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Effect of Ultrasound on Structure and Functional Properties of Antithrombin III and Proteins of PPSB Complex

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Abstract—A combination of gel-permeation HPLC, affinity chromatography on heparin-Sepharose, gel electrophoresis, and estimation of inhibitory activity showed that effect of low-frequency ultrasound (26 W/cm^2, 37°C, pH 7.4) on homogeneous antithrombin III was accompanied by formation of aggregates and a latent form of serpin. Heparin and pentosan polysulfate stabilized antithrombin III; this resulted in decrease in ultrasonic-induced formation of the aggregate and latent forms. The influence of ultrasound was not accompanied by significant changes in the contents of non-activated blood coagulation factors in the PPSB complex.

Key words: antithrombin III, latent and aggregate forms, ultrasound, PPSB complex

The combination of ultrasound with various plasminogen activators is a promising approach for vascular remodeling; it allows therapeutic doses of protein activators to be decreased [1-5]. However, thrombolytic monotherapy with streptokinase or tissue plasminogen activator may cause a preinfarction state, which is accompanied by repeated infarctions in 3-6% of patients subjected to successful thrombolysis. Several mechanisms have been proposed for explaining the prethrombotic state induced by thrombolytic therapy. One of them implies formation of free plasmin, which may trigger blood clotting due to partial activation of coagulation factors. The other possible mechanism involves fibrin-bound thrombin (protected from inactivation by plasma inhibitors) into activation of platelets and acceleration of blood clotting [6-8]. Numerous prethrombotic reactions and their molecular modeling based on the mixtures of enzymes and cofactors of clotting and also consequences of local activation of plasminogen activators which result in restenoses after balloon angioplasty are considered in reviews [9, 10]. Earlier it was shown that ultrasonic treatment in combination with streptokinase was accompanied not only by accelerated thrombolysis due to plasminogen activation, but also activated thrombin system as well [11]. This effect may be attributed to inappropriate regimes of ultrasonic treatment selected on the basis of data on the general state of hemostasis, which does not reflect possible fine structural–functional changes in protein components of the clotting and fibrinolytic systems.

In this study we have used highly purified native inhibitor of serine proteases—antithrombin III (AT III) (serpin)—and a mixture of inactive coagulation factors as the PPSB complex (prothrombin–factor VII–factor IX–factor X) to reveal protein component (or components) exhibiting the highest sensitivity to the ultrasound treatment. X-Ray and biochemical studies suggest an importance of polymer aggregates, native and latent forms of serpin, in the development of some pathological states [12-16]. There is growing interest in AT III due to antiangiogenic and antitumor properties of modified forms of this inhibitor [17]. In the light of the suggestion that local activation of coagulation factors may be the triggering mechanism of the development of prethrombotic state, the PPSB preparation used for replacement therapy of hemophilia B is a convenient model for studies of possible ultrasonic-induced activation of coagulation factors. In general, analysis of specific structural–functional changes in AT III and coagulation enzymes is necessary for determination of threshold regimes for ultrasonic thrombolysis and also for corresponding pharmacotherapy minimizing side effects of acoustic-enzymatic thrombolysis.

Abbreviations: AT III) α-antithrombin III; PPSB) prothrombin complex concentrate of inactive blood coagulation factors (factor II–factor VII–factor X–factor IX).

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MATERIALS AND METHODS

The following reagents were used in the study: PPSB complex for replacement therapy of hemophilia B containing the mixture of coagulation factors IX, II, VII + X (of total activity of 640, 580, and 600 IU, respectively), 25 IU heparin, and 60 mg mannitol per ml (Intersero, Germany); sodium pentosan polysulfate, 100 mg per ml (Bene-Arzneimittel, Germany); heparin (sodium salt), 5000 IE per ml (Belmedpreparaty, Belarus); SDS, 2-mercaptoethanol, Coomassie Brilliant Blue R-250, tris-(hydroxymethyl)aminomethane, phenylmethylsulfonyl fluoride (PMSF) (Serva, Germany); kits for gel electrophoresis (Reanal, Hungary). Heparin-Sepharose was prepared by the method of reductive amination of terminal formyl group of heparin by agarose amino-derivatives in the presence of sodium cyanoborohydride [18].

