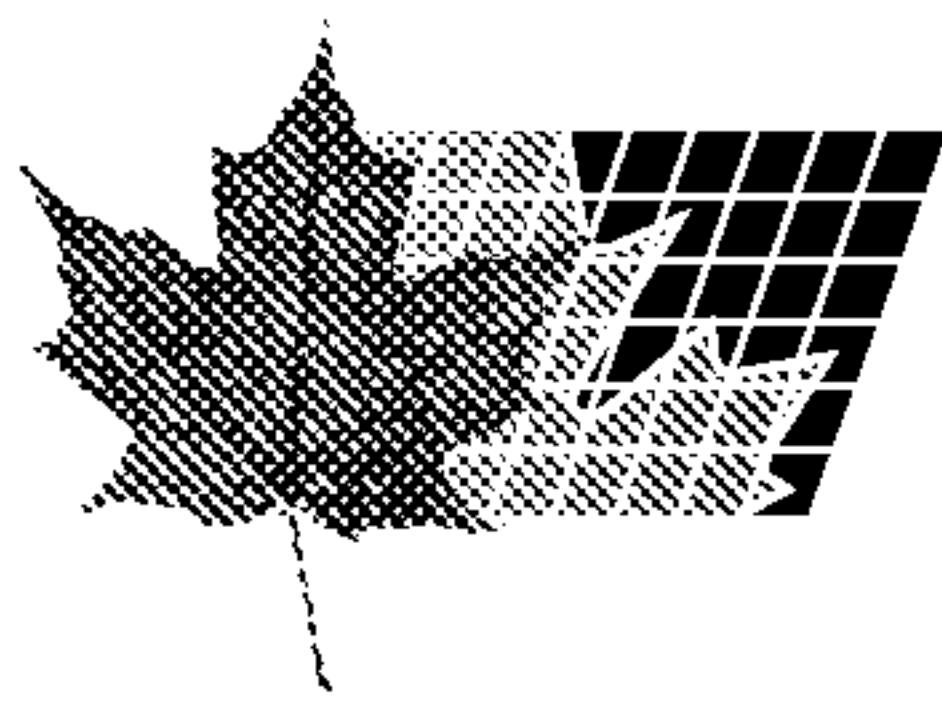




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(54) **FACTEUR SANGUIN ACTIVE DEPENDANT DE LA VITAMINE
K ET PROCEDE DE PREPARATION DUDIT FACTEUR**

(54) **ACTIVATED VITAMIN K-DEPENDENT BLOOD FACTOR AND
METHOD FOR THE PRODUCTION THEREOF**

(57) Procédé d'activation d'un facteur sanguin dépendant de la vitamine K qui consiste à traiter ledit facteur avec une protéase qui est dérivée de plantes ou de procaryotes. La présente invention concerne en outre le facteur sanguin ainsi produit, qui ne présente pas de contamination par des protéases animales, ainsi qu'une préparation pharmaceutique et un kit d'application médicale.

(57) The invention relates to a method for activating a vitamin K-dependent blood factor by treatment with a protease derived from plants or procaryotes. The invention also comprises the blood factor produced according to the invention, which shows no animal protease contamination, as well as a pharmaceutical preparation and a set for medical application.

(57) Abstract

The invention relates to a method for activating a vitamin K-dependent blood factor by treatment with a protease derived from plants or procaryotes. The invention also comprises the blood factor produced according to the invention, which shows no animal protease contamination, as well as a pharmaceutical preparation and a set for medical application.

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**Activated Vitamin K-Dependent Blood Factor and Method
for the Production Thereof**

The present invention relates to an activated vitamin K-dependent blood factor as well as a method for its production.

The complex protease/substrate cascade in the physiological activation of profactors, especially in the area of blood coagulation, requires highly selective proteases. These proteases are present in the human organism itself, particularly in blood plasma. The use of other human proteases for activating profactors, such as for example the digestive enzymes trypsin and pepsin, do not lead to a selective generation of activated blood coagulation factors, but rather to an unspecific degradation of the target substrate. Thus, it is known for example that the tryptic degradation of coagulation factor X activates at the beginning but then leads to low molecular peptides due to unspecific degradation. It is known from AT 397 390 that particular process variations must be adhered to for the activation of prothrombin with the aid of a digestive enzyme in order to prevent a further unspecific degradation of the resulting thrombus.

Therefore, the activation of factor X represents a special case in that a selective factor X activator is available from the venom from *Vipera russelli* (Russell's Viper Venom RVV) which can also be used industrially for obtaining factor Xa. However, since the recognition of the transmission of animal viruses to a less related animal species (prion problem), the use of xenogenic animal proteins as adjuvants for the production of drugs has been viewed with disfavour. But, since suitable human proteases are not available in industrially sufficient amounts, the use of RVV for preparative factor X activation is as before a standard method.

It is known that proteolytic enzymes which can be isolated from prokaryotes, from lower eukaryotes, such as for example fungi, or from higher eukaryotes, such as for example plants, have a low substrate specificity. For this reason, they are mainly used for the total degradation of proteins in crude cell extracts in order to separate or to isolate other cell components, such as for example carbohydrates or nucleic acids in this manner. Additionally, these proteases are also employed for sequencing and characterizing proteins via degradation to small peptides. Up to now, the use of bacterial or plant proteases or proteases from fungi as activators of plasma proteins or plasma protein co-factors or inhibitors from the group of prothrombin complex proteins has not been known.

An object of the present invention is to provide a method for activating pro-factors in the area of blood coagulation with the aid of highly specific proteases which are not of animal origin.

The above problem is solved according to the invention by the method mentioned in the introduction, whereby the activation occurs by means of a protease which is derived from plants or prokaryotes.

The method according to the invention is especially suited for activation of vitamin K-dependent blood factors. Particularly suitable is the activation of human blood factors from the group factor II, VII, IX, X and protein C. The following examples demonstrate the advantages as they are obtained in the activation of factor X to factor Xa- β .

The method according to the invention is equally suitable for obtaining naturally occurring, recombinantly produced as well as chemically or recombinantly modified blood factors.

With respect to the protease used according to the invention, one can start from a proteolytic enzyme from prokaryotes. Here, the bacterial proteases such as for example thermolysin, clostripain proteases IX and X from *Bacillus polymyxa* and protease IX from *Bacillus thermoproteolyticus* are to be especially mentioned.

Additionally, proteases from lower eukaryotes, such as for example fungi, especially protease XXIII from the mould fungus *Aspergillus oryzae*, can also be used in the method according to the invention. Moreover, proteolytic enzymes from higher eukaryotes, such as for example plants, are also suitable. Proteolytic enzymes are especially suitable which belong to the group of cysteine proteases. For example, those to be mentioned here: bromelain (for example from *Ananas comosus*), papain (for example from the milk of *Carica papaya*) or ficin (for example from *Ficus carica*). However, the mentioned proteolytic enzymes from prokaryotes, mould fungi and plants have a low substrate specificity. Therefore, it was surprising to determine that pro-factors of blood coagulation can be highly specifically activated by using these enzymes. These enzymes are either selected native enzymes but can also be modified enzymes and/or enzyme derivatives, especially enzymes produced by recombinant DNA technology.

