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Detection of a novel plasma serine protease during purification of vitamin K-dependent coagulation factors

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Abstract A novel serine protease (PHBSP) was purified from human plasma by two chromatographic steps with a final yield of 1.6 mg/l plasma. The protease consists of two disulfide-bridged chains of about 50 and 30 kDa with the light chain containing the active site of the enzyme. NH_2-terminal sequence analysis revealed identity to the deduced amino acid sequence of HGFA-like mRNA. The activity of PHBSP is strongly dependent on Ca^{2+} ions and is efficiently inhibited by α_2-antiplasmin and aprotinin. Possible functions of PHBSP in the hemostatic system are discussed.

Key words: Plasma serine protease;
Vitamin K-dependent coagulation factor;
Plasma hyaluronan binding protease (PHBP);
Hepatocyte growth factor activator (HGFA)-like

1. Introduction

A large number of proteolytic enzymes circulates in blood as zymogens and is specifically activated by limited proteolysis upon a variety of physiological events. Best known are the proteases involved in coagulation, fibrinolysis and complement activation. Another group of plasma proteins (about 10% of total plasma proteins) belongs to the large group of protease inhibitors, most of them are serine protease inhibitors (serpins) which play a central role in the regulation of coagulation and fibrinolysis. In order to study single steps in the coagulation system, clotting factors have to be separated from other plasma proteins, especially the very abundant serum albumin, immunoglobulins and serpins. A well-established protocol for the enrichment of vitamin K-dependent coagulation factors includes the cryoprecipitation of fresh plasma and the subsequent binding of cryo-supernatant proteins to an anion exchange chromatography matrix followed by elution of proteins with increasing salt concentrations [1,2]. During this type of purification we observed an amidolytic activity in the high salt column regeneration wash towards a thrombin-sensitive chromogenic substrate. Since hirudin did not inhibit this activity, the substrate cleavage was considered not to be caused by thrombin but by another protease which is generated during the purification procedure. Hirudin-insensitive amidolytic activities towards thrombin-selective chromogenic substrates have previously been described to be present in some therapeutically used clotting factor concentrates [3,4]. As the enzyme responsible for the chromogenic activity had not yet been identified, we were interested to clarify the identity of the protease.

Here we describe the purification of this novel serine protease from human plasma, which we call plasma hyaluronan binding serine protease (PHBSP) and present first characteristics of this enzyme. Possible physiological functions of PHBSP are discussed.

2. Materials and methods

2.1. Materials

Materials were obtained from the following sources: Aprotinin-Eupergit from Fluka (Buchs, Switzerland); chromogenic substrates of the S series from Chromogenix (Mölndal, Sweden); chromogenic substrates of the Pefachrome series from Pentapharm (Basel, Switzerland) (cf. Table 1); DEAE Sephadex A50 from Pharmacia (Freiburg, Germany); monoclonal antibodies, extravidin-peroxidase, 3,3′-diaminobenzidine tetrahydrochloride (DAB), 7-hydroxy-7-*O*-(*p*-guanidinobenzoyl)-4-methylcumarin (HBMC), 3,3′,5,5′-tetramethylbenzidine (TMB), α_2-antiplasmin, and trypsin from Sigma (Deisenhofen, Germany); anti-mouse IgG-peroxidase from BioRad (München, Germany); biotinylated D-Phe-Pro-Arg-chloromethylketone (PPACK), α_1-proteinase inhibitor, and C1-esterase inhibitor from Calbiochem (Bad Soden, Germany); aprotinin from Boehringer (Mannheim, Germany). Recombinant hirudin was prepared according to [5].

2.2. Purification of plasma hyaluronan binding protease (PHBSP)

Purification was performed at 4°C. One l frozen plasma was thawed overnight and centrifuged (30 min at $22\,000 \times g$) in order to remove the cryoprecipitate. After addition of DEAE Sephadex A 50 (1.5 g of the dry gel matrix pre-swollen in 10 mM tri-sodium citrate, 125 mM NaCl) and stirring of the suspension for 60 min, the gel was filled into a column and washed with at least two column volumes of washing buffer. Bound proteins were stepwise eluted with buffer E1 (10 mM tri-sodium citrate, 0.5 M NaCl) and buffer E2 (10 mM tri-sodium citrate, 2 M NaCl).