AT III was isolated from freshly frozen plasma of volunteers by the method of [19], using sequential procedures of polyethylene glycol 6000 (20% w/v) fractionation, affinity chromatography (heparin-Sepharose), anion-exchange chromatography (DEAE-cellulose), and gel chromatography (Sephadex G-150). The isolation procedures up to the stage of gel chromatography were carried out in the presence of 0.1 mM PMSF at 5-8°C (excluding the stage of lyophilization). Purity of antithrombin was monitored by SDS-PAGE and the presence of aggregates by gel electrophoresis under non-denaturing conditions in a discontinuous buffer system [20]. Protein molecular weight markers for electrophoresis (phosphorylase *b*, 94 kD; BSA, 67 kD; ovalbumin, 43 kD; carboanhydrase, 30 kD; soybean trypsin inhibitor, 20 kD; α-lactalbumin, 14.4 kD) were purchased from Pharmacia Biotech (USA). Concentration of AT III was determined spectrophotometrically, using molar extinction coefficient ε_{280nm} 37,700 $M^{-1}\cdot cm^{-1}$ [16].

Liquid chromatography with gradient or isocratic elution was carried out on automatic GradiFrac (Pharmacia Biotech) system including a programmed fraction collector, flow UV-detector (λ = 280 nm), gradient mixer, and peristaltic pump.

Ultrasound source. The thrombolytic device Pulsar ART-UN1 developed in the Belarusian State Polytechnic Academy (Minsk, Belarus) was used the source of ultrasound. It consists of an ultrasound generator with controlled power (1-80 W/cm^2), piezoelectric converter of the ultrasound oscillations, and flexible undulator system of variable section 560 mm in length. The diameter of the final stage of the undulator was 0.8 mm, and the diameter of emitting working surface was 1.8 mm. Sonication (except specially specified experiments) was carried out at 26 kHz and ultrasound intensity 26 W/cm^2.

A cuvette containing sonicated solutions of proteins (0.5-1.0 mg/ml) in 0.05 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl was thermostatted at 37°C. During ultrasound (US) treatment, temperature increase did not exceed 0.5°C. Just before US treatment the AT III preparations were subjected to additional chromatography on a heparin-Sepharose column and dialyzed against sonication buffer for separation of spontaneously formed latent forms; PPSB complex was dialyzed against the same buffer to remove mannitol.

Analysis of content of aggregate, latent, and native forms of AT III. The content of aggregate, latent, and native forms was evaluated by affinity chromatography using a heparin-Sepharose column (0.8 × 2.5 cm) followed by subsequent HPLC analysis of the fractions obtained. During affinity chromatography in the presence of protective additives (heparin, pentosan polysulfate), various AT III forms were eluted in the free volume. Under these conditions, the amount of the initial form was determined by residual amidolytic activity of thrombin; content of the latent form was determined by difference between content of monomeric form (HPLC) and the amount of active AT III. Remaining amidolytic activity of thrombin was determined by the initial rate of hydrolysis of the substrate S-2238 (H-D-Phe-Pip-Arg-pNA, Chromogenix, Italy) [21-23], using a Shimadzu 1202 spectrophotometer and software for kinetic measurements. Samples of AT III were incubated at 37°C for 40 min in the presence of heparin (400 nmol) and thrombin (8 nmol) in 200 μl of 0.05 M sodium phosphate buffer, pH 7.4, containing 0.1 M NaCl. This mixture was then added to 1.8 ml of the same buffer system containing 100 μM S-2238. Purified human plasma AT III was used as a standard.

