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Heat Shock Protein DnaK – Substrate of Actin-Specific Bacterial Protease ECP32

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Abstract—It has been found that actin-specific bacterial protease ECP32 cleaves prokaryotic heat shock protein DnaK, which belongs to the family of heat shock proteins with molecular weight 70 kDa. We propose a new one-step method for DnaK purification using heat treatment. The technique yields ~1 mg of partially purified DnaK from 25 g of wet bacterial biomass. Polyclonal antibodies against DnaK were obtained. The degree of ECP32 catalyzed proteolysis of partially purified DnaK and that of DnaK in initial cell extracts was compared.

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Key words: protease ECP32, DnaK, protein purification, limited proteolysis

The method of limited proteolysis is an essential tool in protein structural–functional studies. The approach allows the study of proteins both after purification and in their native environment and conformation [1, 2].

ECP32 is an intracellular neutral metalloproteinase (EC 3.4.24) with molecular weight 32 kDa. It is a highly specific enzyme, and among the wide range of studied substrate proteins it selectively cleaves actin. The protease is accumulated during post-logarithmic stage of *Escherichia coli* A2 original bacterial strain, which recently was identified as *Serratia grimesii* A2 after additional taxonomic studies [3-6]. ECP32 cleaves a single peptide bond in actin between residues Gly42–Val43, and actin modified by proteolysis is commonly used as a unique research model *in vivo* and *in vitro* [7-14]. Except for actin, among several dozens of analyzed proteins the protease also cleaves only melittin and histones. The analysis of substrate specificity indicated that ECP32 hydrolyzes mainly peptide bonds with the carbonyl group belonging to a hydrophobic amino acid residue [5]. But not all peptide bonds formed by hydrophobic amino acids are hydrolyzed by this enzyme. This confirms that not only primary but perhaps secondary and tertiary protein-substrate structure plays a key part in determining the specificity of this protease.

The 70 kDa heat shock proteins contain a highly conservative ATPase domain with tertiary structure similar to the tertiary structure of actin [15, 16]. It has been found that eukaryotic heat shock protein HSC70 with molecular weight about 70 kDa is not cleaved by ECP32 [5]. However, a member of the same family of heat shock proteins with molecular weight 70 kDa, prokaryotic DnaK, was not analyzed as a substrate of ECP32. The search for bacterial ECP32 substrates is important since bacterial actin-like proteins MreB and ParM are much different from actin concerning the primary structure and they do not contain the region where ECP32 hydrolyzes actin [17, 18]. For this reason actin-like proteins cannot be endogenous substrates of ECP32. Starting our study we realized that except for easily available DnaK from *E. coli*, it is essential to study DnaK isolated directly from ECP32 strain-producer as substrate protein since, besides carrying protease, this strain differs from laboratory *E. coli* strains in several culturing characteristics [19].

A unique and rapid way of DnaK isolation was developed during the presented research. In addition, it was found that DnaK of the producer strain is cleaved by ECP32 both in extract and in purified form.

MATERIALS AND METHODS

Chemical reagents were obtained from Sigma and Difco (USA), Serva and Merck (Germany), and Fluka (Switzerland). Carriers for column chromatography were from LKB-Pharmacia (Sweden) and Whatman (USA),

Abbreviations: MWCO, molecular weight cut-off.

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and membranes for filtration and blotting were from Amicon (USA) and Sartorius (Germany).

Microorganism cultures. The following strains were used in this study: strain A2 producing ECP32 (registration number VKPM 3697), *E. coli* strains C600, AB1157, 803-8, XL1blue; *Bacillus subtilis* strain 2054.86 (museum of the Institute of Cytology, Russian Academy of Sciences, St. Petersburg). Bacterial culture was cultivated in 750-ml Erlenmeyer flasks on a rotor shaker at 200 rpm at 35°C in 2LBS medium with the following composition: peptone, 20 g/liter; yeast extract, 10 ml/liter; NaCl, 10 g/liter, pH 7.0. The cells were cultivated in 250 ml of liquid medium or in a two-phase system consisting of 200 ml of agarized (3% agar) and 50 ml of liquid 2LBS [19].