Fractions containing amidolytic activity were pooled and precipitated by addition of ammonium sulfate (66% of saturation). The precipitate was dissolved in 50 mM Tris, 0.5 M NaCl, pH 8.0 and ammonium sulfate was removed by gel filtration. This solution was applied to an Aprotinin-Eupergit affinity column (4 U PHBSP/ml gel, substrate S2765, see below). The matrix was washed with five column volumes of each, equilibration buffer and 20 mM MES, pH 6.0, and the bound protein was eluted with 100 mM sodium acetate, 0.2 M NaCl, pH 4.0. Fractions containing amidolytic activity were pooled, adjusted to pH 5.5 with 2 M Tris-base and stored in small aliquots in liquid nitrogen.

2.3. Amidolytic assays

Enzyme activity was determined in amidolytic assays using various chromogenic substrates. Assays were performed in microplates at 37°C in 25 mM Tris-HCl, pH 8.2, containing 75 mM NaCl, 5 mM $CaCl_2$ and either 0.1% BSA or 0.05% Tween-20 in a total volume of 0.1 ml. Substrates were used at a final concentration of 1 mM. Enzyme activities are expressed as U, which is defined as the activity which cleaves 1 μmole substrate per minute. Inhibition experiments were performed under the same buffer and temperature conditions.

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Abbreviations: PHBSP, Plasma hyaluronan binding serine protease; HGFA, Hepatocyte growth factor activator

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Table 1
Chromogenic substrate profile

Substrate	Selectivity	Formula	Relative activity (%)
Pefa-3297	APC	H-D-Pyr-CHG-Arg-pNA	124
Pefa-5114	Thrombin	H-D-CHG-Ala-Arg-pNA·2AcOH	114
S-2765	FXa, trypsin	Z-D-Arg-Gly-Arg-pNA·2HCl	100
Pefa-5523	FXa	CH_3OCO-D-CHA-Gly-Arg-pNA·AcOH	98
S-2366	APC	Pyr-Pro-Arg-pNA·HCl	73
Pefa-5773	APC	H-D-Lys(Cbo)-Pro-Arg-pNA·2AcOH	70
S-2238	Thrombin	H-D-Phe-Pip-Arg-pNA·2HCl	66
Pefa-5979	FVIIa	CH_3SO_2-D-CHA-But-Arg-pNA·AcOH	65
Pefa-3107	FIXa	CH_3SO_2-D-CHG-Gly-Arg-pNA·AcOH	42
Pefa-5329	Plasmin	H-D-Ala-CHT-Lys-pNA·2AcOH	27
S-2222	FXa, trypsin	Bz-Ile-Glu(γ-OR)-Gly-Arg-pNA·HCl	14

Substrates are listed in the order of PHBSP activity relative to S2765. (Pyr, L-pyroglutamic acid; CHG, cyclohexylglycine; CHA, cyclohexylalanine; CBO, benzyloxycarbonyl; CHT, cyclohexyltyrosine; Bz, benzoyl).

Kinetic constants of the interaction between PHBSP and aprotinin were determined according to the theory of tight binding inhibition by non-linear regression analysis [6,7]. The dependence of PHBSP activity from divalent cations was tested in 50 mM Tris-HCl, pH 8.4, 100 mM NaCl, 0.2% BSA.

2.4. ELISA

Fractions of the DEAE eluate were tested for the presence of the coagulation factors IX and X by ELISA using mouse monoclonal antibodies, anti-mouse IgG-POD and TMB as substrate.