HPLC procedure was carried out on a Shimadzu LC-10AD chromatograph using UV/VIS photodiode detector SPD-M10A. A SPHEROGEL TSK column (7.5 × 300 mm, Beckman, USA) and an ET NUCLEOSIL 4000-7 PEI column (4 × 125 mm, Macherey-Nagel, Germany) were used for gel-permeation and anion-exchange chromatography, respectively. The free volume of the column for gel-permeation chromatography was determined using Blue dextran (2000 kD); the column was calibrated using thyroglobulin (670 kD), γ-globulin (158 kD), ovalbumin (45 kD), and cytochrome *c* (12.5 kD). Chromatographic conditions and treatment of chromatograms (gradient shape, integration of peak areas, eluent flow rate) were programmed by Shimadzu CLASS VP (Japan).

Statistical treatment was carried out using electronic tables of Microsoft Excel 2000.

RESULTS AND DISCUSSION

Effect of ultrasound on structural–functional transitions of AT III: native–latent–aggregate forms. Preliminary experiments revealed that ultrasound treatment (ranging from 5 to 15 W/cm^2) on AT III did not

influence its content (compared with incubation at 37°C). Figure 1 shows chromatograms of AT III samples illustrating more potent ultrasonic effect (26 W/cm^2) on this protein. Incubation at 37°C for 1 h was accompanied by an increase in content of aggregate forms (peak *1*, M_r > 400,000, 6.9%) than in the initial sample (1.2%); total amount of latent and native forms in the mixture was 93.1% (M_r 65,000, peak *2*). For the sample subjected to the ultrasound treatment at the same temperature the content of aggregate polymers was 23.7%, whereas content of latent and native forms was reduced to 76.3%. Inserts of Fig. 1 demonstrate electrophoregrams of AT III samples, which confirm appearance of oligomeric forms compared with the initial sample. The data suggest that in the absence of stabilizers (mannitol, sodium citrate, glycerol, albumin) highly purified AT III may undergo conversion into aggregate forms even at 37°C. Ultrasonic treatment potentiated this effect. HPLC analysis revealed that formation of polymeric aggregates did not involve formation of dimer, which dominated during crystallization of this serpin for X-ray studies [24, 25]. As a rule, protein aggregates are formed due to weak noncovalent interactions such as hydrogen bonds and hydrophobic interactions. Since AT III is a single chain glycoprotein and its six cysteine residues form three disulfide bonds [21], it is possible that aggregates may be stabilized by covalent intermolecular cross-links via disulfide bonds. Gel electrophoresis under denaturing conditions (in the presence of SDS) without protein pretreatment with 2-mercaptoethanol did not reveal the existence of intermolecular disulfide bonds. This suggests that aggregation is not a consequence of thiol-disulfide reactions during folding/refolding processes induced by ultrasonic treatment.

Figure 2 shows the effect of ultrasonic treatment duration on the content of aggregates, monomers (native and latent forms), and active AT III. During different time intervals of ultrasonic treatment, the increase in aggregates (>400,000 daltons) was accompanied by corresponding reduction in monomeric forms (65,000 daltons). This suggests that other aggregate forms (di-, tri-, and tetramers) were intermediates during formation of high molecular weight aggregates. Data of Fig. 2 also show that the proportion of aggregates did not exceed 25% of the initial AT III. At the same time remaining amidolytic activity of thrombin reflecting amount of native AT III was significantly lower than could be attributed to formation of functionally inactive aggregates. This discrepancy might be attributed to appearance of latent AT III form characterized by lower affinity to heparin or proteolytically modified serpin conformer [13, 14, 26]. To test this possibility, we analyzed various AT III samples using affinity chromatography on heparin-Sepharose (Fig. 3). The elution profile of AT III subjected to the ultrasonic treatment was characterized by three clear peaks: the first peak contained material eluted in the free volume, the second peak contained protein eluted at 0.35-0.4 M NaCl, and the third peak (eluted at 0.9-1.0 M NaCl) contained a protein exhibiting the highest affinity to the immobilized ligand.

