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Isolation, Purification, and Study of Properties of Recombinant Hepsin from *Escherichia coli*

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Abstract—A recombinant hepsin-producing strain of *Escherichia coli* was obtained and the conditions for hepsin expression in a bacterial system were optimized. To study the physicochemical properties of the enzyme, a procedure for purification of active recombinant hepsin using metal-chelate affinity chromatography and ion-exchange chromatography was developed. The interaction of recombinant hepsin with various peptide substrates is characterized. The dose-dependent inhibition of the recombinant hepsin enzyme activity by anthralin *in vitro* and an increase in the hepsin enzymatic activity in the presence of resveratrol were revealed.

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Key words: hepsin, proteolytic activity, anthralin, inhibition, cytotoxicity, human prostate adenocarcinoma

In recent decades, considerable attention has been given to research on the family of membrane-associated proteolytic enzymes. The importance of investigations of transmembrane proteases stems from their participation in many biochemical reactions and pathological processes resulting from activity of these enzymes. Studies of this group of proteins are associated with considerable complexity in the recombinant expression and subsequent purification of integral proteins. These difficulties are associated with the toxicity of the protein product in the process of heterologous expression, with the complex procedure for obtaining the native conformation and active form of the enzyme, and with the low yield of the product. In this connection, obtaining of recombinant membrane-associated proteases is a priority in the investigation of their physicochemical, structural, and functional properties.

Hepsin belongs to the family of transmembrane serine proteases [1]. The molecule of full-length human hepsin consists of 417 amino acid residues (a.a.), and the molecular mass of the protein is about 45 kDa [2]. There are the transmembrane, protease, and SRCR (scavenger cysteine-rich receptor domain) domains in the molecule with a small cytoplasmic region at the N-terminus of the protein. The protease domain consisting of 255 a.a. shows significant homology with the protease domains of other members of the chymotrypsin family [3]. The active site of hepsin contains the amino acid triad H203-D257-S353 characteristic of this family of proteases [3]. Hepsin is synthesized as a zymogen within the cell and requires autocatalytic or proteolytic cleavage of the R162-I163 peptide bond for manifestation of enzymatic activity [2, 4]. Hepsin localization at the cell surface is apparently limited to the tight junctions [5].

The substrate specificity of hepsin has been studied in detail during the screening of peptide libraries. The preferred hepsin substrates are peptides with R in position P1; T or N in position P2; K, R, or H in position P3; and K, R, or P in position P4 [6]. The substrate specificity of hepsin is unique and differs from that of other human serine proteases, such as thrombin, plasmin, triptases β1 and β2, matriptase, prostasin, and prostate-specific antigen (PSA) [7, 8].

Normally, the expression of the transmembrane form of hepsin takes place at the cell surface of only a few organs including liver, kidneys, and, to a lesser extent, thyroid and lungs [4, 9]. It is assumed that hepsin is normally involved in cell growth, maintaining of cell mor-

Abbreviations: HAI-1 (2), hepatocyte growth factor activator inhibitor type I (II); IPTG, isopropyl-1-thio-β-*D*-galactopyranoside; P1, P2, P3, and P4 are the specific cleavage site residues enumerated from the C- to N-terminus of the peptide; PSA, prostate-specific antigen; SRCR-domain, scavenger cysteine-rich receptor domain.

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phology, and regulation of the blood coagulation system [10]. However, *in vivo* experiments failed to clearly reveal the physiological role of hepsin. All parameters of hepsin-knockout mice that could by affected by the absence of the enzyme remained normal [11]. At the same time, numerous evidences of the important role of hepsin in the development of certain cancers, such as prostate adenocarcinoma and renal cell carcinoma, have been reported to date [12-16]. Investigations of gene expression profiles in tumor prostate tissue showed that the hepsin gene is one of the most stable overexpressed genes, *viz.*, a more than tenfold increase in hepsin mRNA level was typical of 90% of prostate tumors [17, 18]. It is assumed that hepsin is involved in activation of proteolytic processes in tumor tissue leading to tumor growth and spread of metastases. Apparently, the inhibition of proteolytic activity of hepsin can lead to inhibition of tumor process and thus is of great importance for the treatment of the tumor types related to hepsin activity [19].

