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PURIFICATION AND STUDIES OF COMPONENTS OF THE HAEMOSTATIC SYSTEM BY AFFINITY CHROMATOGRAPHY

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SUMMARY

The application of affinity chromatographic techniques for separations and studies on molecular interactions of the components of the blood coagulation system is reviewed. Most of the components have been purified using processes involving one or several affinity chromatographic steps. Many different kinds of affinity chromatography have been used, including inhibitor interaction chromatography, effector interaction chromatography, immunosorption, hydrophobic interaction chromatography, metal chelate chromatography and covalent chromatography.

Affinity chromatographic techniques have also been used to study molecular interactions such as the fibrin polymerization process. One example of large-scale purification of a clinically used component is given.

INTRODUCTION

The coagulation of blood is a very complex process with a large number of components participating. When a blood vessel is damaged, the clotting process occurs so as to prevent loss of blood and emptying of the circulation. In the formation of the haemostatic plug there is participation from the plasma coagulation system, the vessel wall and the platelets. Initially, formation of a platelet plug occurs, which arrests the bleeding, and subsequently the plug is stabilized and extended through fibrin formation. The plasma coagulation system which is responsible for the formation of fibrin consists of fourteen components, nearly all of them being proteins.

Fig. 1 shows the coagulation–fibrinolysis system with the coagulation on the left-hand side. The coagulation system is often called the coagulation cascade or "waterfall", because it begins with a small triggering effect in a sequence of reactions involving one protease activating another protease, and is then amplified to a large final effect, the formation of the clot. Most of the reactions occur on surfaces such as in the contact activation phase, which is believed to take place on the subendothelium of the damaged vessel or in the activation of prothrombin and Factor X, which occurs on the surface of the aggregated platelets. In the later stages of the process the thrombin formed cleaves off the two fibrino-peptides from fibrinogen, exposing the polymerization sites, resulting in formation of fibrin polymers and a fibrin gel.

As the coagulation cascade begins with a small trigering effect which is strongly

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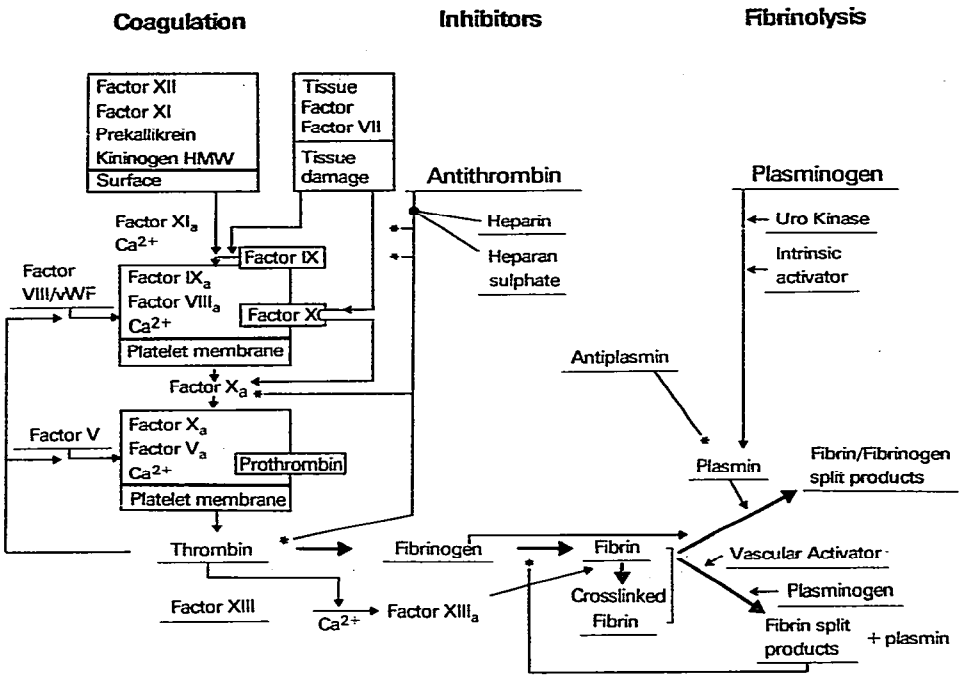

Fig. 1. Coagulation–fibrinolysis System. *, Inhibition; ●, rate enhancement.

amplified, it would be a rather labile system if there were no other controlling factors present. Such factors are present, however, one of the most important being the coagulation inhibitors.

Antithrombin III is the main coagulation inhibitor and it is also identical with heparin cofactor, *i.e.*, the factor in plasma, which is necessary for the anticoagulant activity of heparin. Antithrombin inhibits coagulation enzymes, both early and late in the coagulation cascade.

Another important defence system which protects against thrombosis is the fibrinolytic system. In this system the proenzyme plasminogen can be activated to the protease plasmin, which then degrades the fibrin clot or the thrombus.

