 kg by the usual schedule.

Ion-exchange chromatography was carried out on a column with DEAE-cellulose ("Whatman," England), equilibrated with 0.05 M Na-phosphate buffer, pH 7.6, in a linear NaCl concentration gradient within the range from 0.2 to 1.0 M, at the rate of 40 ml/h.

Affinity chromatography was carried out on a column (1 × 6 cm) with lentil-lectin sepharose 4B ("Pharmacia"), equilibrated with 0.005 M Na-phosphate buffer, pH 7.6, containing 1 mM each of calcium, magnesium, and manganese chlorides, at the rate of 40 ml/h. Proteins were eluted in a concentration gradient of α-methyl-D-glucopyranoside from 0 to 3 M.

For hydrophobic chromatography in a falling linear concentration gradient of ammonium sulfate (from 1 M to zero) a column (1 × 5 cm) with phenyl-sepharose ("LKB," Sweden), equilibrated with 0.05 M Na-phosphate buffer, pH 7.6, containing 1 M ammonium sulfate, was used.

V. P. Serbskii Research Institute of General and Forensic Psychiatry, Moscow. Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 114, No. 7, pp. 36-38, July, 1992. Original article submitted November 6, 1991.

 0007-4888/92/0007-0954$12.50 ©1993 Plenum Publishing Corporation

TABLE 1

Method of Obtaining
Saline extract from human brain
+
Ammonium sulfate 70% saturation
Residue dissolved in ammonium sulfate solution 35% saturation, centrifuged at 24,000 g, and for subsequent work, the supernatant was taken and dialyzed against working buffer.
+
Ion-exchange chromatography on DEAE-52 cellulose
Withdrawal of fractions eluted within NaCl concentration range from 0.25 to 0.30 M. This fraction dialyzed against working buffer, and concentrated on DEAE-52 cellulose to minimal volume.
+
Gel-filtration on Sephadex G-200.
Protein fraction with mol. wt. of 110 ± 10 kD withdrawn.
+
Hydrophobic chromatography on phenyl-sepharose.
Protein fraction eluted within concentration range of ammonium sulfate from 0.45 to 0.12 M withdrawn. This fraction dialyzed against working buffer containing 1 mM each of calcium, magnesium, and manganese chlorides.
+
Affinity chromatography on lentil-lectin sepharose 4B.
Fraction eluted within concentration range of a-methyl-D-glucopyranoside from 0.18 to 0.20 M withdrawn. This fraction dialyzed against working buffer.
+
Isochromatofocusing on PBE gel
Elution with polybuffer 74, then with glycine-HCl buffer, 0.05 M, pH 2.2. During elution protein fraction with isoelectric point within the pH range from 3.1 to 2.9 withdrawn, alkalified with dry Tris, and dialyzed against working buffer.

Isochromatofocusing was carried out on PBE gel with polybuffers 9-6 and 7-4 ("Pharmacia") in a column (1 × 6 cm).

The yield of protein fractions was monitored with a "Uvicord" continuous-flow photometer ("LKB"), followed by immunochemical comparison.

Amino acid analysis was carried out after acid hydrolysis of the preparation in 6 N HCl for 12, 24, and 32 h at 105°C on a "Durrum DS-500" amino acid analyzer (USA). Before analysis the preparation was hydrolyzed with 2 M trifluoroacetic acid at 100°C for 6 h. Comparison of the preparation thus obtained with other NSP known previously was carried out by immunodiffusion. The purity of the preparation was verified by disk electrophoresis [7], crossed immunoelectrophoresis [11], and NH_2-terminal analysis.

EXPERIMENTAL RESULTS

The polyspecific antiserum obtained by the method described above was exhausted with dry plasma, and with freeze-dried extracts of tissues from the kidney, spleen, lung, and testes, and studied by immunodiffusion and crossed immunoelectrophoresis. The investigations showed that this antiserum revealed four antigens in the preparation used for immunization. This same antiserum was additionally exhausted with a purified GFAP preparation, α_2-glycoprotein, and small quantities of the α_1-globulin fraction, so that the exhausted antiserum revealed only brain α_1-globulin in this fraction. This antiserum was used to monitor each stage of our elaborated procedure of purification of brain α_1-globulin, shown schematically in Table 1.