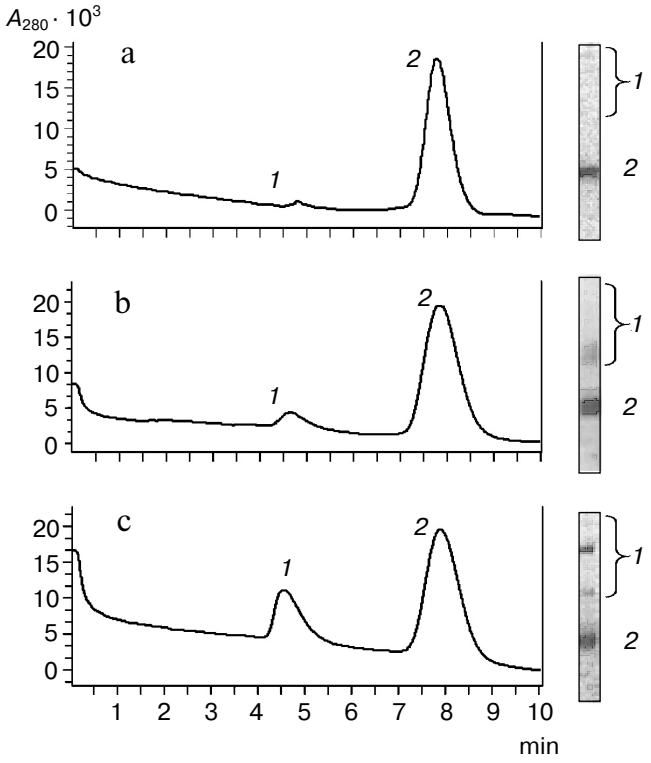

Fig. 1. Analysis of aggregate formation (*1*) and content of monomeric form (*2*) of AT III by gel-permeation HPLC and gel electrophoresis: a) initial AT III; b) AT III treated at 37°C for 1 h; c) AT III subjected to ultrasonic treatment (26 W/cm^2, 26 kHz) at 37°C for 1 h. Inserts on the right show electrophoregrams of corresponding samples of AT III.

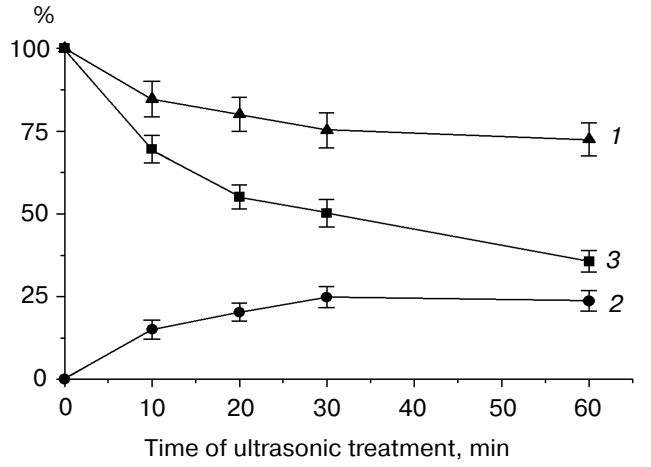

Fig. 2. Content of monomeric (*1*) and aggregate (*2*) forms and inhibitory activity of AT III (*3*) during continuous ultrasonic treatment. Data represent mean ± standard deviation of three independent experiments.

