PHYSIOLOGY

Is the hormone a protease? Proteolytic properties of human recombinant anti-mullerian hormone

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Abstract

Anti-mullerian hormone (AMH) is a glycoprotein of the TGF-β cytokine superfamily that regulates the development of the mammalian reproductive system, as well as the functioning of mature gonads. Recombinant AMH (rAMH) is also able to induce apoptosis of malignant cells bearing AMH type II receptors (MISRII) on the surface. Development of rAMH-based anticancer drugs is hampered by the lack of accurate information about the tissues where the AMH active form is generated and about the enzyme that activates the hormone by specific proteolysis. According to one hypothesis, the proteolytic processing of the hormone is autocatalytic. The goal of this work was to investigate the proteolytic activity of rAMH and its biologically active form — C-terminal AMH fragment (C-AMH). We showed that two forms of the hormone possess both autoproteolytic activity and the ability to influence the structure of other proteins. A full-length molecule of the hormone, as well as C-AMH, also forms complexes with aprotinin, an inhibitor of trypsin-like serine proteases. We determined that aprotinin competes for binding to C-AMH with antibodies blocking C-AMH interaction with MISRII. The obtained data suggest that AMH has proteolytic properties and that the site of specific AMH cleavage is involved in the interaction of the hormone with a specific receptor.

Keywords: anti-mullerian hormone, AMH, aprotinin, autoproteolysis, MIS, protease, recombinant protein.

Introduction

Anti-mullerian hormone (AMH — a substance inhibiting Mullerian ducts, Mullerian inhibitory substance, MIS) is one of the least studied glycoproteins of the TGF-β superfamily. It is both an embryonic and postnatal regulator of the mammalian reproductive system development and functioning (Gukasova and Severin, 2005). Recombinant AMH (rAMH) is known to induce apoptosis in cells of a number of human tumors (Donahoe et al., 2003; Jung et al., 2016). The AMH molecule with a mass of about 140 kDa is a homodimer, both monomers containing a proteolytic cleavage site in their sequence located between the residues Arg427 and Ser428. Specific proteolysis of the full-length hormone molecule at this site leads to its cleavage to N- and C-terminal homodimers with molecular masses of about 115 and 25 kDa, respectively (Gukasova and Severin, 2005). Under physiological conditions, these AMH fragments form a non-covalently associated complex after proteolysis of the prohormone molecules. It has been shown that specific proteolysis is crucial for the activation of the hormone. This fact has been confirmed by experiments with the mutant AMH form without a specific proteolysis site. The mutant protein does not exhibit biological activity in

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the growth inhibition test of the rat Mullerian duct organ culture (Cate, Donahoe and MacLaughlin, 1990). It is also known that, unlike the N-terminal, the C-terminal homodimer of AMH (C-AMH) is biologically active (Pankhurst, Leathart, Batchelor and McLennan, 2016).

The AMH signaling pathway has been well studied; it starts from the interaction of the hormone with AMH type II receptor (MISRII), which further leads to heteromerization of the AMH type I receptor (MISRI) and MISRII and induction of the proapoptotic signaling cascade provided by Smad family proteins (Teixeira, Maheswaran and Donahoe, 2001). The MISRI receptor belongs to the family of transmembrane serine / threonine protein kinases similar to the activin receptor (activin receptor-like kinases, ALKs). Presumably, ALK2, ALK3 and ALK6 (also known as ACVR1, BMPR1A and BMPR1B, respectively) can play the role of MISRI (Orvis et al., 2008; MacLaughlin and Donahoe, 2010; Sèdes et al., 2013). Unlike type I receptors, MISRII is exclusively AMH-specific (Pankhurst, Leathart, Batchelor and McLennan, 2016), MISRII expression is tissue-specific (Lee and Donahoe, 1993; Teixeira, Maheswaran and Donahoe, 2001) and overexpression of this receptor is a characteristic of a number of malignant cells (MacLaughlin and Donahoe, 2010; Kim, MacLaughlin and Donahoe, 2014). For this reason, it can serve as a target for induction of apoptosis in MISRII-positive tumor cells as a result of interaction with rAMH or specific antibodies (Estupina et al., 2017; Deshayes et al., 2018) or be targeted for an attack of AMH complexed with different cytostatics (MacLaughlin and Donahoe, 2010; Kim, MacLaughlin and Donahoe, 2014). Cancer treatment based on a targeted effect on MISRII seems to be a more effective alternative to the traditional one and will significantly reduce the dose of antineoplastic drugs due to their targeted delivery to the malignant cells (MacLaughlin and Donahoe, 2010).

The exact mechanism of hormone binding, as well as the structure of the interaction site, is still unknown. Most researchers take the position that MISRII molecules are not randomly distributed in the cell membrane but form homodimeric complexes even before interacting with the ligand (Hirschhorn et al., 2015). Thus, AMH interacts with the MISRII dimer, which then forms a ligand-receptor complex with the MISRI dimer (Kikuchi and Hamaguchi, 2013). However, it is still unclear which hormone derivatives are capable of triggering this signaling pathway *in vivo*.