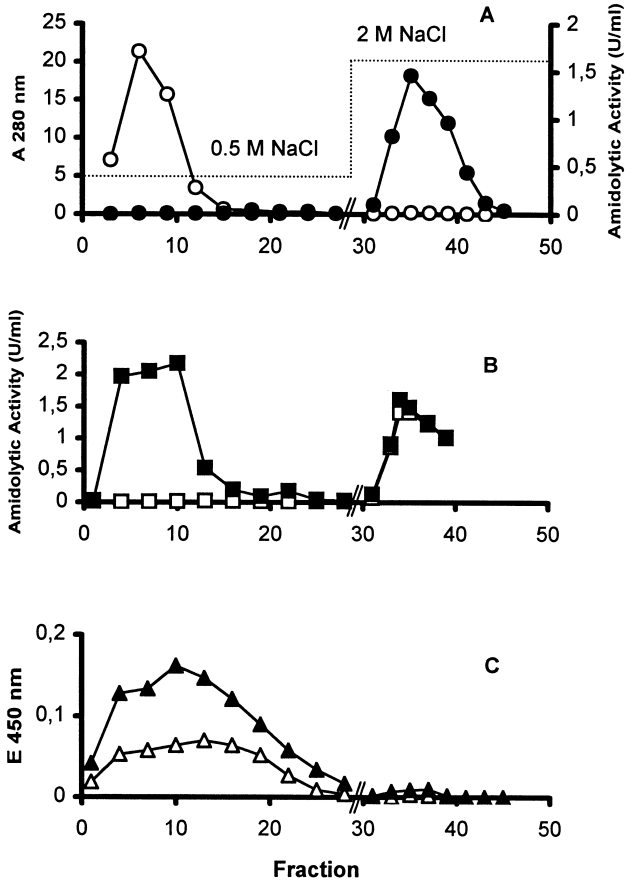

Fig. 1. Elution profiles anion exchange chromatography. A: Protein content (by A_{280}; open circles) and chromogenic activity using S2765 (solid circles). B: Fractions containing FII were detected after activation with ECV. The amidolytic activity is shown before (open squares) and after (solid squares) activation using S2238. C: Detection of FX (solid triangle) and FIX (open triangle) containing fractions was achieved by ELISA.

2.5. Protein concentration

Total protein content was determined by measuring the absorbance at 280 nm. The molar extinction coefficient of PHBSP at 280 nm was calculated from the deduced amino acid sequence of the mature protein according to Gill and von Hippel [8] and amounts to 93 220 M^{-1}.

2.6. Gel electrophoresis and Western blot

Proteins were separated on SDS polyacrylamide gels according to Laemmli [9]. For Western blotting the gel was preincubated for 20 min in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3), followed by transfer to a PVDF membrane (Millipore, Eschborn, Germany) for 40 min at 15 V in a Semi-Dry blotting chamber (BioRad, München, Germany).

2.7. Active site labelling

Affinity-purified PHBSP was incubated with the biotinylated serine protease inhibitor PPACK prior to SDS-PAGE and Western blot as described above. Inhibitor-labelled bands were visualized with extravidin-peroxidase and DAB as substrate.

2.8. Amino acid sequence analysis

After SDS-PAGE proteins were transferred to a PVDF membrane and stained with Coomassie. N-terminal amino acid sequences of the excised blotted protein bands were analyzed by automated Edman degradation with an Amino Acid Sequencer Procise 492 with online PTH-Analyser 140C and UV detector 785A (Perkin Elmer/Applied Biosystems, Weiterstadt, Germany).

2.9. Active site titration

The number of active sites of PHBSP was determined in order to calculate kinetic constants. Since for PHBSP no active site titrant was available the concentration of active sites was calculated as follows: First, the concentration of active sites of a trypsin preparation was determined by titration with the tight binding fluorogenic substrate HBMC in a Perkin Elmer fluorimeter LS50B (excitation: $\lambda = 365$ nm; emission: $\lambda = 445$ nm). Second, a solution of aprotinin was titrated with trypsin (100 nM final concentration) according to the tight binding theory [6,7]. Finally, from tight binding inhibition experiments with aprotinin of known concentrations, the concentration of proteolytically active PHBSP was calculated.

3. Results and discussion

3.1. Isolation of PHBSP during purification of vitamin K-dependent coagulation factors

After binding of the plasma proteins to the anion exchanger DEAE Sephadex A50 and rinsing of the matrix with 0.125 M NaCl, the majority of bound proteins was eluted with buffer E1 containing 0.5 M NaCl (Fig. 1A). In order to regenerate the gel the column was subsequently washed with buffer E2 containing 2 M NaCl. Interestingly, a strong amidolytic activity towards thrombin- and FXa-selective chromogenic substrates was eluted during this step (Fig. 1A, B). As shown by

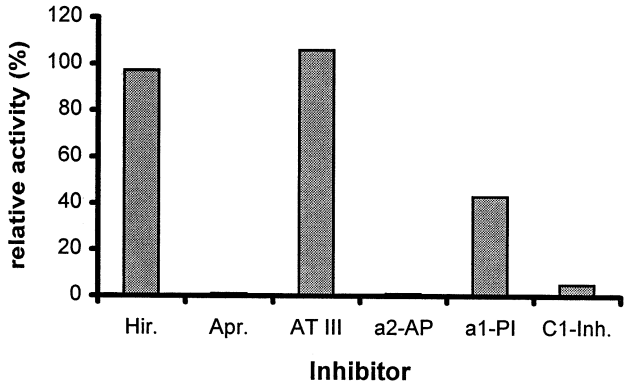

Fig. 2. Inhibition profile of purified PHBSP. Remaining PHBSP activity was determined after 5 min incubation with hirudin (Hir, 1.35 U/ml), aprotinin (Apr, 0.8 μM), antithrombin (AT III, 5 μM), α2-antiplasmin (a2-AP, 0.2 μM), α1-proteinase inhibitor (a1-PI, 1 μM) and C1-esterase inhibitor (C1-Inh, 1 μM).