It is particularly known of cysteine proteases that they completely exhibit their broad protease activity under reductive conditions or in the presence of activators containing sulfhydryl groups. For this reason, SH-donors are frequently added to the incubation buffer as effectors during the incubation with these enzymes. However, the broad activity spectrum of these proteases can be shifted to sub-optimal behaviours by deviation from the optimal incubation conditions, i.e. conditions apart from the pH or temperature optimum or with partial or complete lack of necessary co-factors or effectors, or in the presence of particular effectors, to the point that special substrates such as for

example plasma proteins are no longer completely degraded and, therewith, their activity is not lost as far as this concerns pro-factors which have to be activated. Rather, the activation is stopped at the level of the target substrate and the activation products as such are obtained. This is also demonstrated for the first time using the example of the activation of factor X to factor Xa, especially β -factor Xa.

Moreover, a higher specificity of the cysteine proteases can also be obtained by subjecting the suitable crude enzyme preparations from plants to further purification steps, for example chromatography, in order to isolate those protein fractions whose protease activity exhibits a limited substrate spectrum. Whereas this measure alone is possibly not suitable in order to obtain the desired highly specific activators, it is suitable in connection with a further step for increasing the specificity of proteases, namely by oxidation of these whereby the proteases are reduced in their activity and their activity spectrum as a result of the contact with atmospheric oxygen. It emerges from this that oxidized cysteine proteases are particularly suitable for the method according to the invention, whereby this is preferably the case with chromatographically enriched and/or purified cysteine proteases. Thereby, it is particularly preferred that the protease has an at least two-fold increased specific blood factor activation activity compared to the crude extract from plants or cell cultures and/or compared to the unspecific proteolytic activity.

Furthermore, the substrate spectrum can be narrowed in that defectors are added to the incubation buffer of the enzyme with the substrates to be activated. Heavy metal ions or alkaline earth ions are to be especially mentioned as defectors. The addition of calcium ions is particularly preferred here. Thus, it is shown in the following examples that the activation product β -factor Xa is obtained in the activation of factor X using calcium ions with high yield

whereby other cleavage products only arise in negligible amounts.

However, the activation reaction can strongly proceed without addition of calcium ions and can also be modulated with respect to the obtained activated factor. With suitable experimental arrangements, α -factor Xa and/or β -factor Xa can also be produced according to the invention.

For a preferred industrial application of the enzymes discussed here, these can be employed in an immobilized form. Thereby, a simple separation of the protease from the substrate is made possible, for example by filtration. Additionally, the use of immobilized enzymes, i.e. enzymes bound to a solid phase, ensures the repeated use of the same. In the case of secondarily modified proteins, for example by oxidation, these are irreversibly fixed in their confirmation as it is present after the conversion into a highly specific activator by the immobilization, i.e. by covalent binding of the enzyme to the carrier material.

The method according to the invention is suitable for activating naturally as well as recombinantly produced blood factors. In this connection, the proteolytic cleavage site in a blood factor as a coagulation pro-enzyme can be modified by an amino acid sequence which is then specifically recognized and cleaved by a defined protease such that activation is only possible or also possible with a protease which does not correspond to the physiological activation mechanism. This particularly constructed analogue, for example, is an analogue of a vitamin K-dependent blood factor such as factor X.

Using the example of factor X, the region around the Arg52-Ile53 position which represents the cleavage site for factor IXa can be exchanged by a different amino acid sequence. For example, the amino acids Arg52, Ile53 can be replaced by the sequence Glu-Gly which then excludes a cleavage by FIXa and an

activation by factor IXa but permits the proteolytic digestion with endoproteinase Glu C from *Staphylococcus aureus* V8. Thus, a bacterial enzyme which can be produced in a highly purified manner and does not represent the danger of contamination with an animal or human protein can be employed for the activation of factor X. Analogously, Gly-Ser can also be introduced as a cleavage sequence whereby cleavage with plant proteases is then possible, for example with ficin.

It is preferred that the activated blood factor according to the invention is subjected to further purification steps in order to remove possibly formed proteolytic degradation products in this manner. Chromatographic purification processes or a purification by gel filtration are particularly preferred here.

The invention also comprises a pharmaceutical preparation which comprises a blood factor and a protease derived from plants or prokaryotes with a specificity for the blood factor. The protease can also be a protease derived from mammals, in particular a protease derived from humans. However, in this connection, coagulation factor is not used as a protease according to the invention. Particularly, plasma proteins, for example vitamin K-dependent proteins as well, are contained in this pharmaceutical preparation as blood factors. The pharmaceutical preparation especially comprises an activated vitamin K-dependent blood factor and a protease. This blood factor is preferably human and native.

The protease, especially a purified protease, is to be employed as such for the topical use in hemeostasis, whereby the vitamin K-dependent blood factor in this case originates from a bleeding wound. By applying the factor X activator in a pharmaceutical carrier material, for example in a dressing material or in a powder or in an ointment, the blood coagulation triggering effect of the coagulation activator can be used here. Another pharmaceutical composition also

comprises a blood factor which is present as a pro-protein and contains a pro-protein-cleaving protease isolated from plants, fungi or prokaryotes or recombinantly produced in cells of these species. Proteases of this type with a specificity for pro-proteins are known for example as furin (Van de ven, W. J. et al., Enzyme 45:257-270, 1991) or Paired Basic Amino Acid Residue Cleaving Enzyme, PACE, (Rehemtulla, A. et al., Biochemistry 32:11586-11590, 1993). If the mature protein resulting from the pro-protein is particularly labile in a pharmaceutical preparation, the mature protein can be produced in situ by simultaneous administration of the pro-protein-processing enzyme and is then therapeutically available. Additionally, a further activation with an activator protease is possible as far as the mature protein must be converted into an active form by further proteolytic digestion. Thus, a therapeutic kit consisting of the stable pro-protein and the proteolytic processing and/or activating enzyme also permits the administration of labile proteins as active ingredients for the first time.

Moreover, the invention comprises an activated vitamin K-dependent blood factor as obtained according to the method of the invention. The human activated blood factor obtained according to the invention is distinguished in that it is not contaminated by an animal protease.

The method according to the invention is more closely illustrated by the following examples. These examples relate to the activation of factor X to factor Xa whereby the different process variations according to the depending claims find use.