The question of where specific proteolysis of AMH molecule occurs *in vivo* and what enzyme is required for cleavage also remains open. Some authors suggest that AMH is activated by cleavage exclusively in biosynthesis and secretion, but not in blood. In this case, hormone cleavage occurs in gonads, and both uncleaved prohormone and non-covalently associated complex of the N- and C-terminal AMH fragments enter the circulation (Pankhurst, Leathart, Batchelor and McLennan, 2016). According to another hypothesis, specific proteolysis of AMH can occur both in the bloodstream under the action of plasmin and in the gonads under the action of proprotein-convertase family enzymes such as furin and PC5 (Ragin et al., 1992; Nachtigal and Ingraham, 1996). Finally, the hormone cleavage at a specific site may be associated with its entry into the target tissues and interaction with the AMH receptors type I and type II — MISRI and MISRII, respectively (Di Clemente et al., 2010). In this case, specific proteolysis of the AMH molecule needs to be autocatalytic — that is, this process does not require the involvement of any other enzymes. Many different proteins are known to activate through autoproteolytic processing, in particular, pepsinogen (Dunn B. M., 2001), nucleoporin (Rosenblum and Blobel, 1999), Hedgehog family factors and glycosylasparaginases (Perler, Xu and Paulus, 1997). Clarifying whether the AMH molecule has its own proteolytic activity will make it possible to refresh a look at the biochemistry and pharmacodynamics of this substance, as well as to optimize the rAMH producing method for antineoplastic drugs development.

The goal of this work was to study the stability of highly purified preparations of rAMH and to determine whether prohormone and its biologically active form, the C-terminal fragment, have proteolytic activity.

Materials and Methods

Preparations of prohormone, half-cleaved rAMH (a form containing one monomer protealyzed at a specific site) and C-AMH, characterized by a purity of about 96 %, were obtained previously from the culture fluid of the CHO line producer cells, transfected with the human AMH gene (producer strain CHO-MIS 26 (Rak et al., 2017)), by the method of tandem immunoaffinity and reversed-phase chromatography according to the developed method (Rak et al., 2019). In addition, previously obtained highly purified preparations of recombinant human heat shock protein 70 (HSP70) were used in the experiments (Ischenko et al., 2015). We also used the aprotinin solution for intravenous administration with a concentration of 10,000 U / ml and following previously obtained mouse monoclonal antibodies:

- ACMIS-3 and ACMIS-4 (Rak, Trofimov, Kolobov and Ischenko, 2018), specific to different C-AMH epitopes, but not interacting with the uncleaved hormone. Using these antibodies, ACMIS-3-Px and ACMIS-4-Px conjugates with horseradish peroxidase (Sigma, United States) were prepared according to the attached protocol. An enzyme immunoassay test system (ACMIS-3) — C-AMH — (ACMIS-4-Px) based on these antibodies was used

for quantitative detection of C-AMH in a heterogeneous sandwich enzyme-linked immunosorbent assay (ELISA);

- M2 (Rak et al., 2019), recognizing the full-length AMH and its derivatives, containing the N-terminal fragment, and the M2-Px — derivative of these antibodies. These antibodies were used to develop the ELISA system (ACMIS-3) — AMH — (M2-Px), which was used for the quantitative detection of half-cleaved rAMH;
- I4 (Rak et al., 2017), specific to the Fc-fragment of human IgG1 immunoglobulins;
- CC3-4 (Kartuzova et al., 2016), recognizing the conformational epitope of the human complement C3 component.

The biological activity of rAMH was evaluated by the ability of the hormone to bind to the extracellular part of the specific AMH receptor type II — MISRII (Rak et al., 2017). For this purpose, a previously developed enzyme immunoassay test system (Rak et al., 2019) was used. The keystone of this ELISA system is the recombinant chimeric protein MISRII + Fc, consisting of the extracellular part of MISRII and Fc fragment of human IgG1. Antibody I4 and the peroxidase conjugate ACMIS-4-Px were also used in the test system. At the first stage of the analysis, Corning polystyrene plates (Sigma, United States) were treated with I4 antibodies in the coating solution (20 mM borate buffer, pH 8.0, containing 0.15 M NaCl) at a concentration of 1.5 μg / ml for 20 hours in a humid chamber at room temperature. At the end of the sorption, the plates were treated with wash buffer (20 mM borate buffer, pH 8.0, containing 0.15 M NaCl and 0.05 % Tween-20). At the next stage, the chimeric MISRII-containing construct solution (100 ng / ml) was added into the wells as a specific acceptor for rAMH preparations. After the test samples were added, detection of bound rAMH was performed using ACMIS-4-Px antibodies (0.25 μg / ml). The ELISA was performed by a standard method using tetramethylbenzidine substrate solution (HEMA, Russia) and BioRad Model 680 microplate reader (BioRad, USA) at a wavelength of 450 nm. Schematically, the test system for the rAMH biological activity evaluation can be described as I4 — (MISRII + Fc) — rAMH — (ACMIS-4-Px). We used this ELISA system to accurately determine the concentration of the biologically active hormone in various preparations and biological fluids.

The interaction of C-AMH with aprotinin was also analyzed in solid-phase ELISA. At first, plates were treated with aprotinin in the coating solution at a concentration of 1.5 μg / ml for 20 hours in a humid chamber at room temperature. At the end of the sorption, the plates were treated with wash buffer. Then two-fold dilutions of rAMH in wash buffer containing 10 mg / ml bovine serum albumin (BSA) were added at 100 μl per well. The plates were incubated for 1 hour with stirring at 37 °C. Then a solution of ACMIS-4-Px antibodies (0.25 μg / ml) was added to the washed plates. The plates were incubated for 1 hour with stirring at 37 °C, and finally they were thoroughly washed and stained with a tetramethylbenzidine substrate solution (HEMA, Russia). The ELISA was performed by a standard method using a BioRad Model 680 microplate reader (BioRad, USA) at a wavelength of 450 nm.

