Biochemistry (Moscow), Vol. 66, No. 1, 2001, pp. 83-90. Translated from Biokhimiya, Vol. 66, No. 1, 2001, pp. 105-113.

Bacterial Protease ECP32 Specifically Hydrolyzing Actin and Its Effect on Cytoskeleton *in vivo*

A. V. Morozova, I. N. Skovorodkin, S. Yu. Khaitlina, and A. Yu. Malinin*

Institute of Cytology, Russian Academy of Sciences, Tikhoretskii pr. 4, St. Petersburg, 194064 Russia; fax: (812) 247-0341; E-mail: malinin@mail.cytspb.rssi.ru

Received April 19, 2000
Revision received June 28, 2000

Abstract—A procedure for isolation of bacterial protease ECP32 yielding 100 μg of the enzyme from 10 liters of the *Escherichia coli* strain A2 liquid culture has been developed. The procedure includes chromatography, ultrafiltration, and PAGE under non-denaturing conditions. The purified preparation contained about 80% ECP32 and did not exhibit ATPase activity. Polyclonal ECP32-specific antibodies have been produced, and a two-stage procedure for the isolation of protease ECP32 involving affinity chromatography has been elaborated. Microinjection of the purified ECP32 into *Amoeba proteus* cells caused reversible distortions in amoeba locomotion. The effect was not observed upon inhibition of the protease activity by the ECP32-specific antibodies. The results indicate that bacterial protease ECP32 may be used for the analysis of actin functions *in vivo*.

Key words: protease ECP32, protein purification, limited proteolysis of actin, actin, antibodies, microinjection, amoeboid locomotion

Interactions of pathogenic bacteria with eukaryotic cells include rearrangements of the actin cytoskeleton. Invasive (cell-penetrating) microorganisms induce polymerization of intracellular actin and formation of specific structures on their surface providing their spread [1-3]. Noninvasive bacteria bind to the cell surface and initiate changes in cytoskeleton structures adjacent to the membrane or secrete toxins into the cytosol stimulating the formation of stress fibrils, reorganization of actin filaments, and inhibition of cytokinesis [4-6]. Not only actin polymerization but its degradation as well contribute to the pathogenesis, as reported for the botulinum E-type neurotoxin [7].

Analysis of an unusual actin proteolysis product resulted in the isolation of *Escherichia coli* strain A2; in extracts of this strain, a previously unknown proteolytic activity towards G-actin was detected [8-10]. Actin was cleaved at a single site different from those attacked by commercial proteases [9]; proteolysis was limited, and the proteolysis product displayed unique properties in the polymerization–depolymerization reaction. This peculiarity was successfully used in actin studies *in vitro*: cleaved actin, containing Ca^{2+} as a tightly bound cation, was shown to be incapable of polymerization [10, 11]. The replacement of Ca^{2+} by Mg^{2+} partially restored the ability of actin to polymerize; however, the polymers formed were unstable [11]. In addition, conformational changes induced by cations and nucleotides in globular and fibrillar actin were studied [12]; an allosteric effect of actin degradation was demonstrated [13]. In extracts of *E. coli* strain A2, an intracellular neutral metalloproteinase ECP32 (EC 3.4.24) with molecular weight 32 kD displaying this specific activity was identified [14]. In addition to actin, of several dozens of eukaryotic proteins, protease ECP32 cleaves only histones, this indicating high substrate specificity of the enzyme [10, 14].

In this work, we explored the possibility for using protease ECP32 for cleavage of actin *in vivo*. A model for the proteinase ECP32–cytoskeleton interactions *in vivo* using protozoan cells was developed. The procedure for injection of various compounds into unicellular organisms is rather simple [15], and the effect on the cytoskeleton is clearly manifested by alterations of form and the type of locomotion. *Amoeba proteus* cells provide a convenient model due to dynamic rearrangements in cytoskeleton structures [16]. In the framework of this task, it was necessary to isolate a purified ECP32 preparation and to develop a procedure inhibiting its actin-degrading activity.

MATERIALS AND METHODS

Reagents used for preparation of growth media were of analytical grade; chemicals were obtained from Sigma

* To whom correspondence should be addressed.

0006-2979/01/6601-0083$25.00 ©2001 MAIK "Nauka/Interperiodica"

(USA), Serva and Merck (Germany); sorbents for column chromatography were from LKB-Pharmacia (Sweden) and Whatman (USA).

Bacteria growth and production of cell-free extract. *E. coli* A2 was grown at 35°C with intensive aeration in the following medium: peptone (20 g/liter), yeast extract (5 g/liter), and NaCl (10 g/liter), pH 7.0. Biomass growth was monitored by mass measurements of the bacterial pellet washed with 20 mM Tris-HCl buffer, pH 7.5, (buffer T) and absorption of the culture liquid at 600 nm. From 20 to 30 h after the beginning of the post-logarithmic growth phase, cells were precipitated by centrifugation at 5,000*g* for 20 min. The bacterial pellet was resuspended in 10 volumes of buffer T and centrifuged as described above. Cells were resuspended in 10 volumes of buffer T to wet biomass concentration of 200 g/liter and disrupted in an UZDN-1 U 4.2 ultrasound disintegrator (Russian Academy of Sciences) at a frequency of 22 kHz. The extract was centrifuged at 17,500*g* for 40 min. All procedures were conducted at 4°C.