Since experimental data indicate an important role of proteolytic activity of hepsin in the development of tumors, the search for specific synthetic inhibitors of hepsin activity is currently in progress. One of the most effective inhibitors of hepsin activity is anthralin (1,8,9-anthracenetriol) [20].

In connection with this, it is important to obtain strains producing recombinant hepsin, to develop methods for hepsin isolation, and further study its functional properties. In this paper, we showed the effect of anthralin on the proteolytic activity of recombinant hepsin. Another agent investigated, resveratrol, which exhibits antioxidant and antitumor properties [21], showed no inhibitory properties against hepsin.

MATERIALS AND METHODS

Cloning. cDNA of the hepsin molecule without the transmembrane domain (51-417 a.a) was obtained by RT-PCR from total RNA of LnCap cells using two primers, 5′-ATATACCATGGGCCCGCTGTACCCAG-TGCAGGTC and 5′-GCCGCAAGCTTGAGCTGGG-TCACCATGCCG. For the subsequent activation of the protein, a thrombin cleavage site was introduced into the vector containing the sense sequence. To optimize purification of the protein, a fragment containing the sequence of six histidine residues was introduced into the resulting vector. The resulting DNA fragment was split with the *Nco*I and *Hind*III endonucleases and cloned into the pET28a vector at the *Nco*I and *Hind*III sites, thus resulting in a pET28aHPNc plasmid (Fig. 1).

Expression of recombinant protein in *E. coli*. Transformation of *E. coli* cells with plasmid DNA was performed using a conventional procedure [22]. Transformed cells were grown in Luria–Bertani (LB) broth (Amersham, USA) containing kanamycin (Fluka, USA) at 37°C. Protein synthesis was induced by adding isopropyl-1-thio-β-*D*-galactopyranoside (IPTG) (Helicon, Russia) to final concentration of 0.5 mM (when the optical density of the culture at 600 nm reaches 0.6). To increase the content of the soluble form of the protein, incubation of the IPTG-containing culture was carried out over a period of 12 h at 25°C, and then the cells were harvested and lysed. Lysis buffer (pH 8.3) containing 50 mM NaH_2PO_4, 150 mM NaCl, and 5 mM imidazole was used. The ratio of the lysis buffer volume and the wet weight of the cell pellet was 20 ml buffer per gram of pellet. Then the cells were destroyed by sonication, the cell lysate was centrifuged at 4000*g* for 30 min, and the supernatant was filtered through a cellulose filter (Osmonics, USA) with a pore diameter of 45 μm.

Isolation of the recombinant protein. Chromatographic separation of proteins was performed using the Econo system (Bio-Rad, USA). Affinity chromatography was performed using a Ni-IDA-Sepharose column (GE Healthcare, USA) (column volume 8 ml). The protein was eluted using buffer (pH 8.3) containing 50 mM NaH_2PO_4, 150 mM NaCl, and 20 mM imidazole. Fractions corresponding to the optical density peak during elution were combined.

To activate the resulting zymogen, thrombin (Sigma, USA) was added to the combined fractions while maintaining the ratio 0.2 U of thrombin per 1 mg of total protein, and the mixture was incubated for 14-16 h at 25°C. Then the protein solution was dialyzed against a 100-fold volume of 10 mM Tris-HCl solution (pH 8.3) for 24 h at 4°C.

Ion-exchange chromatography was performed using a Q-Sepharose Fast Flow column (Amersham, USA) (column volume 10 ml). Hepsin was eluted in a NaCl gradient (final salt concentration of 0.25 M). The purified product was analyzed by electrophoresis in 15% SDS-polyacrylamide gel as described by Laemmli [23]. Fractions containing the target protein were combined

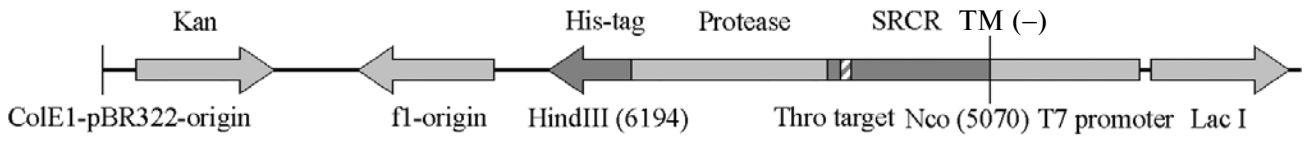

Fig. 1. Expression plasmid pET28aHPNc map. Kan denotes resistance to kanamycin; Thro target is the thrombin cleavage site; Protease is the protease domain; SRCR is the SRCR-domain; TM(–), absence of transmembrane domain.