Mycoplasma cultures were kindly furnished by M. S. Vonsky (Institute of Cytology). Other microorganisms used in the study were grown on beef-extract agar; they were obtained in nonviable condition from the collection of the Western Interdistrict Center of Sanitary and Epidemiological Surveillance in St. Petersburg. The authors thank L. A. Borisova for provided samples.

Heat shock. The procedure was performed in flasks with medium inoculated with culture at the logarithmic phase of growth, which were incubated for 4 h at 28-37°C. Then the temperature was rapidly increased and the flasks were moved to a water bath at 43°C. Simultaneously 1/5 volume of nutrient medium thermostatted at 60°C was aseptically added. The bacterial culture was incubated for 30 min at 43°C and then for 1-2 h at 28-37°C.

For isolating extracts of gram-positive bacteria and yeast, biomass was incubated with 2 g/liter of lysozyme for 40 min at 37°C in medium with 25 mM Tris, 10 mM EDTA, pH 8.0.

Purification of ECP32 protease. The protease was purified according to the protocol described previously [20]. In 20-30 h after the beginning of the logarithmic growth phase, producer cells were precipitated by centrifugation and then sonicated in the presence of ammonium sulfate at the concentration equal to 30% saturation at 0°C. The extract obtained was centrifuged and proteins of a supernatant were precipitated with ammonium sulfate (90% saturation). Then ECP32 was purified by column chromatography on hydrophobic sorbent phenyl-Sepharose CL-4B and on anion exchanger Q-Sepharose Fast Flow. The resulting sample was ultrafiltered in cells with membranes with MWCO 30 and 100 kDa. All manipulations were conducted in buffer T prepared based on Tris (20 mM Tris-HCl, pH 7.5).

Proteolytic activity of the samples was measured according to their ability to hydrolyze G-actin. An equal volume of protease sample (total protein final concentration not more than 1 μg/ml) was added to G-actin solution (1 mg/ml) [21], and the mixture was incubated for 1 h at 20°C and then analyzed using electrophoresis in 12.5% polyacrylamide gel with SDS. Enzymatic activity was determined from the correlation between proteolysis product (36 kDa) absorption bands and intact actin, both stained with Coomassie brilliant blue G-250. The amount of protease hydrolyzing 100 μg of G-actin in 1 h at 20°C was considered as 1 unit of activity. For DnaK proteolysis 1 unit of ECP32 was added to a sample containing 10-1000 μg of total protein, and the mixture was incubated for 1 h at 20°C.

DnaK purification. The strain-producer of ECP32 was cultivated for 20 h. The bacterial precipitate was washed in 10 volumes of buffer TE (buffer T with 1 mM EDTA) after centrifuging, centrifuged again for 30 min at 5000*g*, weighed, suspended in buffer TE to the concentration of 200 g/liter, and then cells were broken in an ice-bath using an UZDN-1 U 4.2 ultrasound disintegrator at 22 kHz during 15 sec cycles (with pauses for cooling). The resulting extract was clarified by centrifugation for 1 h at 17,500*g*. The number of disruption cycles was selected so that protein concentration in the supernatant was about 10 mg/ml.

Clarified extract (2-2.5 g protein) was applied on a column (75 ml) of DEAE-cellulose equilibrated with buffer TE. The column was washed with 200-250 ml of buffer TE and eluted by a linear gradient from 0 to 0.7 M NaCl in buffer TE at flow rate 0.75 ml/min. Fractions containing DnaK were dialyzed against buffer TE. For concentrating, the fractions were applied on a small (2.5 ml) column of DEAE-cellulose, and the protein was eluted by 1 M NaCl (5 ml) prepared in buffer TE. Collected samples were dialyzed against TE buffer and stored at −20°C.

Aliquots were thawed and warmed in a boiling water bath for 10-20 min under different acid-saline conditions while total protein concentration was about 10 mg/ml: 1) 0.8 M $(NH_4)_2SO_4$, 60 mM NaCl in buffer T; 2) 0.1-1.6 M NaCl in buffer T; 3) 0.5 M NaCl in 20 mM Tris-HCl, pH 8.7. The samples were clarified by centrifuging for 30 min at 17,500*g*. The supernatant was collected, concentrated, and transferred into buffer T on a cell with a MWCO 50 kDa membrane. DnaK was isolated similarly from *E. coli* and *B. subtilis*.