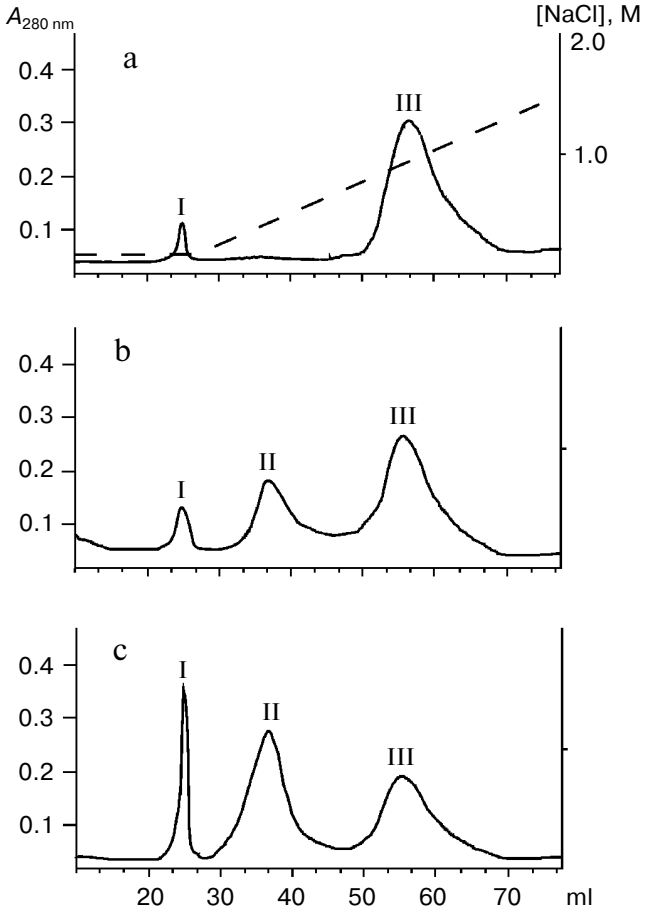

Fig. 3. Affinity chromatography of AT III on heparin-Sepharose: a) initial AT III; b) AT III treated at 37°C for 1 h; c) AT III subjected to ultrasonic treatment (26 W/cm^2, 37°C, pH 7.4) for 1 h. Numbers I, II, and III indicate pooled fractions for subsequent concentrating and analysis by gel permeation HPLC. The solid line shows absorbance at 280 nm, the broken line shows NaCl gradient in 0.01 M sodium phosphate buffer, pH 7.4.

SDS-PAGE revealed that all three peaks contained one monomeric form of protein of M_r 65,000. This suggests lack of cleavage of serpin polypeptide chain or formation of covalent intermolecular cross-links. Pooled fractions were concentrated (~10-fold). Using gel-permeation HPLC it was shown that protein of fraction I (which did not interact with the immobilized heparin) consisted of noncovalent aggregates, whereas low affinity protein of fraction II (the latent form) consisted of protomeric form of serpin (90%) and its aggregates (10%). Fraction III contained native serpin exhibiting high affinity to heparin. Table 1 shows the composition of various forms of AT III (evaluated by affinity chromatography and HPLC). These data suggest that ultrasonic treatment was accompanied by increased formation of the inhibitory-inactive latent form (exhibiting reduced affinity to heparin) and aggregates (which did not form complexes with heparin).

We have also studied the effects of various compounds on ultrasound-induced formation of aggregate and latent forms of AT III. These included physiological complex forming compound heparin [27-29] and semisynthetic analog, pentosan polysulfate, a highly sulfated polyxylose [30]. Sodium citrate was also used as an inhibitor of aggregate formation [13]. In the absence of protectors the content of aggregate, latent, and native forms in AT III samples subjected to ultrasonic treatment was nearly the same (Tables 1 and 2). Addition of heparin caused the highest protecting effect on the native form (85.2%), whereas sodium citrate and pentosan polysulfate were less effective stabilizers (content of the native form was 69.7 and 63.3%, respectively). In the presence of all protectors, the content of aggregates was 6-12%, whereas the content of monomeric forms proportionally decreased. Heparin caused the most prominent effect during formation of latent form (its content was 9.2%), whereas sodium citrate and pentosan polysulfate were less effective in stabilization of the native form (the content of the latent form was 22.3 and 24.4%, respectively) (Table 2).