Analysis of rAMH preparations was performed by electrophoresis of proteins in a polyacrylamide gel with sodium dodecylsulphate (SDS-PAGE) and with or without a reducing agent (β-mercaptoethanol) according to the previously described method (Walker, 1984). In this work, a running gel with a gradient concentration of acrylamide of 4–20 % and a stacking gel with a concentration of 5 % were used. The samples were prepared by incubation of protein solution (1–2 mg / ml) at 95 °C for 5 min in the presence of β-mercaptoethanol (at final concentration 2 %) or without it, then 15 μl of each sample was loaded into the gel. SDS-PAGE was performed by the BioRad MiniProtean Tetra Cell system (BioRad, USA) at a voltage of 40 V per gel for 20 minutes and then at a current of 200 mA until the end of the electrophoresis. To visualize the results, the gel was stained with Coomassie G-250, followed by washing with a 5 % solution of acetic acid at room temperature.

To perform the affinity chromatography of half-cleaved rAMH, an affinity sorbent was prepared by immobilization of aprotinin (with a loading of 10 mg / ml) on cyanbrom-activated sepharose FF (Pharmacia, Sweden) according to the manufacturer's protocol. The column containing the sorbent was equilibrated with 20 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl. At the first stage of chromatography, 100 μg of rAMH was loaded to a 1 ml column for sorption. After washing the column with an equilibration buffer and a buffer containing 1 M NaCl, rAMH was eluted from the sorbent with a solution of 0.1 M glycine, pH 2.5. Detection of components during the chromatography was performed at a wavelength of 280 nm.

The quantitative data are given as mean ± s.e.m. from two independent experiments with three technical replicates (n = 6). Compliance with the normal distribution was checked by Shapiro-Wilk's test. The significance of differences was assessed by Student's t-test for paired data, since all the data were normally distributed. The level of significance was set at $p<0.05$. Statistical analysis was performed using Statistica 10 software.

Results and Discussion

According to previously obtained data (Rak et al., 2019), the uncleaved rAMH (prohormone) contained in the highly purified sterile preparation undergoes proteolysis

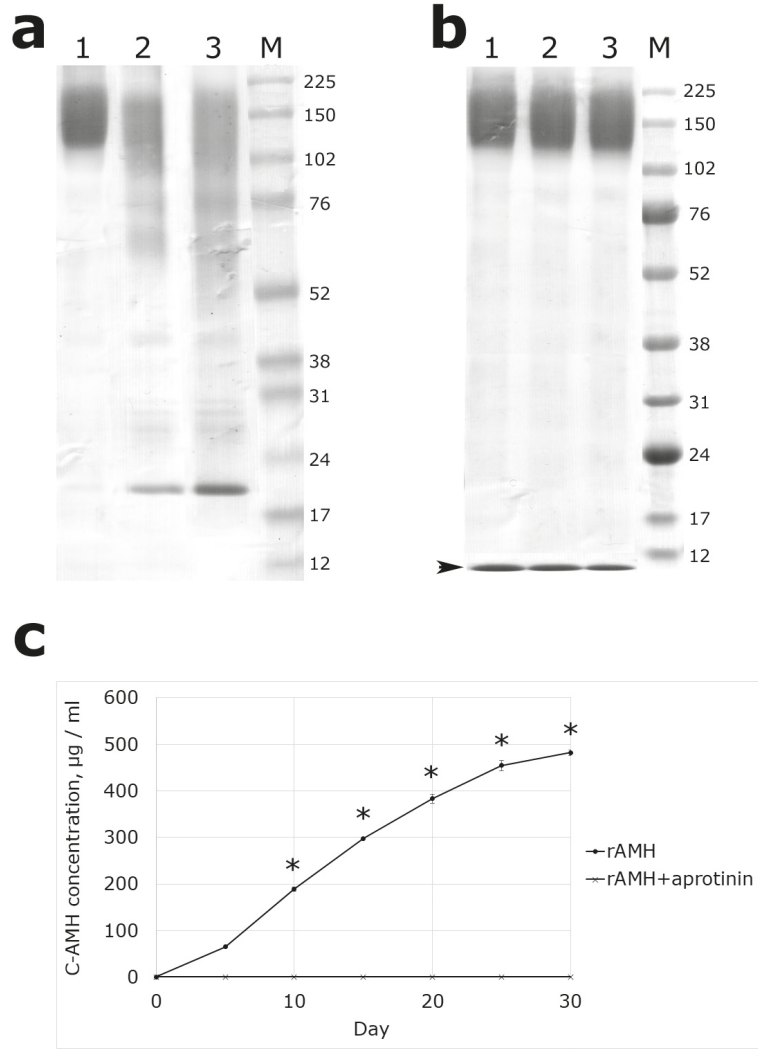

Fig. 1. The effect of aprotinin on the rAMH proteolysis during storage. a — electrophoregram of the initial hormone (1) and rAMH after incubation in 20 mM phosphate buffer, pH 7.4, for 15 (2) and 25 days (3) at + 37 °C, M — molecular weight marker; b — electrophoregram of the rAMH preparation with aprotinin (5 µg / ml) added immediately after the purification: before the storage (1), after incubation in 20 mM phosphate buffer, pH 7.4, at + 37 °C for 15 (2) and 25 days (3); M — molecular weight marker, the arrow indicates the aprotinin localization in the gel; c — the accumulation of the biologically active protein in highly purified rAMH preparation during incubation in 20 mM phosphate buffer, pH 7.4, at 37 °C. The significance of differences was estimated using Student's t-test: * — $p<0.05$ compared to day 5.

during long-term storage; it leads to C-AMH formation. The prohormone is not capable of binding to the receptor; however, as C-AMH accumulates in the solution, the biological activity of the protein contained in the sample increases and reaches a maximum on the 30th day of storage (Rak et al., 2019). Here, we first studied the effect of aprotinin — a nonspecific polyvalent trypsin-like serine protease inhibitor — on rAMH proteolysis during storage. It was shown that the addition of aprotinin at a final concentration of 5 µg / ml blocks the proteolysis of prohormone, the formation of C-AMH and the manifestation of the biological activity of the substance, which is shown in Figure 1. Electrophoretic analysis was performed under non-reducing conditions. To evaluate the rAMH activity, we used ELISA system I4-(MISRII + Fc) — rAMH — ACMIS-4-Px.