Enzyme purification. To obtain crude protease preparation, a 450-ml column (60-mm diameter) filled with 150-200 ml of DEAE-cellulose DE-52 (Whatman) and equilibrated with buffer T was used. The cell-free extract was applied to the column (5-5.5 g protein), and the column was washed with 150-200 ml of buffer T and 150-200 ml of 100 mM NaCl in the same buffer. The protease was eluted with 200-300 ml of 200 mM NaCl in buffer T. Fractions with ECP32 activity were dialyzed against buffer T.

The crude protease preparation (1-1.5 g protein) was applied to a Q-Sepharose Fast Flow (Pharmacia) column (20-mm diameter, anion exchanger volume 70 ml) equilibrated with buffer T. The column was washed with 200-250 ml of 50 mM NaCl in buffer T. The protease was eluted with a linear NaCl gradient (50-200 mM) in buffer T at flow rate 3.5 ml/min. Fractions containing ECP32 were combined and concentrated to 6 ml by ultrafiltration on a Centriprep cell (Amicon, USA) equipped with a membrane with molecular weight cut off (MWCO) 10 kD.

Ammonium sulfate (2.5 M) in buffer T to a final concentration of 1 M was added to the enzyme preparation, and the preparation (50-100 mg protein) was applied to a phenyl-Sepharose CL-4B column (Pharmacia) (10-mm diameter) equilibrated with 1 M $(NH_4)_2SO_4$ in buffer T; the volume of the phenyl-Sepharose CL-4B was 9 ml. The column was washed with 30 ml of 1 M and 50 ml of 0.1 M $(NH_4)_2SO_4$ in buffer T. The protease was eluted with buffer T at flow rate 0.33 ml/min.

The protease-containing fractions were concentrated on a Centriprep cell equipped with a MWCO 10-kD membrane to volume 800-900 μl and separated from high-molecular-weight contaminants by ultrafiltration on a Microcon (Amicon) cell with a MWCO 100 kD membrane. The filtrate was fractionated by preparative electrophoresis in non-denaturing conditions.

Antibodies. Rabbits were immunized with a purified protease preparation. The immune serum was exhausted on cell walls of *E. coli* strain XLMRA. The bacteria were incubated for one day under the conditions described for strain A2, washed with buffer TS (50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl), resuspended in the same buffer, subjected to ultrasound disintegration under the same conditions as for A2, and centrifuged. The pellet of cell walls obtained from 20 g of wet biomass was repeatedly (two or three times) washed with 50 ml of buffer TS, until protein concentration in the supernatant became less than 1 mg/ml, and resuspended in 25 ml of the same buffer. Ten milliliters of the immune serum were added to the suspension. The mixture was incubated at room temperature for 1 h with constant stirring and clarified by centrifugation. The crude IgG fraction was obtained by precipitation with ammonium sulfate (60% saturation) or using DEAE-cellulose DE-52 (Whatman). From 200 to 300 mg of the total exhausted serum protein in 50 mM Tris-HCl, pH 7.2, were applied to 10 ml of DEAE-cellulose. Antibodies eluted in the void volume were dialyzed against 20 mM sodium phosphate buffer, pH 7.0, and the IgG fraction was isolated by affinity chromatography on a HiTrap Protein G column (Pharmacia) according to the manufacturer's protocols.

To produce monospecific anti-ECP32 antibodies, the protease preparation, containing approximately 5,000 activity units and 2 mg protein, was separated by preparative SDS-PAGE in 10% polyacrylamide slab gels (105 × 160 mm) and transferred to nitrocellulose. The protease was visualized by staining the margins of the membrane with anti-ECP32 immune serum. The enzyme-containing strip (from 1 to 2 mm wide) was cut from the membrane stained with Ponceau S (Sigma), incubated at room temperature in 1% BSA in buffer TS for 1 h, placed in 0.5 ml of 0.1% BSA solution in buffer TS containing 1 mg IgG isolated from the anti-ECP32 immune serum, and incubated overnight at 4°C. The unbound antibodies were washed (three times) from the membrane strip with buffer TS containing 0.05% Tween 20 (Sigma). The membrane was washed with water, and monospecific anti-ECP32 antibodies were eluted with 0.5 ml of 100 mM glycine-HCl buffer, pH 2.7, for 5 min at room temperature. The eluate was immediately neutralized with 25 μl of 1.5 M Tris-HCl buffer, pH 8.8.

Antibodies against other polypeptides were produced similarly.