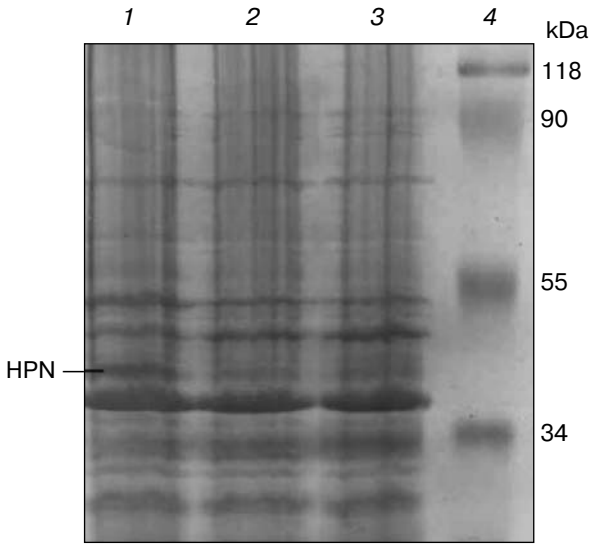

Fig. 2. Electrophoretic analysis of production of the soluble form of recombinant hepsin in different *E. coli* strains transformed with plasmid pET28aHPNc, 12 h after induction of protein synthesis. Lanes: *1-3*) lysed cells of Rosetta pLysS, BL21(DE3), and BL21(DE3) Codon Plus RIL strains, respectively; *4*) molecular mass markers.

and concentrated in an ultrafiltration cell (Amicon 8003 cell; Millipore, USA) on a PES-membrane with a cut-off limit of 30 kDa (Millipore). The resulting protein was stored in 50% glycerol at –20°C.

Determination of proteolytic activity of hepsin and other enzymes. The activity of the protein obtained and other proteases was determined spectrophotometrically. The buffer for incubation with a substrate contained 0.3 M Tris-HCl, 0.3 M imidazole, and 0.5 M NaCl, pH 8.4. A chromogenic substrate (0.19 μmol) was added to each sample. The proteases were incubated with the substrate at 37°C. The optical density in wells was measured at 415 nm at 10 min intervals. When studying the protein inhibition, its activity was determined in the presence of different concentrations of the tested inhibitor. The chromogenic substrates used were KPR (H-D-K (γ-Cbo)-P-R-pNA; American Diagnostica, USA), AAR (H-D-A-A-R-pNA; synthesized at the Chemistry Department, St. Petersburg State University, St. Petersburg, Russia), and thrombin and chymotrypsin (Sigma).

To determine the Michaelis–Menten constants of the proteins, the enzyme reaction rates were measured at different substrate concentrations. The K_m values were calculated from reaction rate versus substrate concentration plots drawn in double reciprocal coordinates.

Protein concentration assay. Protein concentration was determined spectrophotometrically (the extinction coefficient of hepsin calculated using the ProtParam software was 65,400 $M^{-1} \cdot cm^{-1}$) and according to Bradford [24].

RESULTS

Optimization of conditions for expression of recombinant hepsin in *E. coli*. First, we compared the levels of recombinant hepsin expression in the periplasmic space of different *E. coli* strains (BL21(DE3), BL21(DE3) Codon Plus RIL, and Rosetta pLysS). The synthesis of the recombinant protein in cells of all the lines was