Polyclonal antibodies against DnaK were collected according to the protocol described previously [9]. A DnaK sample isolated from *E. coli* A2 and additionally purified by preparative electrophoresis in polyacrylamide gel in the presence of SDS was used for immunization.

Polyclonal antibodies against HSP72 and HSC70, monoclonal antibodies against HSC70, and samples of purified HSP72 and HSC70 were kindly furnished by A. V. Kinev and A. P. Voronin (Institute of Cytology).

Electrophoresis in polyacrylamide gel in the presence of SDS was conducted according to the Laemmli method [22] with slight modifications [23]. The gels were fixed in methanol–acetic acid–water (50 : 10 : 40 v/v) and stained with Coomassie brilliant blue G-250 (Serva). Densitometry was conducted using an Ultrascan laser

densitometer (LKB). Standard protein Low Molecular Weight Calibration Kit (Pharmacia) containing phosphorylase B (94.0 kDa), albumin (67.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (30.0 kDa), trypsin soybean inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) was used as a marker of molecular weights.

Western blot was conducted according to a standard protocol [24] with slight modifications. IgG conjugated to peroxidase was used as the second antibodies. The reaction was visualized using 3,3′-diaminobenzidine tetrachloride.

Protein concentration in the samples obtained after chromatography was measured by microbiuret assay [25].

DnaK concentration was determined using a semiquantitative method. The aliquots from different fractions collected by chromatography were applied into gel wells. The total protein quantity for each well was constant. Different amounts of BSA were applied to the same gel. After SDS-PAGE, the gels were stained with Coomassie G-250 and densitometry was conducted. The correlation between BSA band intensity and amount of protein applied to the gel was determined; this standard curve was used for semiquantitative analysis of DnaK concentration in each analyzed sample.

To determine N-terminal amino acid sequence, proteins were put into PVDF membrane after electrophoretic separation in polyacrylamide gel in the presence of SDS. Sequence analysis was performed using an Applied Biosystems model 477A device (USA) equipped with amino acid analyzer model 120 PTH by standard protocols of producer.

RESULTS

Cleavage of heat shock protein DnaK by ECP32 protease in extract of strain-producer cells. Electrophoretic analysis of proteins from ECP32 strain-producer culture intracellular extract at the logarithmic stage revealed a peptide with molecular weight of about 70 kDa. The intensity of the band corresponding to this protein diminished after treatment of the extract with ECP32 (Fig. 1, lanes *1* and *1′*). Heat treatment of the extract (100°C at pH 7.5 in the presence of 0.2-0.3 M NaCl) was accompanied by denaturation and aggregation of many proteins. Some proteins possessed high stability and remained in the supernatant after heat treatment (Fig. 1, lane *2*). This protein fraction was rich with protein with apparent molecular weight of 70 kDa. However, incubation with ECP32 led to significant decrease in intensity of the band with apparent molecular weight of 70 kDa (Fig. 1, lane *2′*).

The analysis of N-terminal residues of the protein with apparent molecular weight of 70 kDa revealed XKIIGIXLGTTN, which is fully identical to the N-terminal peptide of bacterial heat shock protein DnaK.

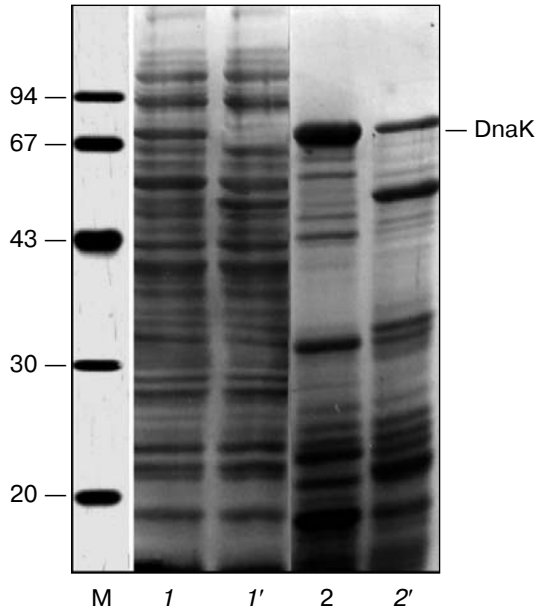

Fig. 1. Cleavage of heat shock protein DnaK by ECP32 in extract of strain-producer bacteria. Electrophoregram in 12.5% SDS-polyacrylamide gel. Lanes: M, marker proteins (their molecular weights are indicated in kDa to the left); *1*) intracellular extract of ECP32 producer after purification on DEAE-cellulose, the fraction contains of 0.2-0.3 M NaCl; *2*) the same sample after heating (for 10 min at 100°C) and centrifuging; *1′*, *2′*) the same samples after ECP32 treatment, respectively.