Affinity chromatography. Sorbents for affinity chromatography of the protease were produced by immobilization of antibodies on Sepharose 4B (Pharmacia) activated by cyanogen bromide according to the manufacturers' protocols or by sodium metaperiodate. Five grams of Sepharose CL-4B (Pharmacia) were washed with water and incubated in aqueous solution of sodium metaperiodate (150 mg in 10 ml water) for 30 min under gentle stirring at room temperature. Glycerol (200 μl) was added,

and the suspension was incubated for an additional 15 min. Sepharose was washed with water and 50 ml of buffer B (100 mM sodium bicarbonate, pH 9.0) on a glass filter. The IgG preparation (15 mg) isolated from the anti-ECP32 immune serum was added at a concentration of 2.5-3 mg/ml in buffer B and stirred at 4°C for at least 48 h. The Sepharose was washed with five volumes of buffer B and treated with one volume of sodium borohydride (3 mg/ml) in the same buffer for 2 h at 4°C under gentle stirring. The Sepharose was washed with five volumes of buffer B, from five to ten volumes of 100 mM sodium citrate buffer, pH 3.5, five volumes of buffer B, and equilibrated with ten volumes of buffer TS.

Approximately 20,000 activity units (up to 30 mg protein) of the crude protease preparation in buffer TS were applied either to 1.0-1.5 ml of the affinity CNBr-activated Sepharose or to 2.5-3 ml of Sepharose activated with periodate and incubated for 1 h at room temperature under mild stirring. Using a column 15 mm in diameter, the Sepharose was washed with six volumes of buffer TS, and the protease was eluted with six volumes of 100 mM glycine-HCl buffer, pH 2.7; each fraction was neutralized with 1.5 M Tris-HCl, pH 8.8.

Microinjection. Microinjection of the protease into *Amoeba proteus* cells was conducted using an Eppendorf Microinjector 5242 (Eppendorf, Germany). The buffer and all preparations for injection except for those containing antibodies were subjected to ultrafiltration on a Microcon cell (Amicon) equipped with a MWCO 100-kD membrane.

For thermal inactivation of the protease, 20-30 μl of the enzyme were heated at 56°C for 30 min in a water bath. For inhibition of activity, the protease was incubated with anti-ECP32 antibodies for 1 h at room temperature.

About 30 pl of buffer T, containing either the protease (0.01-0.015 mg/ml) or IgG (1.3-2.0 mg/ml) or their mixture were injected into the amoebae cytoplasm.

Protease activity assay. The proteolytic activity in the preparations was assayed by the ability to hydrolyze G-actin. Freshly isolated [17], once thawed, or freshly dissolved freeze-dried actin preparations were used. An equal volume of the protease dissolved in buffer T (protein concentration not more than 1 mg/ml) was added to the G-actin solution (1 mg/ml in 0.5 mM ATP, 0.2 mM $CaCl_2$, and 2 mM Tris-HCl, pH 7.5). The mixture was incubated for 1 h at 20°C and analyzed by SDS-PAGE. The enzyme activity was calculated from the absorption ratio of the Coomassie Brilliant Blue stained 36-kD proteolysis product to the intact actin. The amount of the protease hydrolyzing 100 μg of G-actin for 1 h at 20°C was taken for one unit of the enzyme activity.

ATPase assay. To measure ATPase activity, aliquots of the protease solution were incubated with 0.2 mM ATP for 1 h. The reaction was terminated by the addition of an equal volume of 0.6 M $HClO_4$ followed by centrifugation. Inorganic phosphate in the supernatant was determined with malachite green [18].

Determination of protein concentration. Protein concentration was determined by the microbiuret procedure [19]. Actin or BSA (Sigma) was used as standard. The amount of protein in the purified protease preparation was estimated by scanning Coomassie-stained gels on an Ultrascan Laser Densitometer (LKB). Protein concentration was calculated using the manufacturer's program. Actin or carbonic anhydrase (Sigma) was used as standard.

Electrophoresis. SDS-PAGE was conducted according to Laemmli [20] with slight modifications at acrylamide/bis-acrylamide ratio 33 : 1. Gels were fixed in methanol–acetic acid–water (50 : 10 : 40 v/v) and stained with Coomassie Brilliant Blue G-250. Densitometry was carried out on an Ultrascan Laser Densitometer (LKB). The Low Molecular Weight Calibration Kit (Pharmacia) containing phosphorylase *b* (94.0 kD), albumin (67.0 kD), ovalbumin (43.0 kD), carbonic anhydrase (30.0 kD), soybean trypsin inhibitor (20.1 kD), and α-lactalbumin (14.4 kD) was used to estimate molecular masses.

Electrophoresis under non-denaturing conditions was conducted in a slab or tube at 10°C: in slab, 10% polyacrylamide gel and acrylamide/bis-acrylamide ratio of 55 : 1 (w/w) were used; in tube, 9% polyacrylamide gel and acrylamide/bis-acrylamide ratio 36 : 1 (w/w) were employed. The composition of the gel and solutions was the same as for SDS-PAGE; the only difference was the absence of SDS and β-mercaptoethanol.

After electrophoresis, 1.5-mm wide strips of the gel parallel to the gel origin were cut, placed inside dialysis membranes, and the proteins were electroeluted into the electrophoretic electrode buffer at 15 mA for 20 h at 4°C.

Tube electrophoresis was performed in an apparatus for preparative electrophoresis [21]. The time from the beginning of electrophoresis to the migration of the dye bromophenol blue out of the gel was multiplied by 3, and during this time, 60-70 protein fractions migrating from the gel were collected.