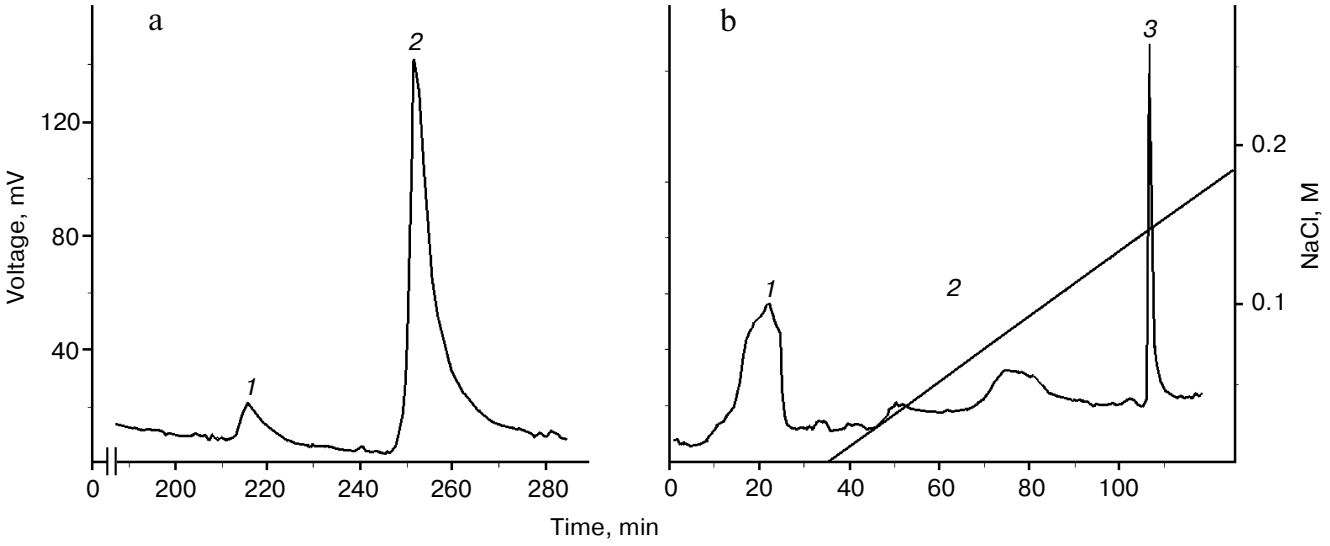

Fig. 3. a) Affinity chromatography of cell lysates: *1*) contaminating proteins; *2*) hepsin. b) Ion-exchange chromatography of recombinant hepsin preparation obtained after affinity chromatography: *1*) proteins not bound to the resin; *2*) contaminating proteins; *3*) hepsin.

induced by adding IPTG to concentration of 0.8 mM. Expression of the recombinant protein was analyzed by electrophoresis in 15% polyacrylamide gel in the presence of SDS. The visible band of expressed hepsin corresponded to a molecular mass of about 43 kDa (*cf.* 42.038 kDa according to ProtParam calculations). As shown in Fig. 2, the highest level of hepsin expression was observed in the Rosetta pLysS strain.

To determine the optimum content of IPTG, the synthesis of the protein was induced in Rosetta pLysS strain cells using different amounts of IPTG. As the IPTG concentration increased from 0 to 0.5 mM, the protein content in cell lysate increased. Further increase in the IPTG concentration led to a decrease in the protein content (data not shown). Subsequent experiments were carried out at the IPTG concentration equal to 0.5 mM.

Isolation, purification, and activation of recombinant hepsin. Recombinant hepsin was purified from supernatant of bacterial cell lysate by affinity chromatography and ion-exchange chromatography. To purify the protein by metal chelate chromatography using a Ni-containing affinity resin, a sequence of six His residues was attached to the C-terminus of the hepsin molecule (Fig. 3).

The resulting hepsin preparation required further purification (Fig. 4). Also, the protein obtained at this stage needed activation by thrombin treatment. The hepsin preparation was purified from thrombin at an intermediate stage; this allowed us to reduce the loss of the target product. The second stage of recombinant hepsin purification involved ion-exchange chromatography using Q-Sepharose (the elution profile is shown in Fig. 3). At this stage of purification, thrombin was separated due to the isoelectric point difference between the proteins (pI = 7.27 for hepsin and 4.25 for thrombin). Thus, the two-stage purification afforded a hepsin preparation with purity of more than 90%.