Additional evidence for the indicated protein actually being heat shock protein DnaK was the fact that the protein was recognized by antibodies against eukaryotic heat shock proteins HSP72 and HSC70. It should to be noted that heat treatment of ECP32 producer and *E. coli* strains AB1157, 803-8, XL1blue according to the scheme 28→43→28°C and 28→43→37°C increased output of a polypeptide with molecular weight about 70 kDa by 3-4-fold, which corresponds to literature data concerning DnaK yield.

Isolation of DnaK. A simple and rapid method for isolation of partially purified DnaK including two stages was developed – chromatography on DEAE-cellulose and heat treatment at 100°C.

During chromatography of proteins from intracellular extract of ECP32 producer culture taken at the logarithmic stage at pH 7.5 on DEAE-cellulose, the main part of DnaK was eluted from the column in 0.2-0.3 M NaCl (Fig. 2, curve *2*), but the resulting sample was heterogeneous. For further purification we used the high thermal stability of DnaK. We selected optimal conditions for heat treatment, when DnaK remained in the solution and the majority of contaminating proteins denatured and aggregated.

Heat treatment at acidic pH (about 5.5) in the presence of 0.8 M $(NH_4)_2SO_4$ was accompanied by DnaK denaturation and aggregation together with the majority of extract proteins, which corresponds to the literature

about the DnaK molecule unfolding at low pH [26]. When heat treatment was performed at alkaline pH, a significant part of the DnaK remained in the solution and the optimal results were obtained while heat treatment was conducted in 0.5-1.0 M NaCl at pH 7.5.

In our developed method for isolation the yield of partially purified DnaK was ~1 mg from 1 g of initial intracellular extract protein or from 25 g of wet bacterial biomass. The amount of DnaK in the final sample was estimated as 10-15% of the intensity of all protein bands stained with Coomassie G-250 (Fig. 3, lane *1*).

Detection of DnaK in different microorganisms. Monospecific polyclonal antibodies against DnaK of ECP32 bacterial strain-producer were collected. The antibodies possessed high specificity and selectively stained only one zone with apparent molecular weight 70 kDa in extracts isolated from different bacteria.

The antibodies that we produced stained DnaK of gram-negative bacteria *E. coli* C600, AB1157, 803-8, *Serratia marcescens*, *Yersinia enterocolitica*, *Proteus rettgeri*, *Klebsiella pneumoniae*, *Citrobacter freundii*, and *Aeromonas dispar* and weakly stained DnaK of gram-positive sporogenous rods *B. subtilis*. The antibodies did not recognize a band with apparent molecular weight 70 kDa in extracts of gram-positive cocci *Staphylococcus aureus* and *Staph. epidermidis* and yeasts *Candida albicans* and *Saccharomyces cerevisiae* as well as in mycoplasmas (*Mycoplasma gallisepticum*, *M. pneumoniae*, *M. capricolum*, *M. fermentans*, *Acholeplasma laidlawii*, *Spiroplasma citri*).

Cleavage of DnaK by ECP32. It was found that ECP32 hydrolyzes DnaK in all listed strains of *E. coli* and *B. subtilis*. However, DnaK cleavage in coarse extracts was accompanied by accumulation of one proteolysis product with molecular weight 46 ± 2 kDa. We revealed several additional rapidly degrading fragments formed during limited proteolysis of DnaK when partially purified DnaK sample isolated through heat treatment was hydrolyzed (Fig. 4a). In further experiments it was found that eukaryotic analogs of DnaK, proteins HSC70 and HSP72, were stable to ECP32 and did not undergo proteolysis.