Western blotting. Western blotting was carried out by a standard procedure [22] with minor modifications. Peroxidase-conjugated IgG were used as secondary antibodies. The reaction with antibodies was visualized by staining with 3,3′-diaminobenzidine tetrachloride in buffer TS.

Ultrafiltration. Microcon, Centricon, and Centriprep cells for ultrafiltration (Amicon) were used. To concentrate the protease solution, ultrafiltration cells equipped with MWCO 10 and 30 kD membranes were used; to remove high-molecular-weight contaminants, MWCO 100 kD membranes were employed.

N-Terminal sequence of proteins was determined on a model 477A sequencer (Applied Biosystems, USA) equipped with a model 120 PTH amino acid analyzer according to the manufacturers' protocols.

RESULTS AND DISCUSSION

Enzyme purification. Cultivation and subsequent ultrasound disintegration provided *E. coli* extract in which the protease of interest accounted for 0.015% of the total bacterial protein. A preparative method for ECP32 isolation including crude fractionation by ion-exchange chromatography, two chromatographic stages, ultrafiltration, and preparative electrophoresis under non-denaturing conditions was developed. In Table 1 and Figs. 1 and 2, the results of one purification cycle are shown. Comparison of the protein composition in the protease preparation (Fig. 2) at different purification stages demonstrates a progressive increase in the intensity of the ECP32 band. By ultrafiltration through the MWCO 100 kD membrane, a sevenfold increase in the purification factor was achieved (Table 1): the protein bands in the upper part of the gel virtually disappeared (compare lanes *2* and *3* in Fig. 2). A considerable decrease in the protease yield at the ultrafiltration stage was not associated with ultrafiltration through the MWCO 100 kD membrane, but occurred during concentration on the MWCO 10 kD membrane; protein loss at this stage was inevitable due to very low protein concentration.

PAGE under non-denaturing conditions showed that the enzyme preparation was heterogeneous. During electroelution from the gel, ECP32 appeared as one protein band upon Coomassie Brilliant Blue staining. To avoid laborious electroelution for the location of ECP32 during each preparative electrophoretic procedure, a marker of protease mobility was found. In the range of acrylamide concentrations from 7.5 to 15.0%, the mobility of ECP32 in non-denaturing gels was similar to that of BSA. We also determined the concentrations of the gels and the degree of cross-linking at which ECP32 migrated slightly quicker or slower than BSA. During slab gel electrophoresis, ECP32 had the same mobility as BSA at acrylamide concentration of 10% and acrylamide/bis-acrylamide ratio 55 : 1. During tube electrophoresis, 9% polyacrylamide gel was used at acrylamide/bis-acrylamide ratio 36 : 1; under these conditions, albumin migrated slightly faster than ECP32. The migration of BSA out of the gel was recorded spectrophotometrically and served as a signal for the following protease elution. In the BSA fractions migrating out of the gel, some increase of protease activity was noted; however, the major ECP32 fraction eluted later. It seemed likely that albumin stabilized ECP32 in highly diluted solutions. Activity assays of the purified protease preparation confirmed this suggestion. At the protease concentrations used in proteolytic activity assays, BSA (1 mg/ml) caused at least 30-fold increase in the ECP32 activity.

The comparison of two preparative electrophoretic procedures (in slab gel with subsequent electroelution and in tube) showed that the enzyme yield was 20-30% for electrophoresis in slabs and 80-90% for tube electrophoresis; however, the latter procedure was more laborious. Low recovery of the enzyme achieved using the first method might be reduced by improving the electroelution procedure.

The purified ECP32 preparation was used for production of polyclonal antibodies. On the immunoblots of the ECP32 preparation after DEAE chromatography (Fig. 3b, lane *2*), three zones corresponding to ECP32 (32 kD) and to two other polypeptides (35 and 22 kD) were revealed. The electrophoregram of the protease preparation obtained by affinity chromatography was similar (Fig. 4, lane *2*). Immunoblotting showed cross-reaction between monospecific antibodies against 35- and 22-kD polypeptides; however, no reaction with ECP32 was observed. The 22-kD polypeptide was always seen as a minor contaminating band in the purified ECP32 preparation, both when our isolation procedure and an earlier developed analytical method were used [14]. This polypeptide exhibited high immunogenicity: its presence in minor quantities in the injected ECP32 preparation induced the production of antiserum with high titer of

Table 1. Purification of the ECP32 protease

Step	Active fractions			Purification factor	Yield, %
	protein, mg	activity × 10^{-3}, units	specific activity, units/mg		
Crude extract	5030	1305	259	—	100
DEAE-cellulose	1220	1024	839	3	79
Q-Sepharose Fast Flow	75	954	12720	49	73
Phenyl-Sepharose CL-4B	20	796	39214	151	61
Ultrafiltration	1.25	342	273600	1056	26
Preparative electrophoresis	~0.12	77	~637500	~2461	6