As can be seen from Figure 1, during storage of rAMH, an accumulation of the C-terminal fragment fraction in the preparation occurs (Fig. 1, a), while in the presence of aprotinin uncleaved rAMH is stable (Fig. 1, b). The quantitative analysis of C-AMH accumulation dynamics is presented in Figure 1, c.

It was previously shown that C-AMH, like the full-length form of the hormone, undergoes spontaneous limited proteolysis at a specific site (leucine motif). In this case, the smallest fragment with a molecular mass of

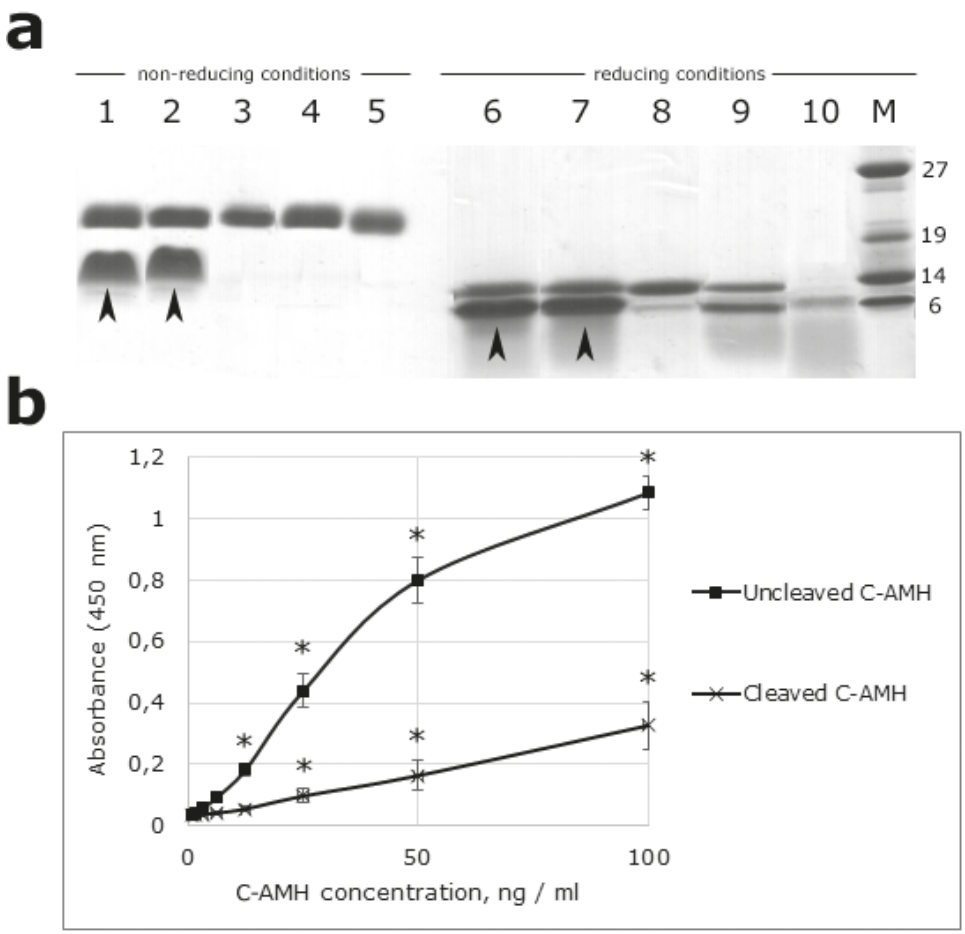

Fig. 2. The C-AMH proteolysis in presence or absence of aprotinin. a — electrophoregram of the initial C-AMH (3, 8) and after incubation in 20 mM phosphate buffer, pH 7.4, for 15 (4, 9) and 25 days (5, 10) at + 37 °C, C-AMH freshly mixed with aprotinin (5 μg / ml) (1, 5) and after incubation for 25 days (2, 6), M — molecular weight marker, arrows indicate the aprotinin bands; b — dependence of the absorbance intensity on the concentration of initial and proteolyzed C-AMH, determined using the test system I4 — (MISRII + Fc) — rAMH — ACMIS-4-Px. The significance of differences was estimated using Student's t-test: * — p<0.05 compared to C-AMH concentration 6.25 ng / ml.