Example 1

Production of the substrate factor X

The purification of factor X was conducted from a prothrombin complex factor preparation which contained the factors FII, FIX, FX, protein C and protein S and which was produced according to the method of Brummelhuis (Brummelhuis HGJ, Preparation of the prothrombin complex. In: *Methods of Plasma Protein Fractionation*, edited by Curling, JM, New York: Academic Press, 1980, p. 117-128) and heat treated for virus inactivation according to EP 0 159 311. A lyophilisate containing the prothrombin complex factors was dissolved in distilled water corresponding to an activity of 50,000 U FX/l and adjusted to pH 7.0. After addition of 12% (v/v) TWEEN 80, this was stirred for 1 hour at room temperature, subsequently diluted with distilled water to the 5-fold volume, mixed with trisodium citrate dihydrate (7 g/l) and adjusted to pH 7.0. Subsequently, this was mixed with 30 g/l $\text{Ca}_3(\text{PO}_4)_2$ and stirred for 1 hour at room temperature. Thereafter, the solid phase was separated by centrifugation and washed twice each with 25 ml/g calcium phosphate of a buffer of 20 mmol/l Tris-HCl, pH 7.0, containing 10 % ammonium sulphate by resuspension and respective renewed centrifugation. Thereafter, a third wash was carried out with 25 ml/g calcium phosphate of a buffer of 20 mmol/l Tris-HCl, pH 7.0, containing 150 mmol/l NaCl by renewed resuspension and subsequent centrifugation. For elution of the adsorbed factor X, the pellet was stirred with 1 mmol/l sodium phosphate buffer, pH 7.0, (25 ml elution solution/g calcium phosphate used) for 1 hour at room temperature. Thereafter, the solid phase was separated by centrifugation and the factor X-containing supernatant was further purified by chromatography over DEAE Sepharose® Fast Flow, Pharmacia. For this, a column filled with swollen, washed DEAE Sepharose® Fast Flow gel and a configuration of column inner diameter: gel-bed height = 1 : 8.3 was used. The chromatography material was loaded with 10 U FX/ml. The FX-containing solution was rebuffered beforehand against a

buffer, 25 mmol/l trisodium citrate dihydrate, 100 mmol/l NaCl, pH 6.0. This factor X solution was adsorbed at a flow rate of 2 ml/min to the DEAE Sepharose® Fast Flow and the column was subsequently washed with a flow of 2 ml/min with 1.7-fold gel volumes of 100 mmol/l NaCl in 25 mmol/l trisodium citrate dihydrate, pH 6.0, and further with 2.6-fold gel volumes of 250 mmol/l NaCl, 25 mmol/l trisodium citrate dihydrate, pH 6.0. The elution of factor X occurred with 5.2-fold gel volumes of a citrate buffer, pH 6.0, containing 280 mmol/l NaCl, pH 6.0. Fractions were collected during the elution which were examined with standard coagulation tests for their content of the prothrombin complex proteins factor X, protein C, factor IX and factor II. The fractions containing factor X which were poor in other prothrombin complex proteins were combined and further purified over a monoclonal antibody purified from ascites which was directed against factor X and was immobilized to Actigel ALD (Sterogene, Bioseparations, Acradia CA). The FX-containing protein solution was adsorbed to the gel at a concentration of 10 U FX/ml gel. Subsequently, this was washed with 5-fold column volumes of a buffer of 20 mmol/l Tris-HCl, pH 7.4. The elution occurred with 10-fold column volumes of a buffer containing 100 mmol 3-[(3-cholamidopropyl)dimethyl-ammonium]-1-propanesulfonate, 25 mmol/l NaCl, pH 10.5. The protein fraction eluting with this buffer was collected and subsequently concentrated to 1/20 of the starting volume by ultra filtration over a membrane with an exclusion limit of 10,000 dalton. Subsequently, the FX-containing solution was diafiltrated against a buffer containing 4 g/l trisodium citrate dihydrate, 8 g/l NaCl, pH 7.0. This factor X preparation was freed from traces of contaminating proteins, especially monoclonal antibody bleeding from the immunoaffinity chromatography column, by adsorption to Phenylsepharose® High Performance, Pharmacia-Biotech. For this, the FX-containing solution was adjusted to 1.8 mmol/l NaCl and a pH value of 7.4 after diafiltration. The hydrophobic interaction chromatography gel was equilibrated in

a chromatography column with 20 mmol/l Tris-HCl, 2 mol/l NaCl, 7.4, and the adjusted factor X solution was applied to the column at a concentration of 30 U FX/ml gel. The FX-containing pass through from the column was collected and concentrated to 1/20 of the starting volume by ultra filtration over a membrane with an exclusion limit of 10,000 dalton and then diafiltrated against a buffer containing 20 mmol/l Tris-HCl, 150 mmol/l NaCl, pH 7.4. The highly pure factor X solution obtained in this manner had a specific activity of approx. 100 U/mg protein.

Example 2

Quantitative determination of factor Xa

Test principle:

The chromogenic peptide substrate $\text{CH}_3\text{OCO-D-CHA-Gly-Arg-pNA}$ was hydrolysed by factor Xa, whereby $\text{CH}_3\text{OCO-D-CHA-Gly-Arg-OH}$ and paranitroaniline resulted. The kinetics of the increase of paranitroaniline is spectrophotometrically determined at 405 nm. The increase of the optical density (OD) is proportional to the content of factor Xa in the sample to be quantified.

Reagents:

Dilution buffer

3.7 g/l Tris- (hydroxymethyl)-aminomethane
2.1 g/l imidazole
18.0 g/l NaCl
1.0 g/l human albumin
pH 8.4

Substrate solution

1.3 mmol/l $\text{CH}_3\text{OCO-D-CHA-Gly-Arg-pNA}$
in dilution buffer

Method:

50 μ l of a sample containing factor Xa are mixed with 50 μ l dilution buffer and incubated for 90 seconds at 37°C. Then, 100 μ l of the substrate solution is added and the increase of the OD per minute at 37°C at 405 nm is determined. The increase of the OD must remain linearly constant over the measurement time period.

For generation of a reference curve, the standard preparation of bovine factor Xa "NIBSC reagent 75/595" is used, whereby an ampulle contains 1 U FXa after reconstitution with 1 ml distilled water (see Datasheet, National Institute for Biological Standards and Controls).