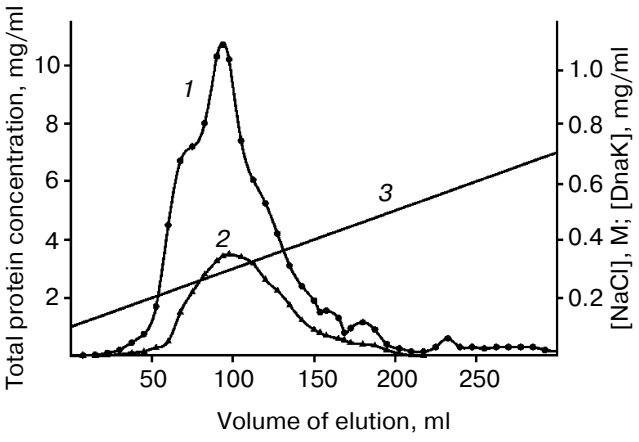

Fig. 2. Chromatography of DnaK on DEAE-cellulose: *1*) total protein concentration; *2*) DnaK concentration (estimated); *3*) NaCl concentration.

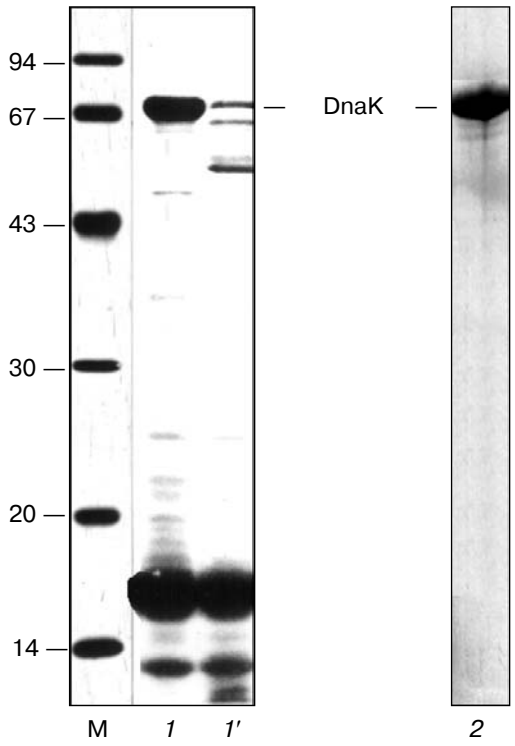

Fig. 3. Polypeptide content of DnaK samples. Electrophoregram in 12.5% SDS-polyacrylamide gel. Lanes: M, marker proteins (their molecular weights are indicated in kDa to the left); *1*) purified DnaK sample after chromatography on DEAE-cellulose and heating in 0.5 M NaCl (10 min, 100°C); *1′*) the same sample after treatment with ECP32; *2*) DnaK sample after preparative SDS-PAGE used for preparation of polyclonal antibodies.

DISCUSSION

More than 100 different methods of purification of heat shock proteins with molecular weight about 70 kDa have been described [27]. Typically these methods are rather laborious and complicated. We developed a new unique method for isolation of partially purified DnaK that involves two steps of purification – chromatography on DEAE-cellulose and heat treatment. The method yields ~1 mg of DnaK from 25 g of wet bacterial biomass.

Until recently there have been no methods for purification of DnaK based on its thermostability described in literature. While this publication was being prepared, a study where *E. coli* extract was heated in phosphate buffer appeared [28]. Unfortunately, the use of heat treatment for DnaK separation from other extract proteins is not