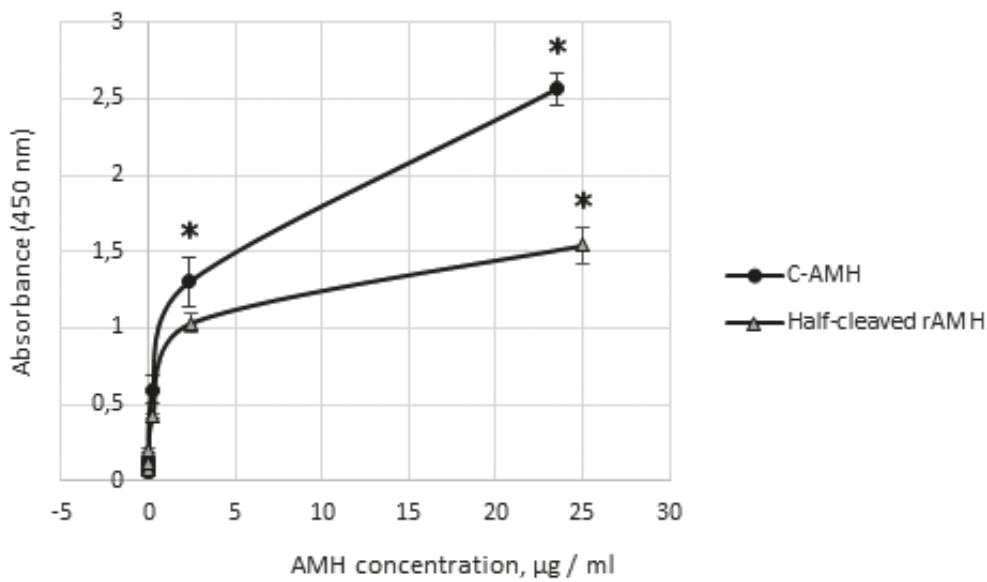

Fig. 3. Dependence of the absorbance intensity on the concentration of AMH forms: half-cleaved hormone (detected by M2-Px) and C-AMH (detected by ACMIS-4-Px) interacting with aprotinin immobilized in the plate (1.5 μg / ml). * — p<0.05 compared to AMH concentration 0.25 μg / ml.

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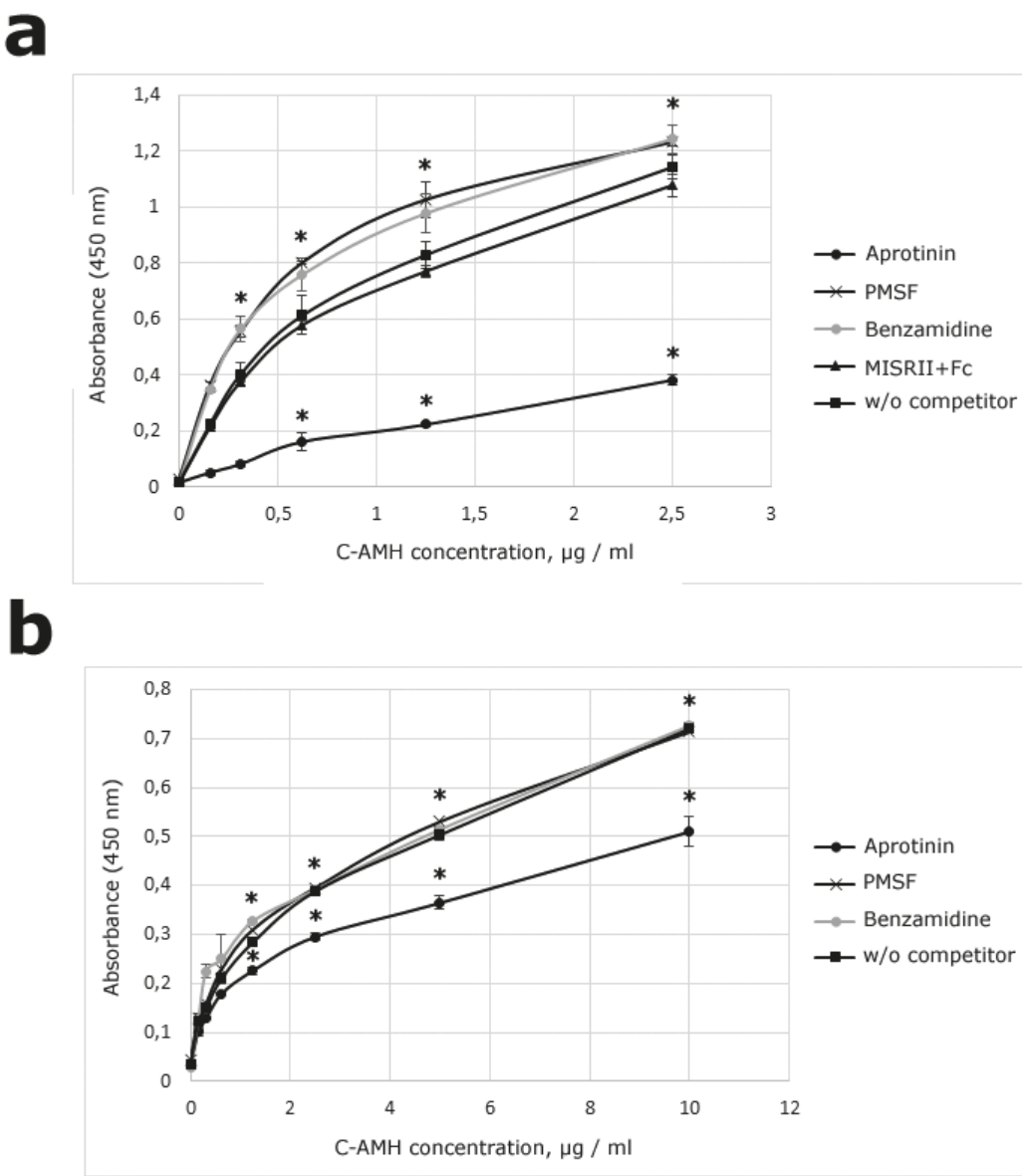