Example 3

Activation of factor X

The activation of purified factor X from example 1 was carried out with the enzymes clostripain (Calbiochem, La Jolla, CA) 10 U/ml, thermolysin (Calbiochem, La Jolla, CA) 200 U/ml, papain (Boehringer Mannheim, Germany) 400 μ g/ml and ficin (Sigma Chemicals Co., St. Louis, MO) 20 μ g/ml, in a buffer containing 20 mmol/l Tris-HCl, 150 mmol/l NaCl, 5 mmol/l CaCl₂, pH 7.4, at 37°C and incubation over several hours. The concentration of factor X was 3.2 U/ml. At the time points 5 minutes, 30 minutes, 1 hour, 2 hours and 19 hours, samples were taken from the respective incubation mixtures and examined for factor Xa activity as described in example 2. The results are to be taken from Figure 1. It was demonstrated that an activation of factor X was possible with all employed enzymes and led to the highest yields of factor Xa with thermolysin after 2 hours. Except for the incubation with ficin, the activation phase which, depending on the enzyme, obtained a maximum between 30 minutes and 2 hours, was followed by an inactivation of the resulting factor Xa. A continual activation with a stable factor Xa activity could be determined also after 19 hours when the activation takes place with ficin.

Example 4

Activation of factor X

Comparison of ficin and Russell's Viper Venom

Factor X activator from *Vipera russellii* (RVV, Pentapharm AG, Basel, CH) is known from the literature as a non-plasmatic factor X activator with a high selectivity which is currently used for in vitro activations of factor X.

In this example, the activation of highly purified factor X from example 1 with RVV was examined in comparison to the activation with the plant factor X activator ficin. For this purpose, the highly purified factor X was used at a concentration of 4 U/ml in a buffer containing 20 mmol/l Tris-HCl, 150 mmol/l NaCl, 5 mmol/l CaCl₂, pH 7.4, and incubated at 37°C either with 2.7 µg/ml RVV (Pentapharm AG, Basel, CH) or 20 µg/ml ficin (Sigma Chemicals Co., St. Louis, MO). Samples were taken at different time points from the incubation mixtures within 22 hours and examined for factor X activity as described in example 2. The result of the examination is presented in Figure 2. It was demonstrated that the incubation of factor X with the factor X activator of ficin led to a factor Xa activity which was comparable with that which could be obtained by incubation with RVV. The activation products of factor X which could be obtained after incubation with both activators after 22 hours were also examined by SDS-polyacrylamide gel electrophoresis in gradient gels of 8-18% under non-reducing conditions. In this connection, factor X before activation, factor X after activation with RVV and after activation with ficin were analysed after electrophoretic separation and subsequent blot to nitro-cellulose membranes and detection with an anti-factor X polyclonal antibody and immunostaining according to standard methods. The result is to be taken from Figure 3. It was demonstrated that the homogeneous factor X before activation was cleaved by the activator RVV as well as by the activator ficin into several factor X-specific protein fragments of

smaller molecular mass than the non-activated factor X, whereby a mixture of α and β -factor Xa resulted after activation with RVV wherein these 2 activation products of factor X were obtained in approximately the same amount and at least 3 further activation products could be identified. In contrast, the activation by ficin showed that β -factor Xa was obtained as the main product (see arrow), whereby three further activation products could also be detected as with activation with RVV. In contrast to the activation with RVV, these fragments had a larger molar weight difference to the main product β -factor Xa such that these could be separated by further simple methods, for example gel filtration.

Example 5

Influence of effectors on the factor X activation with ficin

The incubation conditions given in the literature for the proteolytic degradation with ficin have buffer systems which contain at least cysteine as an SH reagent and activator for the protease. Furthermore, the addition of a metal ion complex former, for example ethylenediamine tetraacetic acid (EDTA), is also described because it is known that such SH-dependent proteases are particularly inactivated by heavy metal ions. In this example, factor X was incubated at a concentration of 4 U/ml in a buffer containing 20 mmol/l Tris-HCl, 150 mmol/l NaCl, pH 7.4, with 2 μ g ficin/ml analogous to example 4 at 37°C for 24 hours, whereby (1) 2 mmol/l CaCl_2 , (2) 1 mmol/l cysteine, (3) 1 mmol/l cysteine and 2 mmol/l EDTA and (4) 1 mmol/l cysteine and 2 mmol/l CaCl_2 were added to the buffer medium. The factor Xa activity was determined according to example 2 after 24 hour incubation. The results are to be taken from Table 1.

Batch	Activity after 24 h (U/ml)
2 mM CaCl_2	42.1
1 mM cysteine	17.5
1 mM cysteine, 2 mM EDTA	10.3
1 mM cysteine, 2 mM CaCl_2	45.1

Table 1: factor X-activation with ficin, influence of effectors

It was shown that a clearly higher yield of factor Xa could be obtained by the addition of calcium chloride, whereas under standard conditions (cysteine + EDTA), only smaller factor X yields could be realized. The batches were also examined for the composition of the activation products by means of SDS-polyacrylamide gel electrophoresis as described above. The result of the electrophoretic examination after staining of the proteins according to the silver staining method as well as after specific immunostaining with an anti-factor X antibody is to be taken from Figure 4. It was shown that the addition of calcium ions to the incubation medium led to a clearly higher portion of β -factor Xa among the factor X cleavage products. The β -factor Xa obtained in this manner can be easily freed from residual ficin still contained in the mixture by conventional chromatography purification methods. Among these are simple gel filtration, ion exchange chromatography or substrate affinity chromatography, for example, on immobilized benzamidine which is capable of selectively binding to factor Xa.

Example 6

Isolation of factor X activator from plant latex

10 mg of a pulverized latex from *Ficus glabrata* containing 2 mg protein were suspended in 2.5 ml of a 5 mmol/l sodium phosphate buffer containing 1 mmol/l EDTA, pH 5.5, suspended and mixed with 100 μ l 50 mmol/l cysteine solution for the activation of the cysteine protease. Subsequently, the free cysteine was removed by a gel filtration over Sephadex® G50 and further diluted with the same phosphate buffer 1 + 3. Then the entire amount was applied to a Mono S HR 5/5 cation exchange chromatography column, Pharmacia, at a flow rate of 1 ml/min. Subsequently, this was washed with 45 column volumes

of the same phosphate buffer and a gradient elution was then carried out whereby the phosphate concentration was continuously increased over 55 column volumes to 185 mmol/l in the same buffer composition. The result is taken from Figure 5. The protein mixture was separated into several individual proteins, as can be recognized in the elution profile by the absorption at 280 nm (- proteins). Subsequently, the individual fractions were examined for FX activating enzymes activity with the factor Xa assay described in example 2 and it was shown that a protein peak which eluted at 49 ml elution volumes and a protein peak which eluted between 68 and 69 ml elution volumes have the highest factor X activator activity (- -x- -). In an analogous manner, the fractions were examined for protease activity with an unspecific protease substrate. For this, the chromogenic protease substrate benzoyl-DL-arginine-p-nitroanilide hydrochloride was used which was employed in a photometric test under the conditions and according to the method of Englund et al., Biochemistry 7: 163-175 (1968). As was recognized from Figure 5, considerable protease activity (Δ) could be found in the column pass through as well as in all further fractions, whereby the peaks at elution volumes 49 ml and elution volumes 68 - 69 ml also had the strongest activities here. However, aside from this, considerable protease activities were also determined in the protein fractions at elution volumes 53 ml and 74-83 ml. It is noteworthy that the factor X activator activity was clearly higher than the unspecific protease activity in the fraction which eluted from the column at 68-69 ml which was due to a separation of the factor X activator from the crude plant extract. The fraction containing the highest factor X activator activity with respect to the protein content was subsequently incubated for 15 hours at 4°C under the influence of atmospheric oxygen. The factor X activator activity could be further increased by this in relation to the unspecific protease activity.