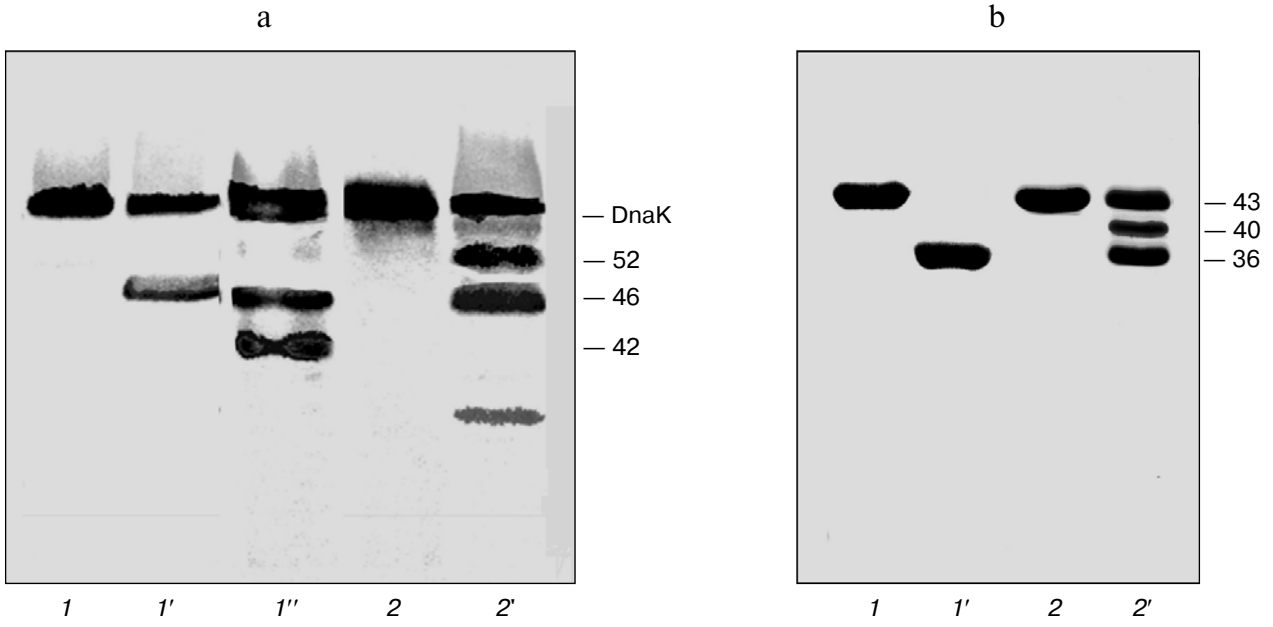

Fig. 4. Immunoblotting of proteolysis products of heat shock protein DnaK hydrolyzed by ECP32 (a) and electrophoregram of products of actin proteolysis by ECP32 (b). a) Lanes: *1*) total intracellular extract of ECP32 producer logarithmic culture; *1′*, *1″*) the same extract after ECP32 treatment and after ECP32 treatment with subsequent heating (100°C, 10 min, 0.5 M NaCl), respectively; *2*) partially purified DnaK sample obtained from ECP32 strain-producer logarithmic culture isolated by chromatography on DEAE-cellulose with subsequent heating (100°C, 10 min, 0.5 M NaCl); *2′*) the same sample after treatment with ECP32. b) Lanes: *1*) intact G-actin; *2*) G-actin inactivated by heating (70°C, 5 min); *1′*, *2′*) the same samples after treatment with ECP32, respectively. To separate proteins, 12.5% SDS-polyacrylamide gel was used. Apparent molecular weights of polypeptides are indicated in kDa to the right.

made possible from the data of this study. Varying salts, pH, and concentration, we managed to select heating conditions when a significant part of DnaK remains in the solution. In addition, the highest DnaK yield was obtained during heat treatment at pH 7.5 in the presence of 0.5 M NaCl.

Using our developed method, we isolated DnaK samples from bacteria of ECP32 strain-producer. Purified DnaK was used for preparing polyclonal antibodies possessing high specificity and able to specifically react with DnaK of gram-negative bacteria and *B. subtilis* during immunoblotting. Using these antibodies, we failed to discover DnaK and fragments of its proteolysis in ECP32 producer stationary culture. It should be specially noted that ECP32 is accumulated during the post-logarithmic stage whereas DnaK, on the contrary, is found only in the logarithmic stage, which argues for the fact that DnaK can act as an endogenous substrate of ECP32 *in vivo*. Experimental data according to which ECP32 is able to hydrolyze DnaK in extracts of strain-producer cells as well as in several strains of *E. coli* and *B. subtilis* are in agreement with this assumption. It is known that the concentration of DnaK co-chaperone, CbpA, is increased 30-fold up to values that are equivalent to DnaK concentration at *E. coli* post-logarithmic stage [29]. But there are no reports about such dramatic changes in concentration of DnaK in enterobacteria.