Testing the purity of the isolated preparation by the above methods revealed no contamination. During immunization of rabbits with the purified preparation, a monospecific antiserum was obtained.

TABLE 2. Physicochemical Properties of Brain α_1-Globulin

Physicochemical properties	Brain $_1$-globulin
Precipitation by ammonium sulfate	Precipitated at 35-70% saturation
Precipitation by butanol	10% butanol denatures antigen
Precipitation by TCA	10% TCA precipitates antigen completely
Adsorption properties	
a) alumina	adsorbed
b) barium sulfate	not adsorbed
c) phenyl-sepharose	bound
Interaction with metallic ions:	
a) zinc	not bound
b) copper	not bound
c) cobalt	not bound
Thermostability	withstands 30 min of incubation at 80°C
Interaction with lectins:	
a) concanavalin A	bound
b) lentil lectin	bound
c) wheat germ lectin	not bound
Relative molecular weight:	
a) measured by gel-filtration	110 ± 10 kD
b) measured by disk-electrophoresis with treatment by β-mercaptoethanol in PAG with SDS	82.0 ± 4.2 36.0 ± 1.2 kD
Relative electrophoretic mobility	0.96 ± 0.01
Isoelectric point	pH 2.9-3.1

TABLE 3. Results of General Chemical Analysis of Brain α_1-Globulin

Chemical composition	Content, mg/100 mg antigen	Method of determination
Protein	81.25	Lowry [12]
Monosaccharides	8.03	Phenol sulforcinate [8]
Sialic acids	1.45	
Hexosamines	1.62	Elson and Morgan [9]
Sulfate	+	IR spectroscopy

Amino-acid composition

Amino acid	Per cent amino acid per 100 amino acids	Amino acid	Per cent amino acid per 100 amino acids
Cys (O_3H)	2.53	Lys	4.12
His	1.92	Arg	2.24
Asp	12.56	Glu	19.11
Tyr	1.82	Ser	5.31
Pro	6.86	Gly	9.32
Ala	7.18	Val	4.78
Met	0.92	Leu	6.15
Ile	5.06	Thr	6.81
Phe	2.42	Trp is destroyed under conditions of acid hydrolysis, and so cannot be determined.	

Some results of our investigation of the physicochemical properties of the brain α_1-globulin, purified to homogeneity, and of its general chemical, amino acid, and carbohydrate composition are given in Tables 2 and 3. The results shown in Table 2 were obtained by means of monospecific antiserum to this protein.

A study of the physicochemical properties of the protein revealed a molecular weight of 110 ± 10 kD and isoelectric point at pH 2.9-3.1. During PAG-SDS disk electrophoresis two bands, evidently corresponding to two subunits with mol. wt. of 82.0 ± 4.2 and 36.0 ± 1.2 kD, were observed, and which under native conditions may be joined by disulfide bonds. As regards the other physicochemical properties, we must draw attention to the high affinity for lentil lectin, suggesting that carbohydrate chains with D-glucose and D-mannose residues are present in its structure. Binding with phenylsepharose suggests the presence of hydrophobic regions, which interact with the phenyl radicals of the sorbent.

The study of the chemical composition of the brain α_1-globulin showed that the antigen is a sialoglycoprotein (81.25% protein and 8.03% carbohydrates, glucose 2.58%, mannose 1.86%, galactose 1.77%, fucose 0.89%, glucosamine 0.65%, galactosamine 0.38%). The amino acid composition is characterized by relative predominance of monoaminodicarboxylic and aliphatic amino acid residues. This determines the acid properties of the antigen, and the strong electrically negative charge of its molecule, suggesting that there is a predominantly amide type of bond with the carbohydrate components. NH_2-terminal analysis revealed two terminal amino acids (Glu and Leu) in the structure of the brain α_1-globulin, in agreement with the results of PAG-SDS disk electrophoresis, which revealed two subunits.

The purified preparation of brain α_1-globulin was compared with certain other previously identified NSP. The results of Ouchterlony's immunodiffusion test with modifications [4] showed that this antigen is not identical with cytoplasmic NSP such as GFAP [10], specific α_2-glycoprotein [15], and proteins 10-40-4, 14-3-2 [2], and S-100. The brain α_1-globulin which we have discovered and purified to homogeneity is thus evidently a hitherto unidentified cytoplasmic neurospecific protein.

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