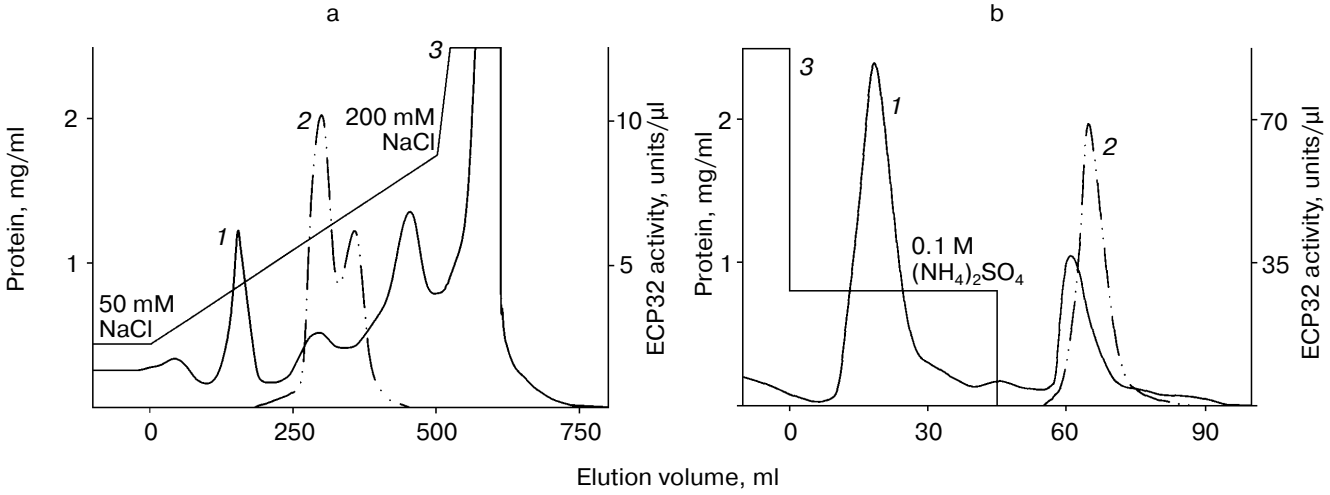

Fig. 1. Chromatography of the ECP32 protease on Q-Sepharose Fast Flow (a) and phenyl-Sepharose CL-4B (b): *1*) protein elution profile; *2*) ECP32 activity; *3*) salt concentration.

anti-22-kD antibodies (compare lanes *3* in Figs. 3a and 3b). The antiserum also revealed the 35-kD protein present in the producer's cell extract in large quantities (1-3% of the total protein). Thus, isolation of the protease using affinity chromatography involved two stages: removal of the 35- and 22-kD polypeptides from the cell extract and affinity chromatography with anti-ECP32 antibodies. However, the protease yield was only 5-6%.

The 32-kD polypeptide was the main component present in the purified ECP32 protease preparation. Its N-terminal sequence was determined (AKTSSAGVV-IRDIFL).

Analysis of properties of ECP32-cleaved actin showed that a crude preparation of the enzyme manifested ATPase activity [11]. To determine whether this activity was associated with contaminating proteins or was specific to ECP32, we assayed ATPase activity in protease preparations at different purification stages. The results shown in Table 2 indicate that the ATPase coeluted with the ECP32 protease during anion-exchange chromatography but was separated from the protease during hydrophobic chromatography. Therefore, the purified enzyme preparation did not show ATPase activity and could be used for injection into cells *in vivo*.

Microinjection. The purified ECP32 preparation was introduced into *Amoeba proteus* cells by microinjections. After injection of 30 pl of the protease solution at a concentration of 0.01-0.015 mg/ml, pseudopodia were not formed and the cytoplasm motility decreased and even stopped (Fig. 5b). Injected individuals sedimented to the bottom of microaquariums and remained spread and immobile for 2-3 h from the beginning of the experimentation. In some instances, monopodial amoebae incapable of locomotion were observed (Fig. 5c). Locomotion was slowly restored and a day after injection became normal. The injection of larger amounts of the protease solution (at least tenfold concentrations) caused disruption of the cortical actin layer in the site of injection and disintegration of the plasma membrane resulting in cell death.

To elucidate whether these changes in amoebae were caused by ECP32 activity, in control experiments heat-inactivated protease preparation or ECP32 complexed

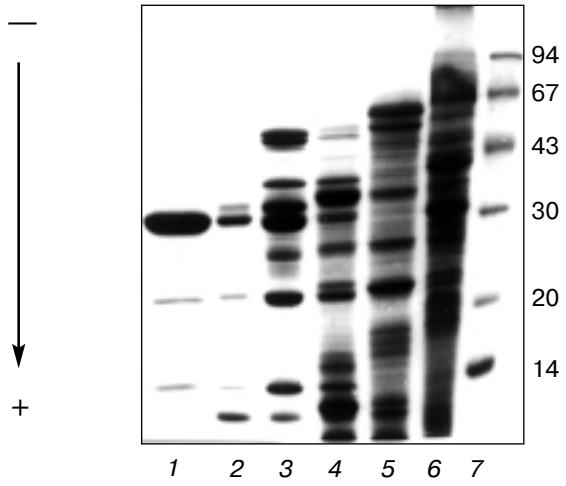

Fig. 2. Polypeptide composition of the ECP32 preparation at different purification stages. SDS-PAGE in a 12.5% gel: *1*) purified protease preparation after preparative electrophoresis under non-denaturing conditions in a 10% slab gel followed by electroelution; *2*) protease preparation after ultrafiltration on Centriprep MWCO 10 kD and Microcon MWCO 100 kD; *3*) protease after chromatography on phenyl-Sepharose; *4*) protease after ion-exchange chromatography on Q-Sepharose Fast Flow; *5*) protease after crude fractionation on DEAE-cellulose; *6*) original intracellular extract of the *E. coli* A2; *7*) protein markers (molecular masses in kD are shown at the right).