Much work has been devoted to purifying the various coagulation factors and other components of the haemostatic system. One reason for this has been the need to gain knowledge regarding the structure and properties of the separate components, which is necessary in order to understand how the whole system is functioning. Another reason has been that for the clinical treatment of hereditary deficiency of certain coagulation factors there is a need for the development and production of concentrated preparations of certain coagulation factors. A good example of this is Factor VIII, which is needed for the treatment of classical haemophilia (haemophilia A).

The work has been fairly successful as most of the components have been purified and more or less well characterized. Affinity chromatographic methods have been used fairly often in the various procedures devised for purification. There are also several reasons why affinity chromatography should be of special value in this

field. First, as evident from Fig. 1, there are a number of interactions between the various components in the haemostatic system that potentially could be used for affinity chromatographic purposes. Second, the concentrations in plasma of most of the factors are very low, which requires the separation procedures used to be very efficient and specific. This is also supported by the fact that many of the coagulation factors have very similar structures and properties and thus are difficult to separate from each other. Finally, several of the coagulation components are labile and it is then advantageous to use gentle purification procedures, a requirement which is often fulfilled by the affinity chromatographic methods.

COAGULATION FACTORS

Fibrinogen

This is the coagulation factor which is present in highest concentration in plasma (2–3 g/l). Methods for its purification were developed early and fibrinogen with a high degree of purity was obtained by various precipitation methods using alcohol or salts as precipitating agents. DEAE-cellulose chromatography has also been used successfully. An affinity chromatographic procedure for the purification of fibrinogen from plasma has been devised[1], based on the affinity between fibrin monomer and fibrinogen. Fibrin monomer is the component formed when the two fibrino-peptides have been split off fibrinogen through the action of thrombin. In fibrin monomer the polymerization sites are exposed and the fibrin monomers rapidly aggregate and form the fibrin network. Fibrin monomer also has affinity for fibrinogen and fibrinogen–fibrin monomer complexes are easily formed.

The procedure used was to couple fibrinogen to cyanogen bromide-activated agarose and then cleave off the fibrino-peptides from the matrix-bound fibrinogen by treatment with thrombin. When plasma was allowed to pass through a column with this fibrin monomer agarose gel, most of the fibrinogen was adsorbed. Elution with buffer containing 2 *M* sodium bromide yielded almost pure fibrinogen with a good recovery. Despite its simplicity, the method will probably not be used very often for the purification of fibrinogen as it has a limited capacity and there are also available a number of good conventional methods for the preparation of fibrinogen in large amounts.

The method of attaching fibrinogen or fibrin monomer to agarose and study[2–4] of the binding of various fibrinogen derivatives or breakdown products to the gel have been of considerable value, however, in studies of fibrin polymerization sites. The data obtained[4] indicated that there are four sets of complementary binding sites involved in fibrin polymerization.

Vitamin K dependent coagulation factors

These are Factors II, VII, IX and X, which have similar sizes and also contain γ-carboxyglutamic acid residues in the amino-terminal part of their sequences. The presence of γ-carboxyglutamic acid residues is necessary for the binding to phospholipid and for coagulant activity. The γ-carboxyglutamic acid residues are introduced after biosynthesis of the peptide chains in a vitamin K dependent process.

Factor II (prothrombin) is normally present in plasma at a concentration of 90 mg/l. A number of methods have been devised for its purification, most of them using

adsorption–elution from barium sulphate, calcium phosphate or DEAE-Sephadex. One affinity chromatographic procedure, based on immunosorption, has been developed for the purification of bovine prothrombin[5]. Antibodies against prothrombin were adsorbed and eluted from matrix-bound prothrombin and the specific antibodies were then coupled to Sepharose. The immunosorbent showed a good capacity for adsorption of prothrombin and a 74% yield of pure prothrombin was obtained following desorption with buffer containing 2 *M* sodium bromide and 5 m*M* EDTA (pH 7.4). Despite the high yields obtained with this method, it does not seem to have been used very often. The conventional methods for preparing prothrombin still predominate. Recently it was shown[6] that human prothrombin could be adsorbed to matrix-bound phospholipid vesicles in the presence of Ca^{2+} and eluted by citrate-containing buffer.

The enzyme form of prothrombin, thrombin, has been purified by a number of affinity chromatographic methods. Substrate analogues or inhibitors were used early and matrix-bound lysine[7], arginine[7], benzamidine[8] and *p*-chlorobenzylamide-ε-amino-caprylic acid[9,10] have been used successfully in the purification of thrombin. The desorption agents used were buffers containing lysine[7] or benzamidine[9].