ELISA, the vitamin K-dependent coagulation factors FIX and FX (Fig. 1C) as well as FVII and protein C (data not shown) were predominantly eluted with buffer E1. This is also valid for FII, since incubation of prothrombin with the specific activator ECV revealed the generation of thrombin activity, whereas the cleavage of the thrombin-selective substrate in the E2 eluate was not enhanced by ECV (Fig. 1B). The latter activity could not be inhibited by hirudin or AT III but by aprotinin (inhibition experiments with the purified protease are shown in Fig. 2), indicating that thrombin is not responsible for the observed activity. Thus, from the elution profile and the inhibition experiments it was concluded that the amidolytic activity was not caused by a protease of the coagulation system.

Since aprotinin was an effective inhibitor of the amidolytic activity, aprotinin affinity chromatography using Aprotinin-Eupergit was applied for further purification of the protease. Another aprotinin matrix, Aprotinin-Sepharose was not suitable, probably because of lacking a spacer. The amidolytic activity was strongly reduced in the aprotinin column flow-through but could be retrieved in the eluate (data not shown). The final yield of purification was about 1.6 mg protein/l plasma with a specific activity of 50 U/mg (substrate S2765).

SDS gel electrophoresis of the proteins obtained from each purification step revealed the accumulation of a dominant 70 kDa band under non-reducing conditions (Fig. 3A2). Under reducing conditions two bands of about 50 kDa and 30 kDa were observed (Fig. 3A1) suggesting that the unknown proteolytic activity is caused by the 70 kDa protein which comprises a two-chain, disulfide linked molecule. Occasionally, under reducing conditions two additional bands of 40 and 17 kDa could be observed. As shown by amino acid sequence analysis, these additional bands were fragments of the 50 kDa and 30 kDa polypeptide chains, respectively (Table 2). In order to identify the active site containing chain of the molecule, the affinity-purified protein was treated with the biotinylated serine protease inhibitor PPACK prior to SDS gel electrophoresis. As shown in Fig. 3B, the 70 kDa protein is strongly labelled under non-reducing conditions, whereas under reducing conditions the inhibitor is exclusively associated with the 30 kDa subunit. These data demonstrate that the protein purified from plasma is an active serine protease which consists of a heavy and a light chain covalently linked by disulfide bond(s) and that the proteolytic activity is located on the light chain.

3.2. Amino acid sequence analysis

In order to unequivocally identify the enzyme N-terminal amino acid sequence analyses of the protein bands separated by SDS-PAGE under reducing conditions were performed (Table 2). The 30 kDa and the 17 kDa fragments exhibited identical N-termini. This implies that the smaller fragment is the N-terminal part of the 30 kDa chain. The 50 kDa and the 40 kDa fragments have N-termini that are distinct from each other. A homology search in a data bank revealed that these sequences were not known on the level of protein sequence analysis. However, nearly complete identity was found to the putative amino acid sequence deduced from the hepatocyte growth factor activator (HGFA)-like mRNA (registered in DDBJ, accession number D49742). Only the Lys at position 6 of the 50 kDa chain is different from the predicted amino acid Trp. In our analysis there is also a minor signal of Trp at this position (about 10% of the signal for Lys). Furthermore, we found two equivalent signals for Ala and Asp at position 9 of the same chain, whereas the deduced amino acid is Asp. Whether these differences are due to a sequencing error or to an amino acid polymorphism is not yet clear. The N-terminus of the 50 kDa fragment is located eight amino acids downstream of the N-terminus of the putative mature HGFA-like protein. Whether this terminus arises from another cleavage site of the signal peptidase or results from cleavage related to the activation of the proenzyme or whether this terminus is caused by partial degradation remains to be elucidated.