Fig. 4. Interaction of C-AMH with solvated aprotinin. a — the effect of C-AMH concentration and the presence of protease inhibitors or competitor on the binding of C-AMH with immobilized aprotinin (1.5 μg / ml); a decrease in the intensity of C-AMH interaction with immobilized aprotinin in the presence of dissolved one is showed. b — the effect of C-AMH concentration and the presence of protease inhibitors on the binding of C-AMH with immobilized chimeric construct MISRII + Fc (1.5 μg / ml); decrease in signal intensity in the presence of aprotinin, caused by the competition between MISRII+Fc and aprotinin for binding to C-AMH, is shown. The significance of differences was estimated by Student's t-test: * — $p<0.05$ compared to the controls (w/o competitor).

about 6 kDa is formed, and C-AMH biological activity disappears (Rak et al., 2019).

In this work, the inhibitory effect of aprotinin on C-AMH specific proteolysis was also studied. In order to show whether the inhibitory effect of aprotinin extends to C-AMH degradation and to study the effect of the inhibitor on the activity loss of this AMH derivative, the sample was incubated with or without the aprotinin. It was found that aprotinin, as well as in the case of a prohormone, prevents the proteolysis of C-AMH (Fig. 2).

The interaction of aprotinin with C-AMH and half-cleaved full-length hormone (containing one monomer proteolyzed) was studied in solid-phase ELISA. It was shown that C-AMH, as well as half-cleaved rAMH, is able to form the specific complexes with aprotinin immobilized in the plate (Fig. 3). Note, that the intensity of C-AMH binding with immobilized aprotinin was significantly higher.

In order to find out whether C-AMH is capable of interacting not only with immobilized aprotinin, but

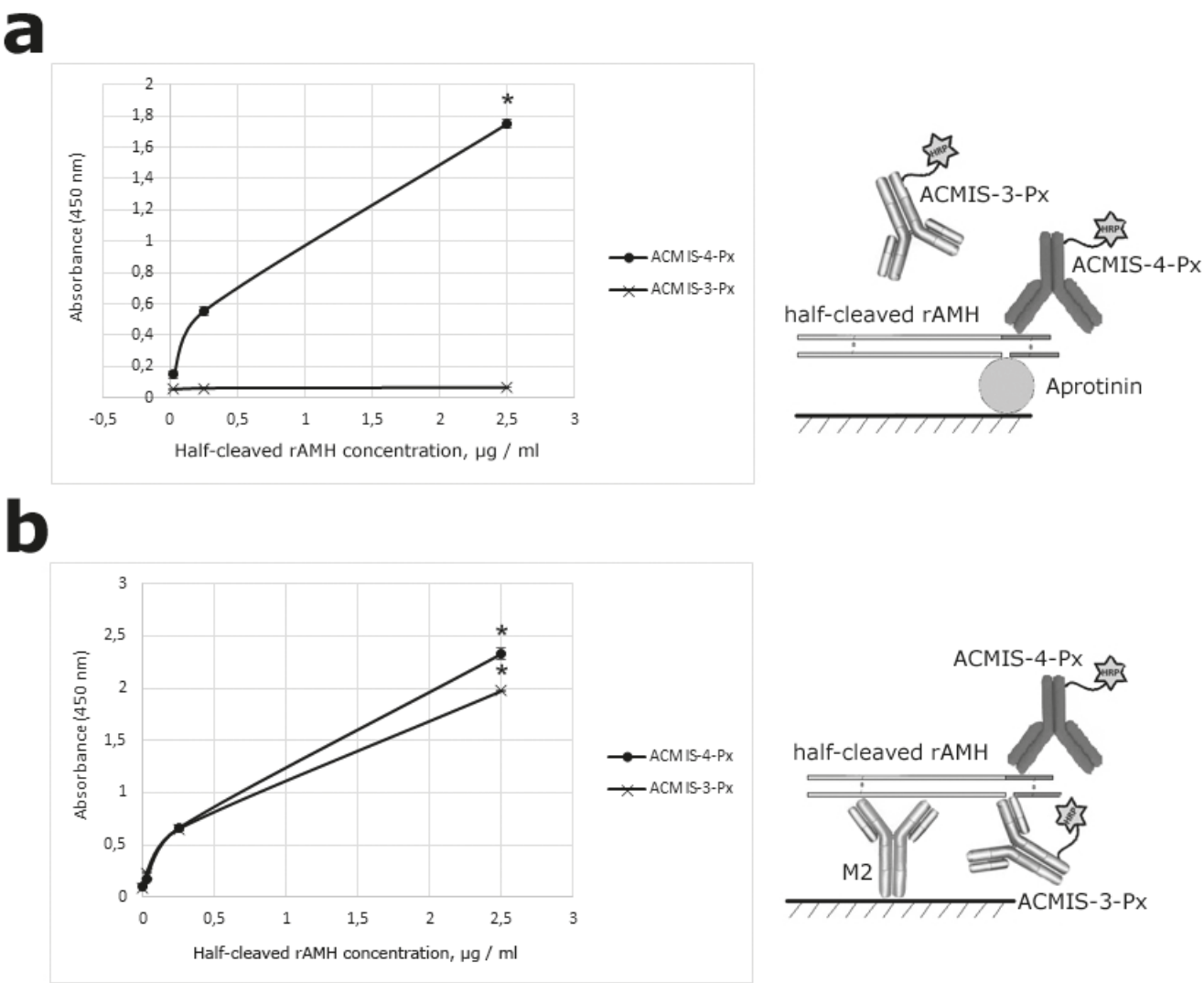

Fig. 5. Detection of half-cleaved rAMH by peroxidase conjugates ACMIS-3-Px and ACMIS-4-Px with immobilized: a — aprotinin (1.5 µg / ml); b — M2 antibodies (1.5 µg / ml). * — $p<0.05$ compared to half-cleaved rAMH concentration 0.25 µg / ml.