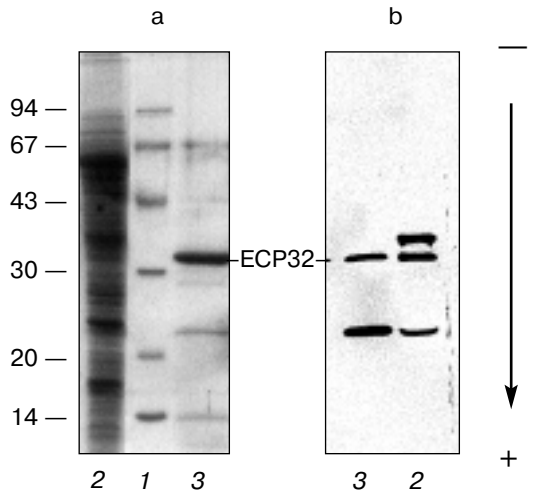

Fig. 3. Immunoblotting of ECP32 preparations with ECP32-specific antibodies (b); blotting of the same preparations stained with Ponceau S (a): *1*) protein markers; *2*) crude protease preparation after DEAE-cellulose; *3*) purified ECP32 after complete purification cycle (at the left, molecular masses of protein markers in kD are shown).

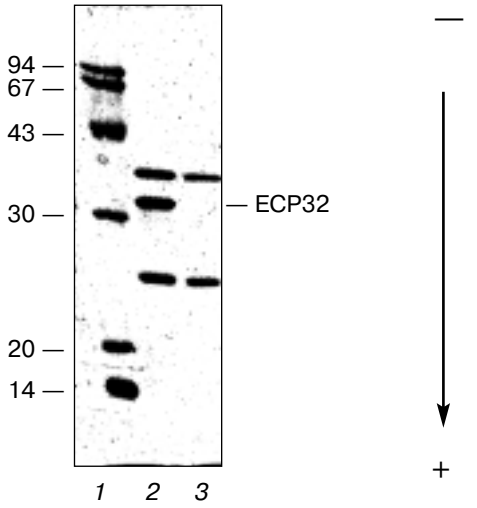

Fig. 4. SDS-PAGE (13.5% gel) of fractions obtained by affinity chromatography with ECP32-specific antibodies: *1*) protein markers; *2*) first ECP32-containing fraction; *3*) subsequent fractions lacking ECP32 (molecular masses of protein markers in kD are given at the left).

with antibodies inhibiting the enzyme activity were injected into cells (Fig. 5d). Heating of the protease solution at 56°C led to complete enzyme inactivation. However, in this instance degradation of the enzyme probably occurred, since in heated preparations the protease band was not seen both after electrophoresis and immunoblotting with anti-ECP32 antibodies.

A preparation without ECP32 activity but containing the intact enzyme molecule was obtained by treatment of the protease with anti-ECP32 antibodies. Figure 6 shows that complete inhibition of ECP32 by the antibodies occurred at the following ratio: 0.15 μg IgG per unit of ECP32 activity or higher. To produce inactive preparations, the following ratios were used: 0.3-0.4 μg IgG per protease activity unit. Injection of preparations without ECP32 or with the enzyme inactivated by antibodies caused no morphological changes in the amoebae. Ten-fifteen minutes after injection, the locomotion of amoeba did not differ from control.

The abnormalities observed in amoebae in our work were similar to morphological and locomotor changes induced by injection of actin-binding proteins such as DNase I or fragmin described in the literature [15]. DNase I and fragmin are known to inhibit the formation of actin filaments [23, 24]. Since ECP32-cleaved actin fails to polymerize [9-11], we suggest that injection of the protease inhibits assembly of amoeba cytoskeleton and disturbs locomotion. In all likelihood, the disruption of the cortical cytoskeleton was also related to the specific effect of ECP32 on fibrillar actin and not to introduction

Table 2. ATPase activity of fractions during ECP32 purification

ECP32 purification step	Proteolytic activity, units/mg	ATPase activity, μmol P_i/min per mg protein
After complete purification cycle	637,500	no
Phenyl-Sepharose after DEAE-cellulose, protease activity peak	13,393	no
DEAE-cellulose, protease activity peak	1635	2.92
DEAE-cellulose, eluate after the protease peak	<10	2.53
DEAE-cellulose, eluate before the protease peak	no	0.48
Phenyl-Sepharose after DEAE-cellulose, void volume	no	2.36
Phenyl-Sepharose after DEAE-cellulose, eluate before the protease peak	no	0.11