In 1996 the purification and characterization of a novel hyaluronan binding protein from human plasma (PHBP) was published, which also had identical amino acid sequences to the putative HGFA-like protein [10]. The strategy for the purification of PHBP revealed a protein which gave a single 70 kDa band on SDS-PAGE under non-reducing conditions,

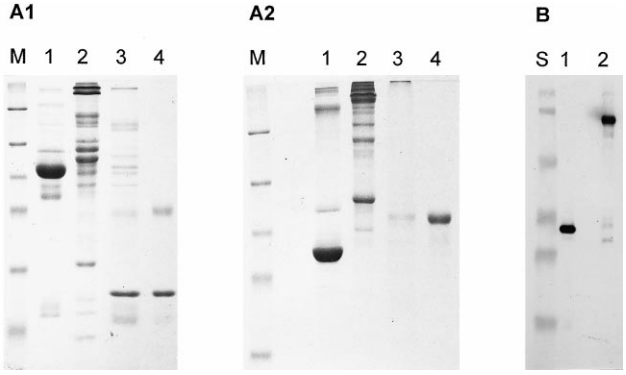

Fig. 3. SDS-PAGE and Western blot analysis of samples on various steps of purification. A: Samples were run on a 12.5% gel under reducing conditions (A1) or on a 10% gel under non-reducing conditions (A2) and stained with Coomassie. Lane M, molecular mass marker (175, 83, 62, 47.5, 32.5 and (only in A1) 25 kDa); lane 1, plasma; lane 2, 0.5 M NaCl eluate of the DEAE chromatography; lane 3, 2 M NaCl eluate; lane 4, purified PHBSP after aprotinin affinity chromatography. B: Affinity-purified PHBSP was labelled with biotinylated PPACK prior to electrophoresis and transferred to a PVDF membrane. Labelled bands were visualized with extravidin-peroxidase. Lane S, prestained molecular mass marker (104, 82, 48.3, 33.4, 28.3, and 19.4 kDa); lane 1, reducing conditions; lane 2, non-reducing conditions.

Table 2
N-terminal amino acid sequences

Peptide	Sequence
p50	SLDPDKXPA(D)Q
p40	HAENPDWYYTE
p30	IYGGFKSTAG
p17	IYGGFKSTAG

Sequence analysis was performed on peptides derived from the enzyme by SDS-PAGE under reducing conditions.

and 50 kDa and 17 kDa under reducing conditions. For the following reasons we suggest that PHBP and the protease described here are several forms of the same precursor protein. PHBP and PHBSP have comparable features regarding the origin from human plasma, the apparent molecular mass under non-reducing conditions, the heterodimeric composition, the amino acid sequences, the hyaluronan binding properties (data not shown) as well as the yield of the purification. The latter point is so far surprising as with our purification procedure using aprotinin affinity chromatography only an active form of the enzyme was isolated, whereas the purification by hyaluronan affinity chromatography should also be capable to isolate the zymogen of the protease. The difference regarding the apparent molecular mass of the light chain can be explained by further proteolytic degradation of the fragment as was suggested by Choi-Miura et al., since the 17 kDa fragment is much smaller than the molecular mass calculated from the amino acid sequence [10]. We also observed a C-terminal degradation product of the light chain with a comparable molecular mass during the storage of the purified protease. This degradation is accompanied by a loss of amidolytic activity. This could also be the reason for the observation that PHBP exhibits only a weak proteolytic activity with inter-α-trypsin inhibitor as substrate [10]. Until now the mechanism of degradation is not yet clear. Since no further proteases than PHBSP are detectable in our preparations, an autoproteolytic mechanism seems likely.

In consideration of the above mentioned observations we believe that PHBP with its 17 kDa light chain is an inactive degradation product of the protease described here. Therefore, in order to distinguish the novel protease of this study from PHBP and to emphasize the enzymatic activity of the former, we called the protein plasma hyaluronan binding serine protease (PHBSP).

3.3. Substrate/inhibitor profile

Substrates with selectivity for thrombin, factor Xa, Factor VIIa and activated protein C were all hydrolyzed with a comparable velocity (Table 1). Kinetic analysis according to Michaelis–Menten revealed a K_m of 0.58 mM for S2238, which is in the range usually found for chromogenic substrates of serine proteases involved in blood coagulation and fibrinolysis. The specificity constant k_{cat}/K_m amounts to 125 mM^{-1} s^{-1}. For Pefa-3297, the substrate with the highest cleavage rate of the substrates under investigation, a K_m of 0.56 mM and a specificity constant of 250 mM^{-1} s^{-1} was estimated.