Another way of purifying thrombin is to use chromatography on heparin–agarose. In contrast to prothrombin[11], thrombin is strongly bound to heparin–agarose and this has been used for its purification[12,13]. Elution was accomplished by using a salt gradient of sodium chloride and the degree of purification obtained was very good. This method seems to be advantageous compared with the methods based on substrate analogues or inhibitors[12]. The biological relevance of the thrombin–heparin interaction is probably related to the observation[14,15] that thrombin binds strongly to the endothelium of the blood vessel wall where the heparin-related component heparan sulphate is also found.

Factor VII has been difficult to purify, one reason being its very low concentration in plasma (*ca.* 0.2 mg/l). Bovine Factor VII was isolated by Jesty and Nemerson[16] and by Kisiel and Davie[17] using similar methods involving barium sulphate adsorption and DEAE-Sephadex chromatography and one affinity chromatographic step on benzamidine–agarose. Factor VII was eluted from the benzamidine–agarose with a gradient from 0.3 to 0.9 *M* guanidine hydrochloride. The purification obtained in this step was good (*ca.* 20-fold). The benzamidine was coupled to the agarose through a spacer. Human Factor VII has been isolated[18] using a similar procedure but not involving benzamidine–agarose affinity chromatography. In this procedure contaminating albumin and prothrombin were removed by immunoadsorption and Factor IX by adsorption to heparin–agarose.

In the purification of Factor IX affinity chromatography on heparin–agarose has been a key step. Factor IX is strongly bound to heparin–agarose but can be eluted by buffers of high ionic strength. In the purification of human[11,19–21] and bovine Factor IX, affinity chromatography on heparin–agarose using salt gradient elution is the main separation step. Good separation from the other vitamin K dependent coagulation factors is obtained. One problem in the purification of Factor IX, as with several of the other coagulation factors, is to prevent activation, that is, conversion of proenzyme to enzyme. This problem was solved for Factor IX either by having benzamidine present in the buffer[22] to inhibit traces of activating enzymes or by performing[11] the affinity chromatography at around pH 5, where these enzymes have lower activities.

It is possible to speculate about a biological role for the Factor IX–heparin interaction, but none is known at present. As in the case with prothrombin, Factor IX is bound to matrix-bound phospholipid vesicles[6] in the presence of Ca^{2+} and can be eluted with citrate-containing buffers. The binding of Factor IX to phospholipid seems to be stronger than the corresponding binding of prothrombin and some separation of Factor IX and prothrombin can be obtained under suitable conditions.

In the isolation of human Factor X a number of affinity chromatographic methods have been used. DiScipio *et al.*[19] separated Factor IX from Factor X by benzamidine–Sepharose chromatography and the remaining prothrombin was separated from Factor X by polyhomoarginine–Sepharose chromatography. The polyhomoarginine–Sepharose was prepared by coupling polylysine to cyanogen bromide-activated Sepharose followed by guanidylation with O-methylisourea. The elution of Factor X from the polyhomoarginine–Sepharose was performed by desorption with buffer containing 2.9 *M* guanidine hydrochloride.

Heparin–agarose chromatography has been used in several studies[23,24] to separate prothrombin, Factor IX and Factor X. The separation obtained between prothrombin and Factor X is not very satisfactory, however. Considerably improved separation can be obtained[25] by using dextran sulphate–Sepharose instead of heparin–Sepharose.

Purification of Factor X by adsorption–elution from blue dextran–Sepharose has been reported[26]. This procedure, however, is probably sensitive to small changes as other workers have experienced difficulties in reproducing the results.

Factors V and VIII

These two proteins are thought to have effector functions in the coagulation cascade. They are present in the circulation in proeffector forms, but can be activated by thrombin. Activated Factor V strongly enhances the speed of the prothrombin activation step and activated Factor VIII speeds up the rate of Factor X activation.

Factor V has been isolated by conventional separation methods, and so far no affinity chromatographic procedure has been published.

Factor VIII is present in plasma as a series of high-molecular-weight complexes with Von Willebrand Factor. These complexes have been isolated by several methods, but most commonly by using gel filtration on agarose gels as the main separation step. The main component (>90%) of the complexes is Von Willebrand Factor. This factor is necessary for normal platelet adhesion and is absent or present in decreased amounts in Von Willebrand's disease. Factor VIII coagulant activity can be separated from Von Willebrand Factor related activities by, among other methods, agarose gel filtration in the presence of 0.25 *M* calcium chloride or by ion-exchange chromatography.