Example 7

Immobilisation of plant factor X activator

Crystallized ficin (Sigma) in crystal suspension was diluted to a concentration of 2.5 mg/ml and rebuffered against a buffer containing 20 mmol/l Tris-HCl, 150 mmol/l NaCl, pH 7.4, by gel filtration over Sephadex® G50 (Pharmacia). A pre-activated gel, Actigel ALD Superflow (Sterogene Bioseparations, Arcadia, CA), was washed with a buffer containing 20 mmol/l Tris-HCl, 150 mmol/l NaCl, pH 7.4, and subsequently mixed in a ratio 1 + 1 with the solution containing ficin to be immobilized and incubated with 1/15 of the volume of coupling solution (Sterogene Bioseparation, Arcadia, CA) for 3 hours at room temperature. Subsequently, the immobilizate was separated on a sinter nutsch and freed from non-bound ficin and the coupling reagent by excessive washing alternative with a 20 mmol/l Tris-HCl, 150 mmol/l NaCl, pH 7.4, buffer and a 20 mmol/l Tris-HCl, 2 mmol/l NaCl, pH 7.4, buffer. The ficin immobilisate was subsequently employed for activation of factor X as follows.

Highly purified factor X from example 1 was adjusted to 2 U FX/ml in a buffer containing 20 mmol/l Tris-HCl, 150 mmol/l NaCl, pH 7.4, and mixed with μ l/ml moist ficin immobilisate. Subsequently, this was incubated for 60 minutes at 37°C under continuous thorough mixing and, thereafter, the factor Xa activity was measured as described in Example 2. Thereby, a factor Xa activity of 4 U/ml could be determined. It can be derived from this that ficin also maintains the factor X activator activity in a solid phase bound form and, therefore, is suitable as an immobilizate for producing factor Xa.

Example 8

An industrial ficin preparation from *Ficus glabrata* latex was purified according to the conditions and the method of Englund et al., Biochemistry 7:163-175 (1968), whereby those fractions

were collected which had the highest specific factor X activator activity compared with the unspecific protease activity against benzoyl-DL-arginin-p-nitroanilide hydrochloride. The preparation accumulating from the chromatographic purification was subjected to a 15 minute activation with 10 mM cysteine at 37°C and the activation mixture was subsequently freed from excess activator by a group separation by means of gel filtration over Sephadex® G-25 Superfine. The activation was carried out in incubation mixtures containing 4 U/ml factor X and 3 µg/ml of the activated ficin preparation in a buffer system of 20 mmol/l Tris-HCl, 150 mmol/l sodium chloride, pH 7.4, in the absence and presence of calcium and manganese ions. The batches contained either 2 mmol/l calcium ion, 1 mmol/l manganese(II) ions or a combination of the two metal ions. After 5 h incubation at 37°C, the batches were examined by means of SDS-polyacrylamide gel electrophoresis for the composition of the activation products. The result of the electrophoretic separation was made visible according to the silver staining method and the intensity of the separated bands was densitometrically analysed. The result of this analysis is to be taken from the following Table.

Activation batch	Portion factor Xa densito (%)	Portion cleavage products meteric (%)
Buffer	15.3	84.7
2 mM Ca ²⁺	44.4	55.6
1 mM Mn ²⁺	30.3	69.7
2 mM Ca ²⁺ + 1 mM Mn ²⁺	65.5	34.5

It was shown that the activation by the purified, activated ficin preparation led to an increased degradation of the formed factor Xa without addition of the two metal ions. The alternative addition of Ca²⁺ and/or Mn²⁺ ions reduced the

production of various cleavage products, whereas the combination of both ions almost reversed the ratio between activated factor X and its degradation products.

Additionally, it could be shown that the activation under addition of Ca^{2+} and Mn^{2+} ion resulted in a activated factor X which is not subject to any further degradation process after the activation process. In order to elucidate this evidence, an activation batch analogous to the conditions given previously was carried out under addition of 2 mmol/l calcium ions and 1 mmol/l manganese(II) ions in which samples were taken at certain time points from the incubation mixture in order to record the activation kinetics. The activity of factor Xa was determined according to Example 2 and the portion of degraded cleavage products of factor Xa were determined in a manner analogous to the techniques described above. The results of these experiments are summarized in the following Table:

Sample taking after	Factor Xa activity amidolytic (Units/ml)	Portion factor Xa densitometric (%)	Portion cleavage products (%)
1 h	18.5	84.4	15.6
3 h	30.0	65.1	34.9
5 h	34.0	65.4	34.5
26 h	33.6	64.1	35.9

Further purification of the factor Xa obtained can then be conducted according to Example 5.

New Claims 1 to 20

1. Method for the activation of a vitamin K-dependent blood factor by treatment with a protease, characterized in that the protease is derived from plants, fungi or prokaryotes and has at least 2-fold increased specific blood factor activation activity as compared to the crude extract of plants or cell cultures.
2. Method according to claim 1, characterized in that the blood factor is selected from the group of human factors II, VII, IX, X and protein C.
3. Method according to claim 2, characterized in that the factor Xa- β is obtained after activation of human factor X.
4. Method according to claims 1 to 3, characterized in that the protease is selected from the group ficin, bromelain, papain, thermolysin and clostripain.
5. Method according to one or more of claims 1 to 4, characterized in that the treatment is carried out under conditions which are sub-optimal for the protease.
6. Method according to one or more of claims 1 to 5, characterized in that the protease is an oxidized cysteine protease.
7. Method according to one or more of claims 1 to 6, characterized in that the protease is chromatographically purified.
8. Method according to one or more of claims 1 to 7, characterized in that the activation is carried out in the presence of defectors.

9. Method according to one or more of claims 1 to 8, characterized in that the activation is carried out in the presence of heavy metal ions or alkaline earth ions.
10. Method according to claim 9, characterized in that the activation occurs in the presence of calcium ions.
11. Method according to one or more of claims 1 to 10, characterized in that the protease is immobilized to a solid carrier.
12. Method according to one or more of claims 1 to 11, characterized in that the protease is produced by recombinant DNA technology.
13. Method according to claim 1, characterized in that a recombinant blood factor is activated.
14. Method according to claim 13, characterized in that a blood factor analogue with a proteolytic cleavage site specific for the protease is activated.
15. Method according to claim 14, characterized in that the proteolytic cleavage site is modified and the activation is performed with the protease which does not correspond to the physiological activation mechanism.
16. Pharmaceutical preparation comprising a blood factor and a protease specific for the blood factor which is derived from plants, fungi or prokaryotes.
17. Pharmaceutical preparation according to claim 16, characterized in that blood factor is an activated vitamin K-dependent protein.