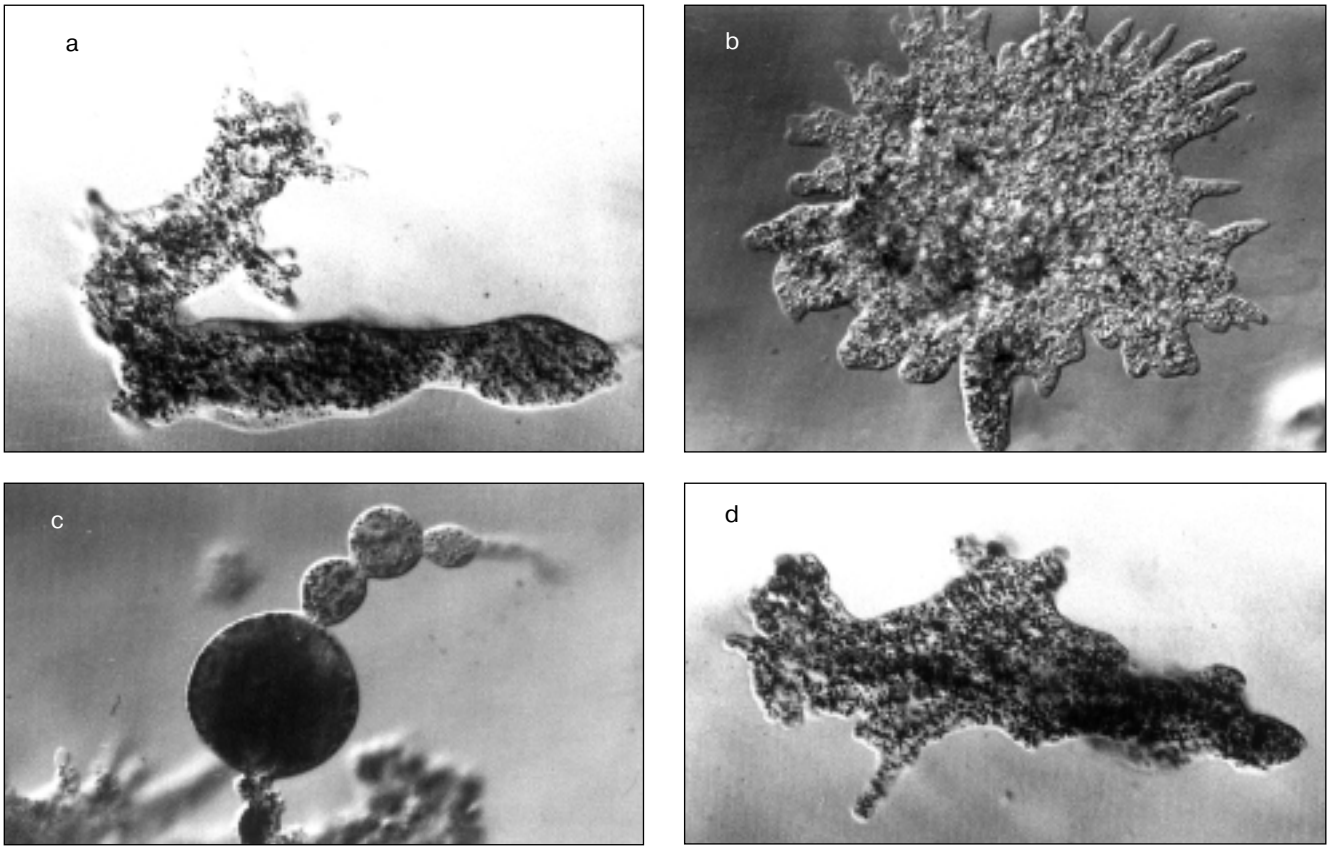

Fig. 5. *Amoeba proteus* cells: a) control; b, c) after injection of the ECP32 protease; c) after injection of inactivated ECP32 protease.

of large amounts of alien protein. A 100-fold increase in the amount of the injected protein at the expense of antibodies (or BSA) did not cause visible morphological or locomotor changes in amoebae. Fibrillar actin is rather stable to proteases [25]; its degradation by ECP32 is inconsiderable in comparison with G-actin [26]. However, at high enzyme/substrate ratios, F-actin may be cleaved by proteases such as ECP32 [27]. Upon injection into cells, local ECP32 concentrations in the site of injection may be very high. In addition, it should be taken into consideration that actin in lower eukaryotes has a specific monomer structure and polymerization properties [16, 23]. This is essential for protease action: proteolysis of F-actin by ECP32 increases upon its modification [27]; β-F-actin is less stable to degradation by subtilisin than α-F-actin from skeletal muscle [28, 29]. Thus, our data suggest that the disruption of the cortical layer in the microfilaments induced by injection of high ECP32 concentrations was due to degradation both of globular and fibrillar actin in amoebae.