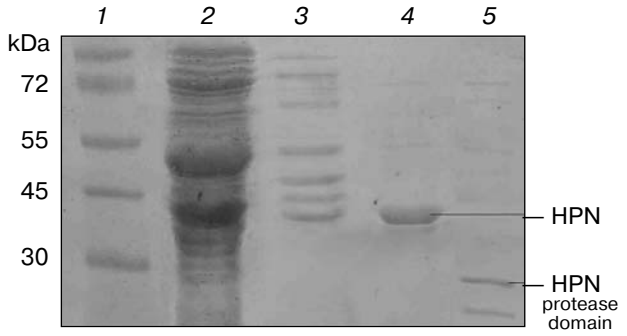

Fig. 4. Electrophoretic analysis of hepsin preparations at different stages of purification. Lanes: *1*) molecular mass markers; *2*) cell lysate; *3*) partially purified protein preparation obtained after affinity chromatography; *4*) purified inactive hepsin preparation obtained after ion-exchange chromatography and concentration of the protein; *5*) purified, thrombin-activated hepsin preparation obtained after ion-exchange chromatography and concentration of the protein.

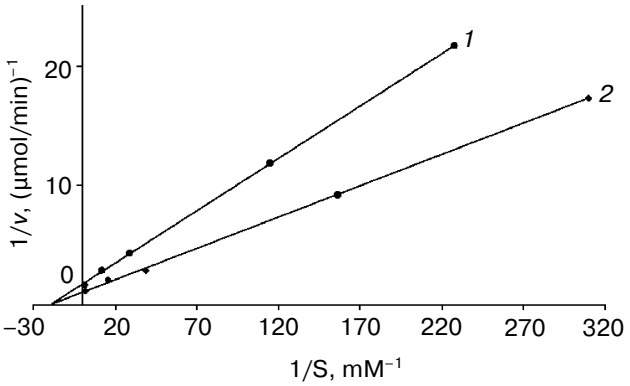

Fig. 5. Rate of hepsin-catalyzed enzymatic reaction plotted versus substrate concentration in double reciprocal coordinates. Substrate: *1*) AAR; *2*) KPR.

Figure 4 shows non-activated (not treated with thrombin) and thrombin-activated preparations of hepsin after purification and concentration (Fig. 4, lanes *4* and *5*, respectively). As can be seen, electrophoretic analysis of non-activated hepsin revealed a protein band at 42 kDa, whereas the activated protein in the presence of SDS shows two bands at around 26 and 16 kDa. The yield of the purified hepsin preparation was 0.7-1.0 mg/liter of bacterial culture.

Activity of hepsin preparation. The activity of recombinant hepsin was determined using a commercially available peptide substrate with a chromogenic group (*p*-nitroaniline). *p*-Nitroaniline has an absorption maximum at 405 nm; as a consequence, the optical density measured at 415 nm was slightly underestimated. However, the difference between the optical densities of the samples at these wavelengths is negligible (about 5%) in the initial time interval. The reaction rate was determined in the linear portion of the optical density curve. The specific activity of the hepsin preparation was 1.2 mmol/mg per minute.

Determination of Michaelis–Menten constants of proteases. To verify the specificity of the available substrates and to study the interaction with an inhibitor, purified recombinant hepsin was used. To determine the Michaelis–Menten constants of hepsin and other enzymes, peptide substrates with a chromogenic group attached to the N-terminus were used. We compared the specificity of hepsin to two tripeptide substrates, HDK-(γ-Cbo)-PR-pNA·2AcOH (KPR) and Cbo-AAR-pNA·2AcOH (AAR). In both cases the K_m values were equal to 50 μM despite the fact that cleavage of these substrates occurred at different rates (Fig. 5).

In subsequent experiments, the proteolytic activity of proteins was determined using the KPR substrate. The Michaelis–Menten constants of thrombin and chymotrypsin with respect to the substrates investigated were determined analogously; the results are presented in the table.

Michaelis–Menten constants of hepsin, thrombin, and chymotrypsin for chromogenic peptide substrates KPR and AAR

Enzyme	K_m, mM	
	KPR	AAR
Hepsin	0.05	0.05
Chymotrypsin	3.8	2.9
Thrombin	0.44	8.3

Note: The constants were determined from the enzymatic reaction rate versus substrate concentration plots drawn in double reciprocal coordinates.