The data suggest that ultrasonic treatment destabilized the AT III molecule due to impairments in intermolecular noncovalent bonds resulting in formation of aggregate and latent forms. The latent form of AT III is the most stable conformation of the serpin [13]. The mechanism responsible for polymer aggregates remains unknown. By analogy with dimer formation [15], we may assume that aggregates are linear molecules: the central reactive loop of one AT III molecule "enters" into another molecule (and this results in formation of an inactive form). The latter "delegates" its central reactive loop into the next molecule, etc. Possible intermolecular cross-links, which might stem from modification of cysteine or aromatic amino acid residues by reactive oxygen radicals, were not detected by HPLC and gel electrophoresis. In the presence of heparin, AT III is more resistant to ultrasonic treatment (in terms of aggregate and latent form appearance) than in the presence of sodium citrate or

Table 1. Content of aggregate, latent, and native forms of AT III evaluated by affinity chromatography on heparin-Sepharose and gel-permeation HPLC*

Sample	Aggregates, %	Latent form, %	Native form, %
Initial AT III	1.2 ± 0.5	—	98.8 ± 4.4
AT III, 1 h at 37°C	6.9 ± 1.6	27.3 ± 4.5	65.8 ± 2.9
AT III, US treatment, 1 h at 37°C	23.7 ± 2.1	40.6 ± 5.3	35.7 ± 3.2

* Data represent mean ± standard deviation of three independent experiments.

Table 2. Content of aggregate, latent, and native forms of AT III during ultrasonic treatment in the presence of various effectors*

Sample	Aggregates, %	Latent form, %	Native form, %
AT III, US treatment, 37°C, 1 h	22.2 ± 2.4	37.8 ± 4.7	40.0 ± 3.1
+ heparin (0.2 mM)	5.6 ± 2.0	9.2 ± 3.3	85.2 ± 2.5
+ pentosan polysulfate (0.2 mM)	12.0 ± 1.5	24.4 ± 4.0	63.6 ± 4.6
+ sodium citrate (0.25 M)	8.0 ± 3.1	22.3 ± 2.9	69.7 ± 3.8

* Data represent mean ± standard deviation of three independent experiments.

pentosan polysulfate. Complex of AT III and heparin is stabilized by many hydrogen bonds formed between sulfate or carboxyl groups of pentasugar sequence of the heparin molecule and Arg and Lys residues of the inhibitor molecules [27]. Stabilization may also involve indirect hydrophobic interactions of the polysaccharide with Phe121 and Phe122 residues [31]. Being rather large polyanions, sulfated polysaccharides block (with varying effectiveness) conformational changes of AT III promoting aggregate association. Thus, the data suggest that under conditions of ultrasonic treatment of thrombi *in vivo* ultrasound may be the factor responsible for formation of conformationally-inactive forms of the serpin, and, consequently, for increased local activity of blood coagulation factors.

Effect of ultrasonic treatment on PPSB complex. Activated coagulation factors, thrombin (FIIa) and factor Xa, are molecular targets of AT III. X-Ray and biochemical studies revealed the mechanism of conformational activation of AT III by heparin followed by subsequent "capture" of activated coagulation factors by serpin molecules. For evaluation of possible direct ultrasonic effect we used PPSB complex containing coagulation factors IX, II, VII, and X. Changes in the content of non-activated factors induced by ultrasonic treatment were determined by HPLC after separation of protein factors of PPSB complex under non-denaturing conditions. Use of a strong cation exchanger carrying sulfonic acid residues (Nuckleogel SCX 1000-8/46) or hydrophobic sorbent containing phenyl residues (TSK Phenyl-5PW) did not result in satisfactory resolution under various chromatographic regimes. As suggested (on the basis of isoelectric points of separate factors), the best results were obtained during anion-exchange HPLC on sorbent containing polyethyleneimine (Nucleosil 4000-7 PEI). Figure 4 shows chromatograms of control (37°C, 1 h) and sonicated (26 W/cm^2, 37°C, 1 h) samples of PPSB complex. Peak integration revealed (with correction of coefficients of molar absorbance at 280 nm for individual factors) relative content of coagulation factors in the sonicated sample: factor VII (15%), factor X (22%), factor II (28%), and factor IX (35%). These values corresponded to specification of the commercial preparation by relative activity and they were close to the control sample of PPSB complex (within the accuracy of the HPLC method). It should be noted that the HPLC procedure was carried out under denaturing conditions and so formation of "light" and "heavy" chains as products of proenzyme activation was not fixed due to the existence of disulfide bonds. Similar behavior of elution profiles suggest that acoustic-chemical reactions did not alter protein structure (modification of side chains of amino acids, covalent or noncovalent aggregation). During gel electrophoresis, control and sonicated samples of PPSB complex were characterized by similar qualitative and quantitative distribution of polypeptide chains (see inserts in Fig. 4). Additional rather insignificant accumulation of "heavy" and "light" chains compared with initial PPSB complex suggested minor activation of proenzymes by traces of endogenously activated proteases [18]. Ultrasonic treatment did not promote endogenous proteolysis and activation of coagulation factors in PPSB preparation. Tolerance of blood