Intact DnaK was cleaved by ECP32 after chromatography on DEAE-cellulose of cell extract forming a stable fragment with apparent molecular weight 46 ± 2 kDa (Fig. 4a, lane *1′*). It is possible to estimate that formation of this product is determined by breaking of an extremely flexible linker region connecting ATPase (44 kDa) and substrate-binding DnaK domains (25 kDa) [30, 31]. During subsequent heating of incubation medium obtained after proteolysis using ECP32, an additional proteolysis product with apparent molecular weight 42 ± 2 kDa appeared at the electrophoregram (Fig. 4a, lane *1″*). It conforms with previously collected data indicating that during the first minutes of reaction mixture heating, ECP32 is rapidly activated, which leads to the rapid degradation of substrates inactivated by heating and even to autoproteolysis of the enzyme [20].

Partially purified DnaK samples collected using heat treatment were cleaved by ECP32 forming a fragment with molecular weight 46 ± 2 kDa and an intermediate fragment with molecular weight 52 ± 2 kDa (Fig. 4a, lane *2′*). Comparable results were collected during proteolysis of a protein from the heat shock family with molecular weight 70 kDa by chymotrypsin [32]. In this case an intermediate fragment with molecular weight about 60 kDa, which was the product of proteolysis in the substrate-binding domain, was formed during proteolysis at first. Then fragments forming after linker hydrolysis

between the ATPase and substrate-binding domains appeared. Perhaps, a 52-kDa DnaK fragment forming during hydrolysis by ECP32 is also the product of cleavage in the heat shock protein substrate-binding domain.

The comparison of DnaK and actin proteolysis processes under the action of ECP32 is of interest (Fig. 4, a and b). In the case of actin, thermal treatment of the substrate leads to denaturation and aggregation of the protein. Probably this is the reason why heated and aggregated actin was less susceptible to ECP32 and after hydrolysis two fragments possessing almost similar intensity and molecular weights 36 and 40 kDa were formed (Fig. 4b, lanes *2* and *2′*). In the case of intact (unheated) actin ECP32 hydrolysis leads to selective and rapid cleavage of only one peptide bond and to the accumulation of one stable product with apparent molecular weight 36 kDa (Fig. 4b, lanes *1* and *1′*). Different results were obtained during hydrolysis of DnaK. In this case the substrate has high thermal stability and therefore heat treatment does not result in protein substrate aggregation. Partial protein denaturation observed during heat treatment leads to an increase in DnaK sensibility to proteolysis, and consequently several different protein fragments are detected among proteolysis products (compare wells *1′* and *1″* or wells *1′* and *2′* on Fig. 4a).

Actin and heat shock proteins from the family with molecular weight 70 kDa have various primary structures and serve absolutely different functions in the cell. Nevertheless, tertiary structure of actin and ATP-binding domains of heat shock proteins with molecular weight 70 kDa share significant similarities [31, 32]. Noted similarities in tertiary structure are specific not only for DnaK and actin but also for hexokinase (and probably for other sugar kinases) and prokaryotic proteins FtsA, MreB, StbA [15, 16, 33]. But DnaK structure lacks the region homological to the region cleaved by ECP32 in actin. Possibly, this circumstance explains the fact that ECP32 is not able to hydrolyze the ATPase domain of DnaK. The ability of ECP32 to hydrolyze the linker region and substrate-binding domain of DnaK suggests that ECP32 can not only be a useful tool for determining the structure and properties of these proteins, but also can be applied in biotechnology.

Heat shock proteins play an important part in the industry of recombinant protein production using bacteria. Abnormally high concentration of exogenously introduced proteins in the cell results in its misfolding and incorrect aggregation. The producer uses chaperone systems including DnaK as a defense mechanism. For this reason it is often desirable to increase chaperone concentration for obtaining soluble and correctly formed target product. Strains-superproducers both in target protein and in heat shock proteins are often used to that end [34, 35]. There are also two-step systems for sequential target protein and heat shock proteins synthesis [36]. On the other side, heat shock proteins synthesized even in usual (not increased) amounts can contaminate the target product or complicate its full purification. For this reason producing strains deficient in DnaK (or in other heat shock proteins) are sometimes used [37, 38]. Recombinant systems in which the synthesis is induced by temperature are particularly complicated cases [39]. Considering the fact that ECP32 has high substrate specificity, this protease can be used for purifying target proteins from certain heat shock proteins with molecular weight 70 kDa contaminating them.

Our studies suggest that heat shock protein DnaK may be a native substrate of ECP32. In addition, ECP32 can be applied for studying prokaryotic heat shock protein structure and properties as a highly specific tool that has proved itself during investigations of actin.

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