18. Pharmaceutical preparation comprising a purified protease specific for a blood factor, characterized in that it is formulated for topical use and the protease is derived from plants, fungi or prokaryotes.
19. A kit for medical use, comprising
 - a) a proteolytic enzyme derived from plants, fungi or prokaryotes with a specificity for a proform of a protein, and
 - b) the proform of a protein.
20. Activated vitamin K-dependent blood factor characterized in that it was obtained according to the method of claims 1 to 15.

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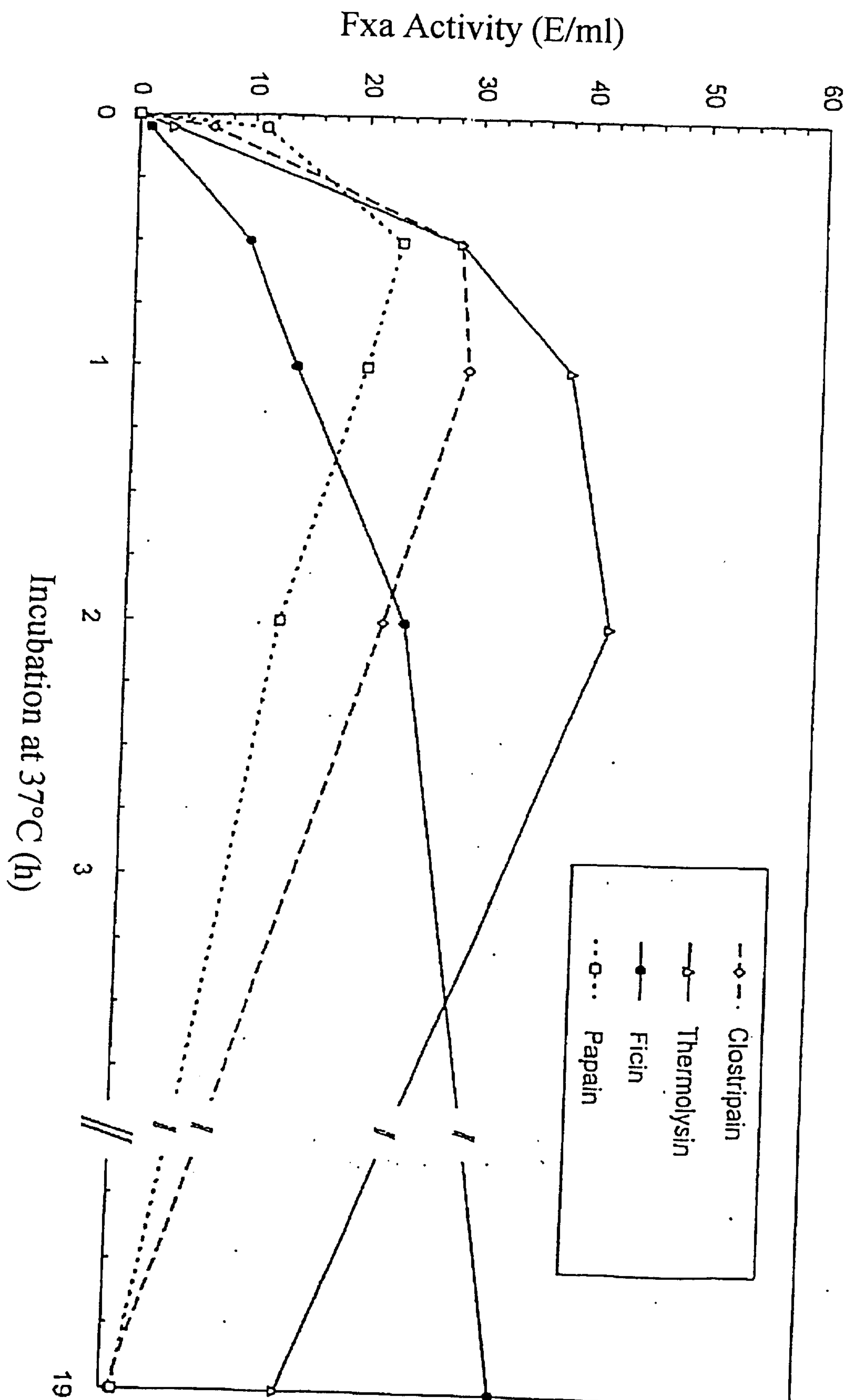