Although the substrate specificity was not investigated systematically, we assume that Arg at position P1 is a better cleavage site than Lys (Pefa-5329). Furthermore, all substrates exhibit an uncharged amino acid residue at P2. The size of the side chains of these amino acids appears to have no influence on the reactivity (Pefa-3297, Pefa-3107). S2222 has the same P1 and P2 residues as S2765, Pefa-5523 and Pefa-3107, but is less reactive. This could be due to the acidic properties of the Glu(γ-OR) (R is each 50% CH_3 and H) at P3 or the Ile at P4.

PHBSP activity was strongly suppressed by a variety of natural serine protease inhibitors, most efficiently by aprotinin and α_2-antiplasmin (Fig. 2). For aprotinin, an inhibition constant K_i of 0.88 nmol/l was determined in tight binding experiments. C1-esterase inhibitor and α1-proteinase inhibitor also inhibited PHBSP, but were less effective, whereas AT III did not influence the activity. Nevertheless, human plasma possesses a considerable inhibition capacity for PHBSP as was demonstrated by the loss of activity after spiking of plasma dilutions with PHBSP (data not shown).

3.4. Influence of bivalent metal ions on PHBSP

Since many enzymes depend on bivalent cations as cofactors we investigated the influence of eight different bivalent metal ions onto the chromogenic activity of the protease. Fig. 4 shows that PHBSP activity was strongly enhanced by calcium ions, yielding a 3.5–4-fold increase of activity when 2 mM Ca^{2+} was present in the reaction mixture. Two other cations, Mn^{2+} and Mg^{2+}, showed also an activating effect. All other metal ions had a rather inhibitory effect at higher concentrations. The result of this experiment implicates the presence of a Ca^{2+} binding site on PHBSP. The typical calcium ion binding site of vitamin K-dependent coagulation factors, the Gla domain, is not present on PHBSP as can be concluded from the deduced amino acid sequence of PHBP cDNA [10]. Another Ca^{2+} binding structure, the epidermal growth factor (EGF) domain which is present in many extracellular proteins including those of the coagulation and fibrinolytic system [11], is found in PHBSP in triplicate. However, a proposed consensus amino acid sequence for calcium ion binding EGF domains D/N X D/N E/Q X_m D/N X_n Y/F (m, n: variable numbers of amino acids) [12] is not found in any of the PHBSP EGF modules. Whether EGF domains on PHBSP have indeed Ca^{2+} binding properties or whether there is a yet unknown Ca^{2+} binding motif on PHBSP remains to be elucidated.

3.5. Investigations on the physiological function of PHBSP

In order to investigate the influence of PHBSP on the hemostatic system we performed global clotting tests (prothrombin time, activated partial thromboplastin time) and platelet aggregation studies (final PHBSP activity: 0.016–1.6 U/ml, substrate Pefa-5114). The results of these experiments revealed

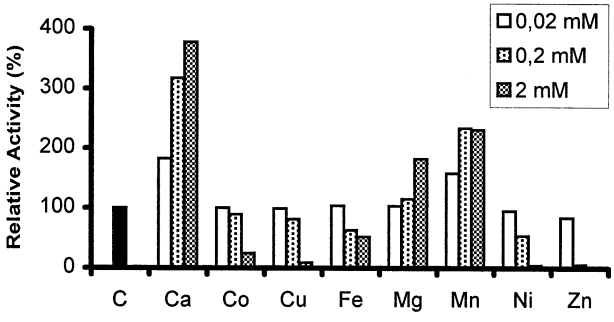

Fig. 4. Dependence of PHBSP on divalent cations. Cations were added as chloride salts except Co (nitrate), Cu and Fe (sulfates); C, control.

no hints for any effect of PHBSB neither on the intrinsic or extrinsic coagulation system nor on the platelet function. However, another research group, which became interested in PHBSP after presentation of our data on a scientific meeting [13], recently described an activation of FVII and an inactivation of FVIII and FV by PHBSP [14]. We could confirm these findings in so far as we could also demonstrate a shortening of the clotting time of FVII depleted plasma using the STACLOT VIIa-rTF assay (Roche Diagnostics, Mannheim, Germany) after addition of a partially purified FVII concentrate, which was preincubated with PHBSP compared to control experiments without PHBSP or after inactivation of PHBSP by aprotinin. Furthermore, besides the possible pro- and anticoagulatory properties a profibrinolytic activity of PHBSP by activating single chain plasminogen activators was described [15].

Nevertheless, the physiological relevance of these findings remains to be clarified. Taking the strong inhibitory properties of human plasma into consideration it is of primary interest to evaluate whether PHBSP exhibits the functions described above also in vivo. Thus, further work is needed to elucidate the role of PHBSP in the hemostatic system.

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