Effects of anthralin and resveratrol on recombinant hepsin activity. To study the effect of these substances on the hepsin activity, the rate of enzymatic reaction was determined in the presence of different concentrations of these compounds. In the experimental samples, the anthralin and resveratrol concentrations ranged from 0.5 to 67.0 μM.

Figure 6 shows the dependences of the relative activity of recombinant hepsin on the anthralin and resveratrol concentrations. The relative activity was calculated as the percentage ratio of the protein activity in the presence of a compound to the protein activity in control sample. It was found that an increase in the anthralin concentration in the sample caused the enzymatic activity of hepsin to decrease, and at the anthralin concentration of 67 μM the hepsin activity was reduced by more than 70%. The effect of resveratrol on the hepsin activity is opposite in character, namely, the hepsin activity increased in dose-dependent manner in the presence of resveratrol.

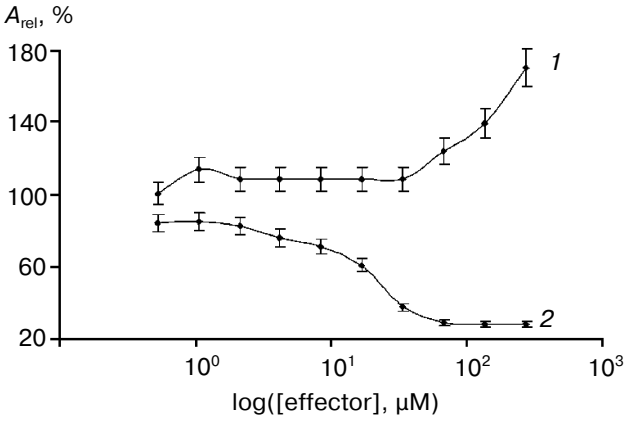

Fig. 6. Dependence of relative activity of recombinant hepsin on the concentration of resveratrol (*1*) and anthralin (*2*). Relative activity of recombinant hepsin was calculated as percentage ratio of enzymatic activity in the experimental sample to the activity in the control sample. Shown are the activity values averaged over three independent experiments.

DISCUSSION

Transmembrane proteins perform many important functions in eukaryotic cells and are therefore of considerable interest for research. Despite the development of cell biotechnology, the problem of heterologous expression of eukaryotic proteins is still topical. Earlier, the protein under study was expressed only in eukaryotic systems [3, 25]; however, some features of hepsin allowed us to efficiently express active hepsin in *E. coli* cells. A characteristic feature of hepsin is glycosylation of its molecule at a single site (Asn112) located in the SRCR-domain. Moreover, the protease domain of the protein is localized at the cell surface, which made it possible to express the extracellular part of the hepsin molecule and to increase significantly the yield of the soluble form of the protein product. Here we obtained a bacterial strain producing recombinant hepsin capable of producing the active form of the protein [26].

In this work we have developed a two-stage method for isolation and purification of recombinant hepsin from *E. coli*. Since the hepsin preparation obtained in the first stage of purification contained a large amount of contaminating proteins (Fig. 4), we elaborated the second stage of the purification procedure, which included ion-exchange chromatography on Q-Sepharose in a NaCl gradient. Replacement of the native amino acid sequence of the hepsin activation site by the sequence that could be cleaved by thrombin gave us a tool for hepsin activation by treatment with thrombin. Since thrombin can cleave chromogenic substrates of hepsin, the presence of thrombin in the hepsin preparation led to overestimation of the activity of the product. We managed to combine the removal of thrombin from the hepsin preparation with the second stage of purification, this significantly reducing the overall loss of the product. In this work, we for the first time demonstrate the possibility of obtaining the active form of recombinant hepsin in a bacterial expression system. The properties of the recombinant protein thus obtained are comparable with those of hepsin obtained in eukaryotic expression systems [3, 25].