also with that present in the solution, a competitive ELISA was performed. The C-AMH double dilutions were preincubated for 15 min with fixed concentrations of aprotinin (10 µg / ml) and possible competitors of C-AMH binding with aprotinin (protease inhibitors PMSF and benzamidine, and chimeric protein MISRll + Fc; 1 mM, 5 mM and 10 µg / ml, respectively). Then the samples were transferred into the plate wells containing immobilized aprotinin. C-AMH was detected by the subsequent addition of the peroxidase conjugate ACMIS-4-Px. The ELISA results presented in Figure 4 indicate that not only immobilized, but also solvated aprotinin is capable of binding to C-AMH present in the solution. Competition between the solvated aprotinin and immobilized one for C-AMH binding results in a decrease in the C-AMH detection intensity by ACMIS-4-Px conjugate. It is shown (Fig. 4, a) that other protease inhibitors, namely PMSF and benzamidine, present in the solution do not have this effect.

In another variant of the analysis, the presence of competition between aprotinin and the chimeric receptor-containing protein MISRII + Fc for binding to C-AMH was found. In this case, double dilutions of C-AMH were preincubated with aprotinin, PMSF and benzamidine at the same fixed concentrations as in the previous experiment, and then the mixes were added to the immobilized construct MISRII + Fc (Fig. 4, b). At the last stage of this ELISA, peroxidase conjugate ACMIS-4-Px was used to detect C-AMH. It was shown that in the presence of aprotinin, the intensity of C-AMH interaction with ACMIS-4-Px decreases, which was not observed when adding other non-specific protease inhibitors PMSF and benzamidine.

An analysis of ACMIS-3 specificity, which was performed in a previous study (Rak et al., 2017), showed

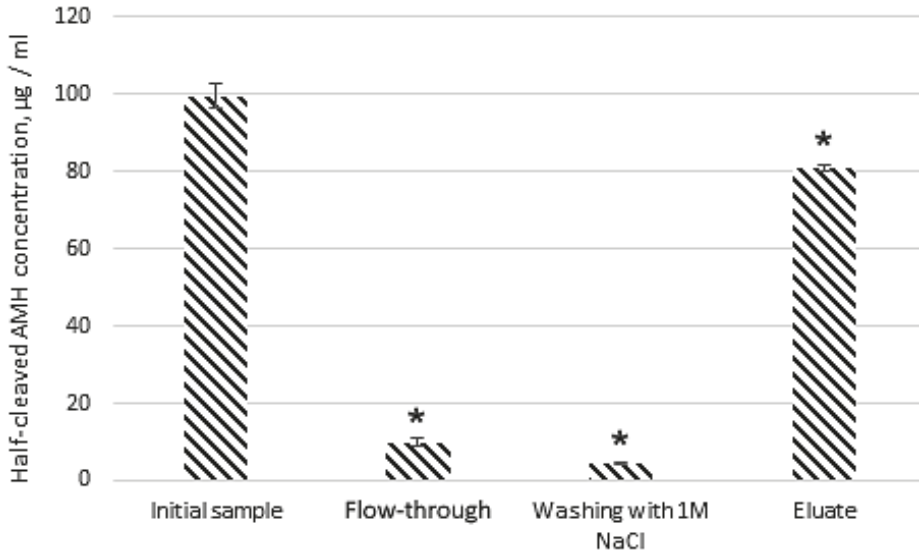

Fig. 6. Affinity chromatography of half-cleaved rAMH on aprotinin-sepharose. For quantitative detection of the hormone, the ELISA system (ACMIS-3) — rAMH — (M2-Px) was used. The significance of differences was estimated using Student's t-test: * — $p<0.05$ compared to initial concentration.