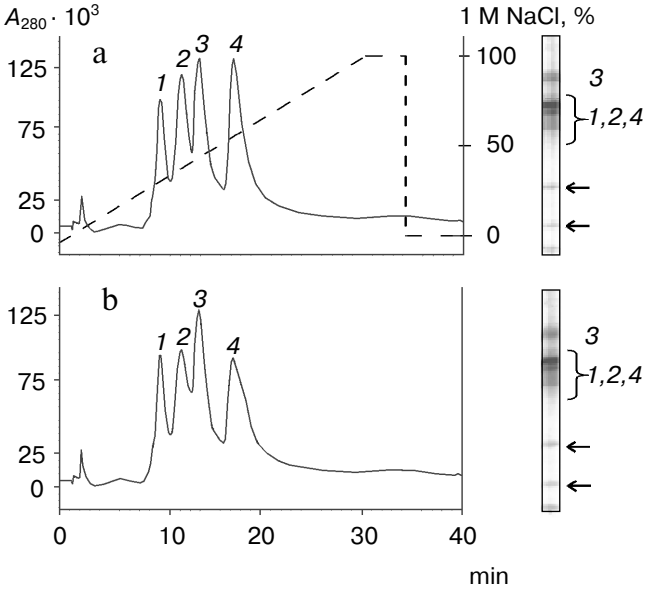

Fig. 4. Analysis of proteins of PPSB complex by anion-exchange HPLC and SDS-PAGE after the incubation at 37°C for 1 h (a) and continuous ultrasonic treatment at 37°C for 1 h (b): *1*) factor VII; *2*) factor X; *3*) factor II; *4*) factor IX. Arrows on electrophoregrams indicate "heavy" and "light" chains of the coagulation factors. The solid line shows absorbance at 280 nm; the broken line shows NaCl gradient in 0.01 M sodium phosphate buffer, pH 7.4.

coagulation proenzymes to the ultrasonic treatment may be attributed to unique structural organization of these proteins [32]. Due to the presence of carbohydrate components, these four-domain glycoproteins are resistant to damages by free radicals generated by ultrasound [33]. Molecules of these proteins are also stabilized by numerous intrachain disulfide bonds, which might prevent unfolding and denaturation of native protein globules during ultrasonic treatment. Commercially available PPSB complex also contains heparin, which may stabilize proteins due to complex formation and act as effective "scavenger" of free radicals. Particularly, sulfated polysaccharides may act as complex forming ligands and intermolecular noncovalent cross-linking agents for proteins of PPSB complex [34]. Although direct effect of ultrasound on activation of blood coagulation proenzymes was not detected, the effect of ultrasound on kinetics of their activation under various conditions requires further investigations because the functional models may be more informative during evaluation of acoustic-chemical effects. For example, we found that supratherapeutic doses of ultrasonic treatment of a protein plasminogen activator, streptokinase, caused irreversible aggregation and more intensive proteolysis by plasmin; during activation of equimolar mixture of trypsinogen and chymotrypsinogen, trypsin was the component most sensitive to the ultrasonic treatment [35].

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