As it was found earlier, peptides in which the first position is Arg are best suited to the substrate specificity of hepsin due to the structure of its active center and substrate-binding loops [6]. We compared the specificity of hepsin with respect to two substrates (tripeptides KPR and AAR) in order to assess the possibility of using them in further studies of the inhibition mechanisms of hepsin enzymatic activity. Since both peptides met the major condition for hepsin specificity, it was necessary to study the possible effect of other amino acid residues on the affinity of hepsin to these substrates. The results are con-

sistent with data about lesser importance of the amino acid residues that are not in the first position for the hepsin affinity to the substrate [6]. KPR was chosen for further research due to the higher rate of cleavage by hepsin compared to AAR. Moreover, K is more preferred in position P3 compared to A, while P and A are approximately equivalent in position P2 of the studied substrates [6].

The substrate specificity of hepsin differs from that of related proteolytic enzymes [7]. To explore the possibility of KPR cleavage by other enzymes, chymotrypsin (a representative of the same family of proteases) and thrombin capable of cleaving arginine peptide bonds only were chosen [27]. A comparison of the K_m values for all three proteases shows that the affinity of hepsin to these substrates exceeds the affinity of chymotrypsin and thrombin by more than an order of magnitude. These data suggest that the substrates used are more specific to hepsin rather than to thrombin and chymotrypsin.

Due to the important role of the proteolytic activity of hepsin in the neoplastic process, the inhibition of hepsin seems to be a possible way to affect progression of the tumor types mentioned above. Overexpression of hepsin in intercellular contacts of prostate adenocarcinoma cells can lead to a breach of cell adhesion and integrity of the basement membrane owing to hepsin enzymatic activity and thus contribute to local invasion of tumor cells. Also, hepsin can probably activate inactive forms of the enzymes directly involved in degradation of the basement membrane [25]. Recently, two potential inhibitors of hepsin proteolytic activity, HAI-1 and HAI-2, were reported [28]. It is noteworthy that, in contrast to hepsin, the level of HAI-2 expression remained unchanged or decreased with the development of prostate tumors [29, 30].

In addition to the study of macromolecular inhibitors of hepsin activity, the search for specific synthetic inhibitors of hepsin has been carried out. Screening of drug libraries and various chemical compound libraries revealed a number of substances capable of inhibiting the proteolytic activity of hepsin in specific manner [20]. To study the inhibition of the proteolytic activity of recombinant hepsin, anthralin (a strong antioxidant) and resveratrol, which also exhibits antioxidative properties [21], were chosen. It should be noted that anthralin is carcinogenic during systemic administration and is used only as a topical drug; in contrast to this, resveratrol possesses antitumor properties [21]. In this work we have shown that anthralin can inhibit the enzymatic activity of recombinant hepsin expressed in a bacterial system in dose-dependent manner. At the same time, resveratrol enhanced the hepsin activity (Fig. 6). The results reported here demonstrate the opposite effects of anthralin and resveratrol on the proteolytic activity of hepsin. The inhibitory effect of anthralin is probably a consequence of its ability to bind to the active center of the enzyme.

Since anthralin inhibits recombinant hepsin, one can assume that its effect on native hepsin is similar. Since intercellular communication and contacts are necessary conditions for the existence of cells both *in vitro* and *in vivo*, violation of these interactions can lead to cell death. In this regard, target action of drugs on hepsin molecules localized in desmosomes might cause damage to these structures and result in death of tumor cells. Thus, anthralin might have a specific cytostatic effect on cells expressing hepsin on their surface. From this point of view the transmembrane localization of hepsin makes it a convenient target for therapeutic agents due to location of its protease domain at the cell surface. Yet another advantage of hepsin as a molecular target is the fact that hepsin is apparently not an essential protein for functioning of the organism [11].

In this paper we for the first time demonstrated the possibility of obtaining the active form of recombinant hepsin in a bacterial expression system. A technique of two-stage separation and purification of the recombinant protein from *E. coli* cells was developed, and the enzymatic activity in the resulting protein preparations is shown. These results can have implications for further *in vivo* studies of the functional activity of hepsin. Investigations of inhibition mechanisms of the enzymatic activity of hepsin are promising for the development of new anticancer drugs and treatment of the tumor types characterized by hepsin overexpression. Such features of hepsin as high specificity of its expression in tumor cells and surface localization make it a promising target for therapeutic intervention.

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