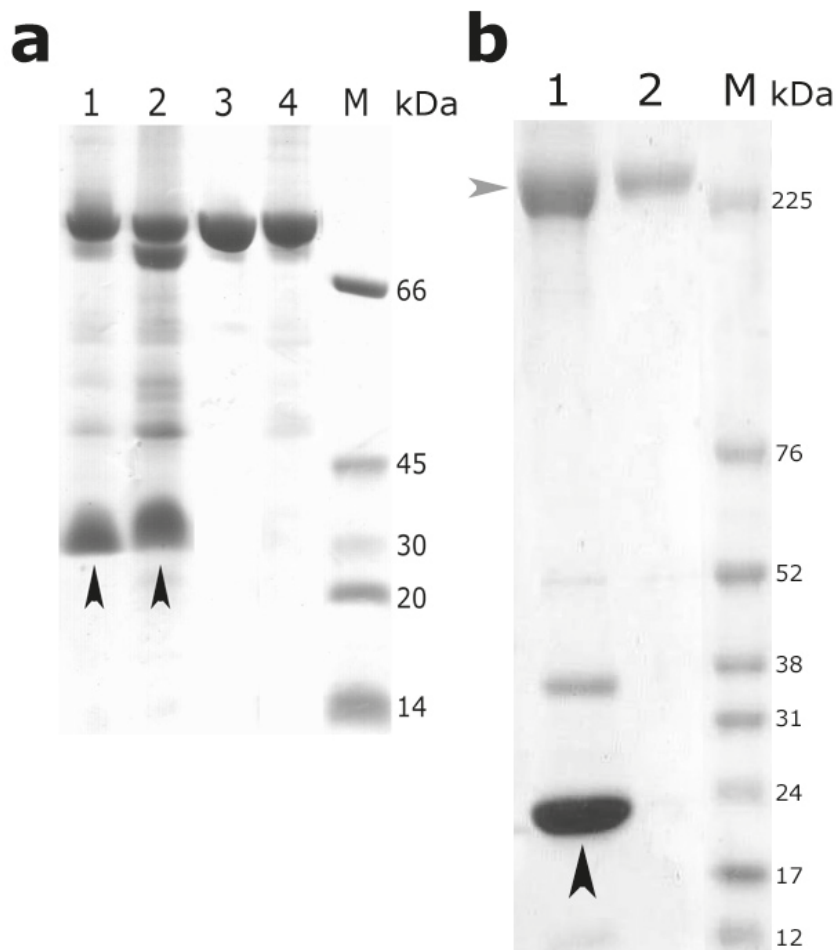

Fig. 7. C-AMH is able to catalyze the proteolysis of other proteins. a — electrophoregram of HSP70 and C-AMH mix after incubation for 7 (1) and 15 days (2), as well as the initial HSP70 preparation (3) and HSP70 after 15 days of incubation (4), M — molecular weight marker; b — electrophoregram of CC3-4 and C-AMH mixture after incubation for 15 days (1) and the initial CC3-4 preparation (2), M is the molecular weight marker. Electrophoretic analysis was performed under the non-reducing conditions. The black arrows indicate the aprotinin localization in the gel, and the grey arrow indicates the area corresponding to immunoglobulins.

that these antibodies against C-AMH are able to inhibit the interaction of the hormone with AMH receptor type II (MISRII). As can be seen from Figure 4, b, aprotinin has a similar effect.

To test the hypothesis that ACMIS-3 antibodies and aprotinin bind to the same C-AMH epitope involved in interacting with the MISRII, the ELISA using full-length one-chain cleaved (half-cleaved) rAMH was performed. A series of the hormone dilutions were added into the wells with immobilized aprotinin (Fig. 5, a) or M2 antibodies recognize the epitope within N-terminal fragment of rAMH (Fig. 5, b). The bound hormone was detected by two conjugates: ACMIS-3-Px and ACMIS-4-Px.

It can be seen that in the case of M2 antibody immobilization, full-length rAMH cleaved in one chain can be detected using both the ACMIS-3-Px and ACMIS-4-Px conjugates (Fig. 5, b). However, under immobilization of aprotinin in the wells of the plate, hormone detection is possible only with the aid of the ACMIS-4-Px conjugate, since the epitope recognized by the antibodies ACMIS-3-Px and involved in the interaction of rAMH with MISRII is occupied by aprotinin. (Fig. 5, a). These data suggest that the sites of the AMH molecule involved in the interaction of the hormone with MISRII and aprotinin are identical.

To study the ability of rAMH to bind to aprotinin in a flow system, 100 μg of the half-cleaved hormone was analyzed by affinity chromatography on an aprotinin-sepharose column. Such sorbents have often been used for the purification of various proteins to remove the serine proteases from preparations (Shikimi and Kobayashi, 1980; Schmitt, Goetschel, Römmelt and Adler, 1990). The results of affinity chromatography are presented in Figure 6. It can be seen that the content of half-cleaved rAMH in the eluate is about 80 % of hormone applied on the column. These data confirm the specificity of the interaction of the hormone with aprotinin.

In order to test the C-AMH ability to catalyze the proteolysis of other proteins, the hormone was added to the solution of highly purified human recombinant heat shock protein 70 (HSP70) in a weight ratio of 1:10. The mixture was incubated for 30 days at + 37 °C. The result of the experiment is shown in Figure 7, a. The appearance of additional bands on the electrophoregram indicates that C-AMH is involved in the proteolysis of the HSP70 molecule (Fig. 7, a).

A similar experiment was performed with mouse immunoglobulins CC3-4, specific to human complement C3 component. C-AMH was added to the CC3-4 solution (also in a weight ratio of 1:10), and this mixture was incubated at + 37 °C. As follows from the results shown in Figure 7, b, in the presence of C-AMH: new bands appear, and the area corresponding to immunoglobulins becomes more diffuse (Fig. 7, b). These data prove the presence of the proteolytic effect of C-AMH on CC3-4 antibodies. Affecting factors, as well as the sequence of the fragments that are formed and the site of C-AMH action will have to be clarified in the course of further experiments.

Conclusions

In this study, the proteolytic properties of the activated AMH (C-AMH) and full-length hormone were first described *in vitro*. Inhibition of the proteolytic activity of both hormone forms by aprotinin — the inhibitor of trypsin-like serine proteases — was found. The proteolytic activity of C-AMH against human HSP70 and mouse immunoglobulins CC3-4 was shown. We demonstrated that half-cleaved rAMH and C-AMH are able to interact with both immobilized and dissolved aprotinin; this protease inhibitor also competes for binding to the hormone with antibodies that block the interaction of C-AMH with MISRII. The data obtained support the autocatalytic mechanism of AMH cleavage and prove the ability of AMH to catalyze the proteolysis of other proteins as well as the fact that the site of AMH specific proteolysis may be involved in the interaction of the hormone with type I and II receptors exposed on the surface of target cells.

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