Figure 1

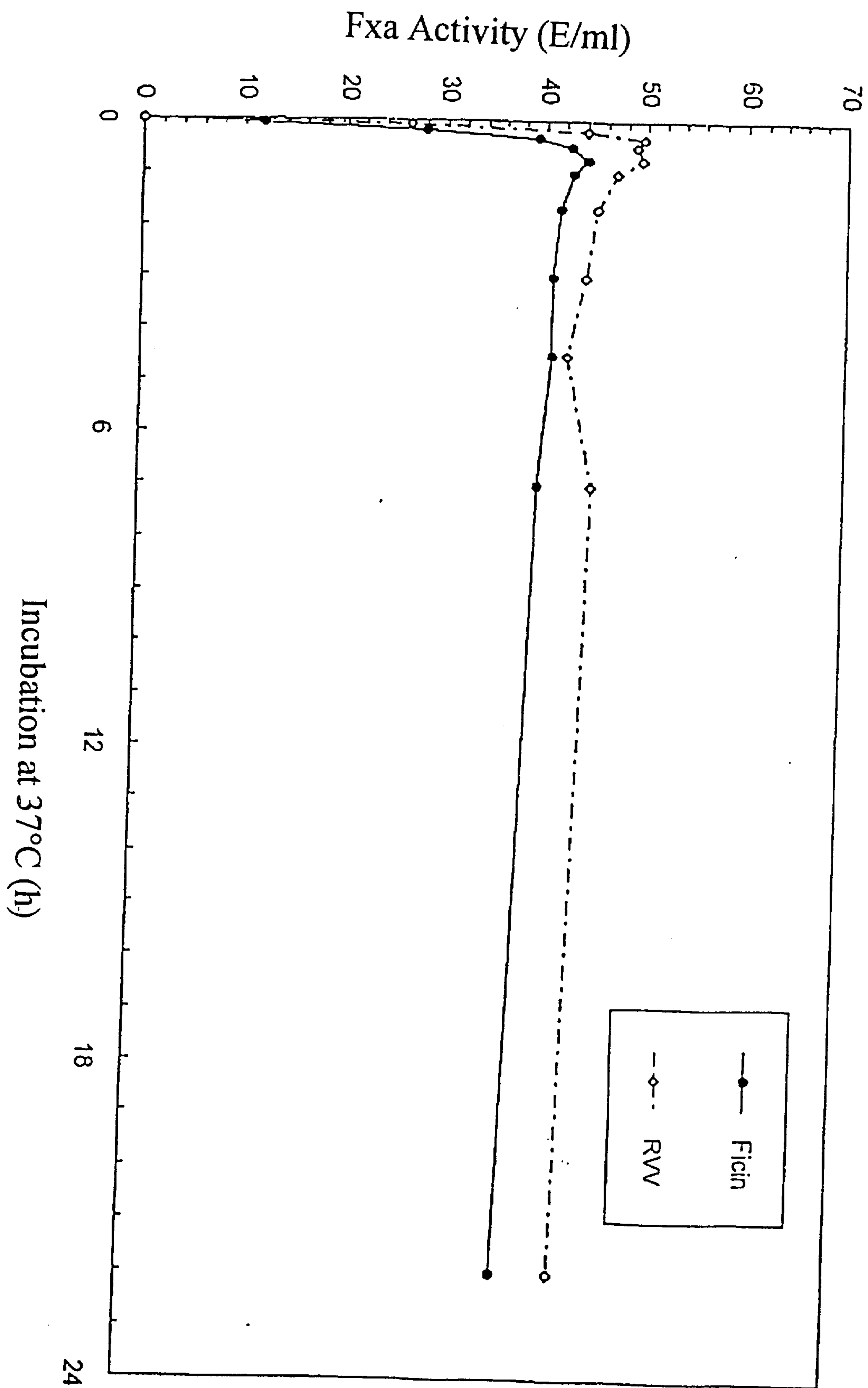


Figure 2

Figure 3

SDS-PAGE (8-18%) - not reduced

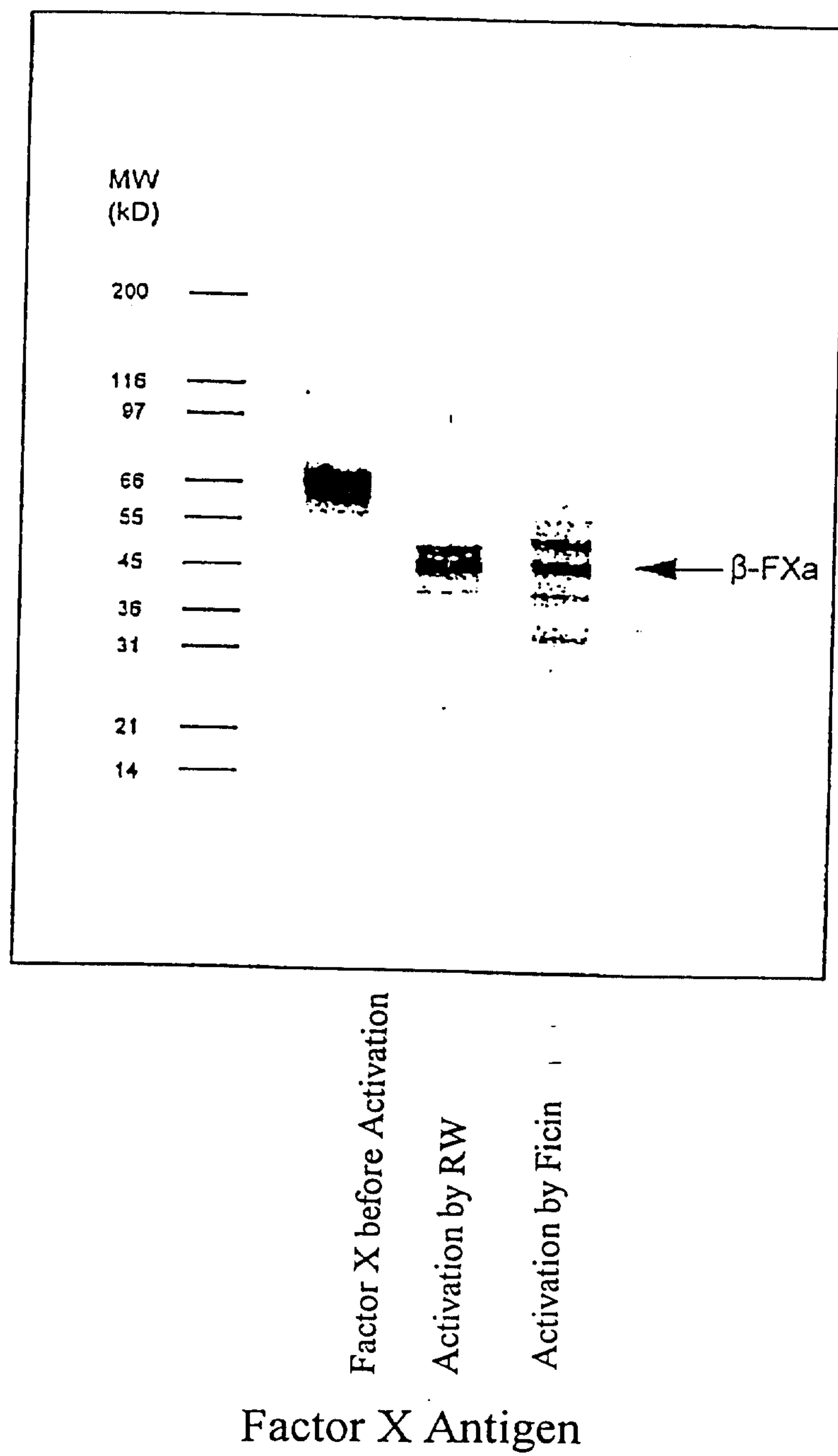
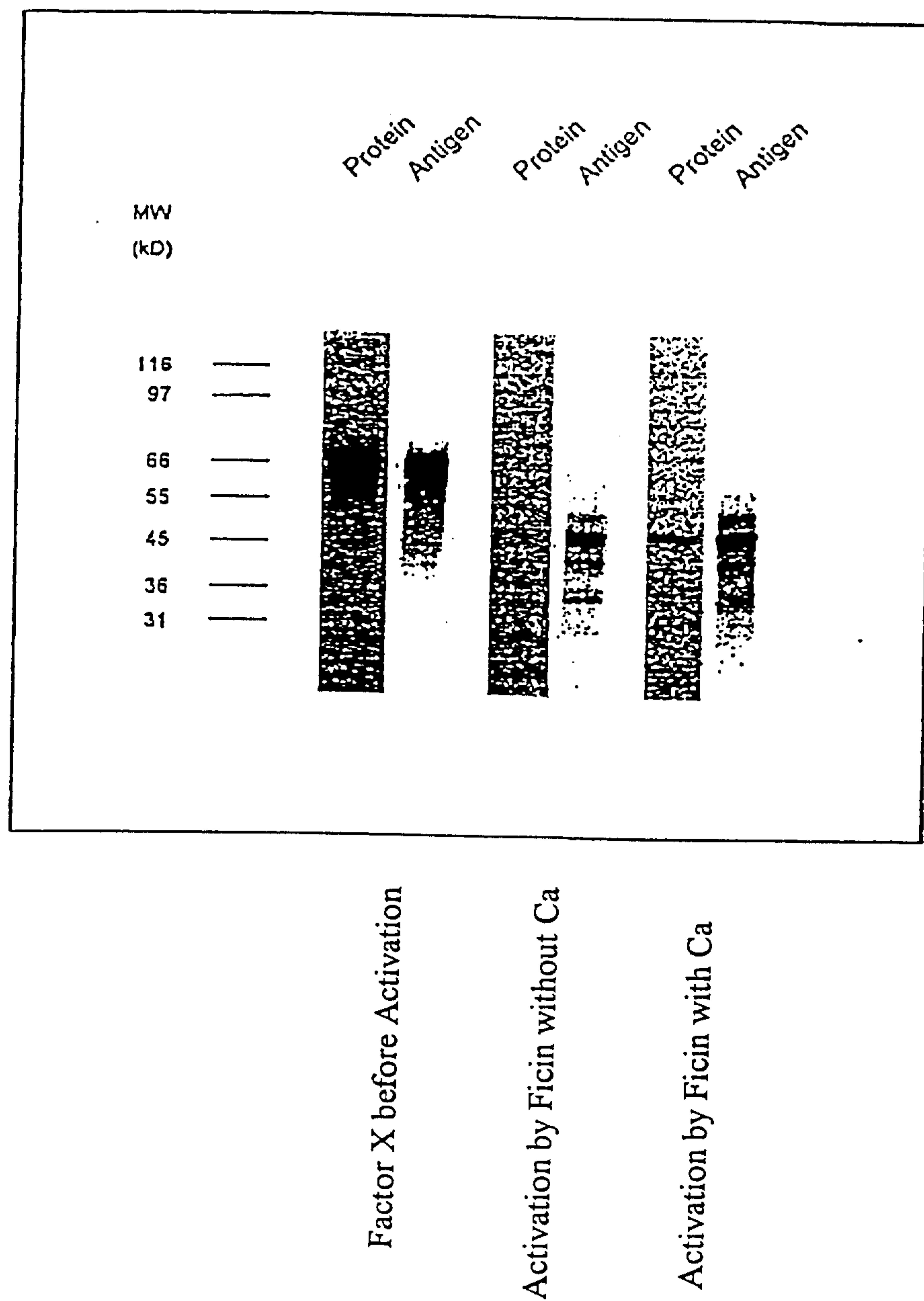