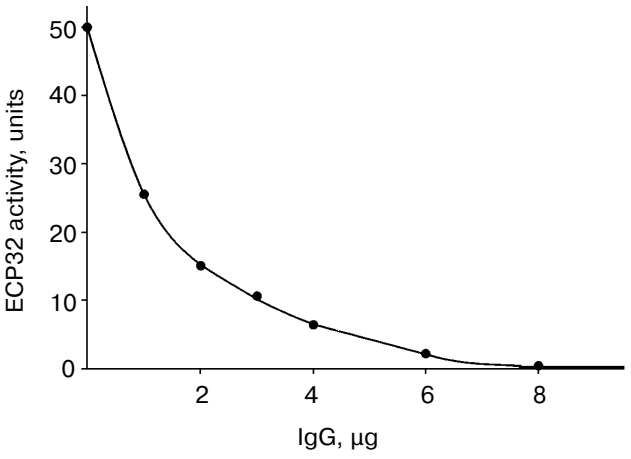

Fig. 6. IgG-concentration dependence of ECP32 activity. To 10 μl of ECP32 (50 activity units), different amounts of anti-ECP32 IgG in 80 μl of buffer TS were added, incubated for 1 h at 20°C, and then actin-degrading activity was assayed.

Since motility in amoebae is provided by the cytoskeleton, our data on the irregular locomotion induced by ECP32 prove that the protease can be used in studies of the cytoskeleton *in vivo*.

This work was supported in part by the Russian Foundation for Basic Research (grants No. 96-04-49659 and 99-04-49482).

REFERENCES

1. Tilney, L. G., Conelly, P. S., and Portnoy, D. A. (1990) *J. Cell Biol.*, **111**, 2979-2988.
2. Prevost, M. C., Lesourd, M., Aprin, M., Vernel, F., Mounier, J., Hellio, R., and Sansonetti, P. J. (1992) *Infection Immunity*, **10**, 4088-4099.
3. Lasa, I., and Cossart, P. (1996) *Cell Biology*, **6**, 109-114.
4. Higley, S., and Way, M. (1997) *Curr. Opin. Cell Biol.*, **9**, 62-69.
5. Oswald, E. M., Sugai, A., Labigne, A., Wu, A. C., Fiorentini, C., Boquet, P., and O'Brien, A. D. (1994) *Proc. Natl. Acad. Sci. USA*, **912**, 3814-3818.
6. Fiorentini, C., Arancia, G., Caprioli, A., Falbo, V., Rugger, F. M., and Donelli, G. (1988) *Toxicon*, **26**, 1047-1056.
7. DasGupta, B. R., and Tepp, W. (1993) *Biochem. Biophys. Res. Commun.*, **190**, 470-474.
8. Khaitlina, S., Smirnova, T., and Usmanova, A. (1988) *FEBS Lett.*, **228**, 172-174.
9. Usmanova, A. M., and Khaitlina, S. Yu. (1989) *Biokhimiya*, **54**, 1074-1079.
10. Khaitlina, S., Collins, J. H., Kuznetsova, I., Pershina, V., Synakewich, I., Turoverov, K., and Usmanova, A. (1991) *FEBS Lett.*, **279**, 49-51.
11. Khaitlina, S., Moraczewska, J., and Strzelecka-Golaszewska, H. (1993) *Eur. J. Biochem.*, **218**, 911-920.
12. Strzelecka-Golaszewska, H., Khaitlina, S., and Mossakowska, M. (1993) *Eur. J. Biochem.*, **211**, 731-742.
13. Kuznetsova, I., Antropova, O., Turoverov, K., and Khaitlina, S. (1996) *FEBS Lett.*, **383**, 105-108.
14. Matveyev, V. V., Usmanova, A. M., Morozova, A. V., Collins, J. H., and Khaitlina, S. Yu. (1996) *Biochim. Biophys. Acta*, **1296**, 56-62.
15. Stockem, W., and Klopochka, W. (1988) *Int. Rev. Cytol.*, **112**, 137-183.
16. Sonobe, S., Takahashi, S., Hatano, S., and Kuroda, K. (1986) *J. Biol. Chem.*, **261**, 14837-14843.
17. Spudich, J. A., and Watt, S. (1991) *J. Biol. Chem.*, **246**, 4866-4871.
18. Kodama, T., Fukui, K., and Kometani, K. (1986) *J. Biochem.*, **99**, 1465-1472.
19. Itzhaki, R. F., and Gill, D. M. (1964) *Anal. Biochem.*, **9**, 401-407.
20. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
21. Naryzhny, S. N. (1996) *Anal. Biochem.*, **238**, 50-53.
22. Harlow, E., and Lane, D. (1988) *Antibodies*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
23. Korn, E. D. (1982) *Physiol. Rev.*, **62**, 672-737.
24. Pollard, T. D., and Cooper, J. A. (1976) *Ann. Rev. Biochem.*, **55**, 987-1035.
25. Rich, S. A., and Estes, J. E. (1976) *J. Mol. Biol.*, **104**, 777-792.
26. Mantulenko, V. A., Khaitlina, S. Yu., and Shelud'ko, N. S. (1982) *Biokhimiya*, **48**, 61-66.
27. Strzelecka-Golaszewska, H., Mossakowska, M., Wozniak, A., Moraczewska, J., and Nakayama, H. (1995) *Biochem. J.*, **307**, 527-534.
28. Kim, E., Miller, C. J., and Keisler, E. (1996) *Biochemistry*, **35**, 16566-165762.
29. Khaitlina, S. Yu., Antropova, O. Yu., Kuznetsova, I. M., Turoverov, K. K., and Collins, J. H. (1999) *Arch. Biochem. Biophys.*, **368**, 105-111.