Figure 4

SDS-PAGE (8-18%) - not reduced



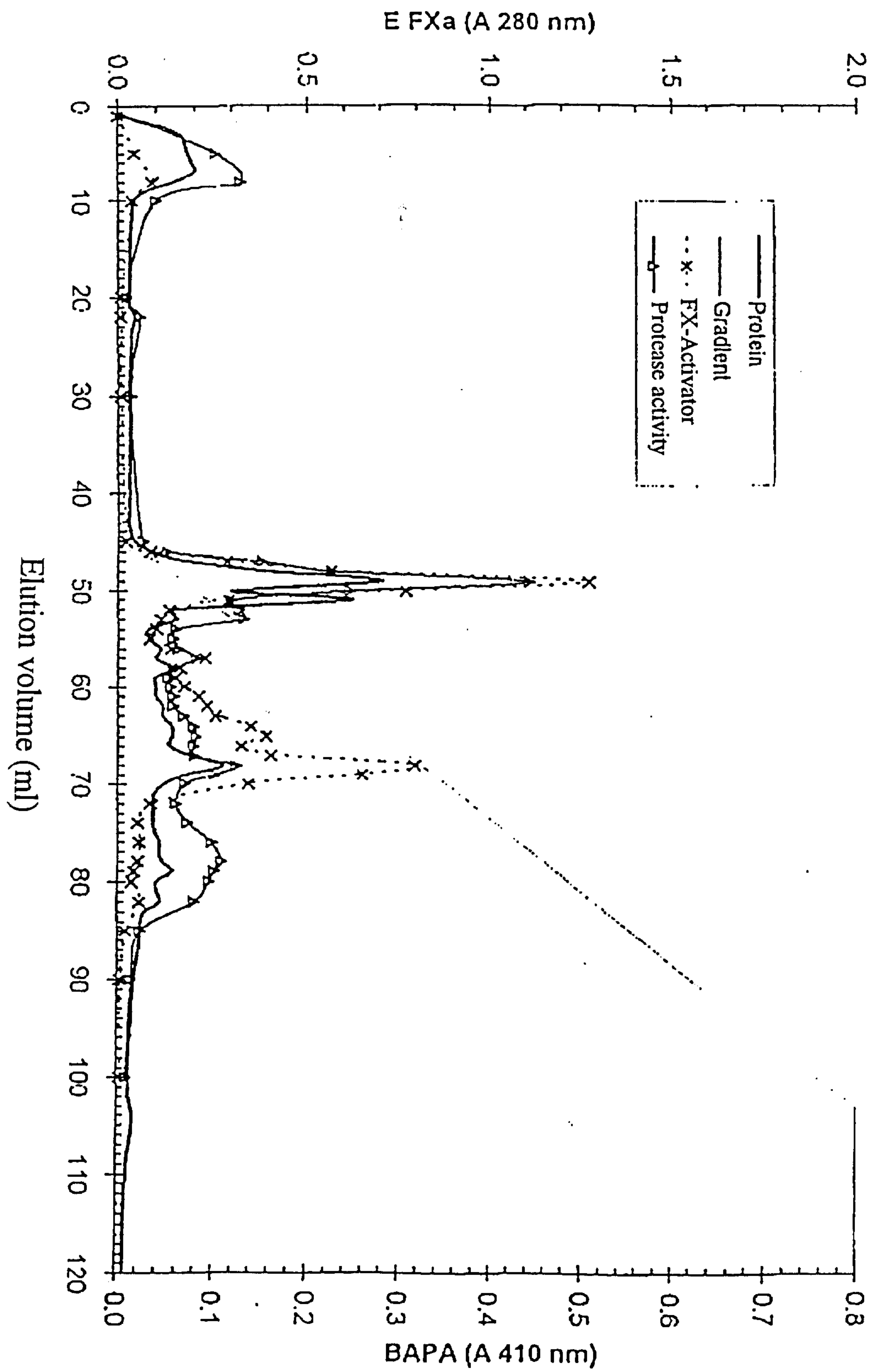


Figure 5