Immunosorption techniques have been used both for separation purposes and for studies of the nature of the Factor VIII/Von Willebrand Factor complexes. When antibodies against Von Willebrand Factor are coupled to agarose and plasma is run through this immunosorbent, both Von Willebrand Factor related activities and Factor VIII activity are usually bound to the gel[27–30]. This procedure has been used to prepare artificial Factor VIII deficient plasma[30] for testing purposes, but in order to obtain sufficiently low Factor VIII values the immunosorbent also had to contain antibodies against the Factor VIII coagulant part. These antibodies can be obtained

from haemophilia patients who have developed antibodies against Factor VIII. It is possible[28,29] to separate the Factor VIII from the Factor VIII/Von Willebrand Factor complex by elution of the immunosorbent with 0.25 *M* calcium chloride solution and this then makes a one-step procedure for purification of Factor VIII from plasma corresponding to a purification of more than 5000-fold. In one study[31] sequential immunosorption was used, where in the first step matrix-bound rabbit antibody against Factor VIII related antigen adsorbed this antigen from the applied plasma, but left both Factor VIII and a Von Willebrand Factor related activity in the plasma. Factor VIII activity was subsequently adsorbed by passage through a second immunosorbent column containing human antibodies against Factor VIII. Some of these results are surprising as it is usually assumed that Von Willebrand Factor and Factor VIII related antigen are properties of the same molecule.

Despite extensive efforts, human Factor VIII has not yet been isolated. In a recent study, however, the isolation of bovine Factor VIII was reported[32]. The highly purified preparation migrated as a triplet in SDS–urea polyacrylamide gel electrophoresis and the degree of purification was 300,000-fold. The last step in the purification was affinity chromatography on matrix-bound Factor X, which did bind Factor VIII, as might have been expected from the known participation of Factor VIII in the activation of Factor X. Elution was accomplished with buffer containing 0.5 *M* sodium chloride.

Other affinity chromatographic methods that have been used for purifying Factor VIII are as follows. Matrix-bound Factor VIII/Von Willebrand Factor complex can be used[33] to purify Factor VIII, as this complex reversibly binds more Factor VIII, which then can be dissociated and eluted with buffer containing 1.3 *M* sodium chloride. Dextran sulphate–agarose[34] and aminohexyl-Sepharose[35] chromatography have also been utilized to separate Factor VIII and Von Willebrand Factor. No affinity chromatographic procedure has so far been published for preparing Factor VIII concentrate for clinical use.

Coagulation factors participating in contact activation

These are Factor XI, Factor XII, prekallikrein and high-molecular-weight kininogen.

Bovine Factor XI has been purified by Koide *et al.*[36], employing a procedure with precipitation and ion-exchange chromatographic steps and also involving affinity chromatography on heparin–agarose. The heparin–agarose chromatographic step was repeated three times and performed at two different pH values. In the first heparin–agarose step a 200-fold purification was obtained. The buffer solutions used contained diisopropyl fluorophosphate and Polybrene in order to inhibit protease activity and prevent activation. Human Factor XI and XIa have been prepared in partly purified form by heparin–Sepharose chromatography[37]. Completely pure human Factor XI was prepared using a method[38] similar to that used for purification of the bovine factor. Affinity chromatography on benzamidine–agarose was used as an additional step, however.

Bovine Factor XII was isolated by Fujikawa *et al.*[39] in a process involving a number of steps and in which heparin–agarose, arginine–agarose and benzamidine–agarose chromatography were used.

Factor XII binds to heparin–agarose and this affinity chromatography was

utilized twice, at pH 7.2 and 6.0. Arginine–agarose did not bind Factor XII but approximately 75% of the contaminating proteins did bind and could thus be separated. Factor XII binds to benzamidine–agarose and was eluted by a salt gradient. Activated Factor XII can be separated[40] from Factor XII by benzamidine–agarose chromatography as it is more strongly bound to the gel and elutes later with the salt gradient. Human Factor XII has been purified by several methods, in one of which[41] contaminating proteins were removed by immunosorption.

The isolation of bovine prekallikrein has been accomplished[42] using, among other things, heparin–agarose,benzamidine–agarose and arginine methyl ester–agarose chromatography. The last step resulted in good separation as prekallikrein was bound to the arginine methyl ester–agarose and eluted with a salt gradient. The enzyme form of prekallikrein, kallikrein, has been purified[43] by affinity chromatography on soybean trypsin inhibitor–agarose and benzamidine–agarose. Elution was accomplished using a buffer 1.0 *M* with respect to benzamidine.

Factor XIII

In contrast to most of the other coagulation factors, the enzyme form of Factor XIII is not a protease but a transaminase. Activated Factor XIII catalyses the formation of ε-(γ-glutamyl)lysine cross-links between fibrin monomers so as to produce insoluble fibrin. Factor XIII contains SH groups, and this has been exploited to purify it by affinity chromatography on organomercurial agarose[44]. Elution was accomplished using a gradient from 1 to 10 m*M* mercaptoethanol.

COMPONENTS OF THE FIBRINOLYTIC SYSTEM

Plasminogen

The main protease of the fibrinolytic system is plasmin, which proteolytically degrades fibrin and can dissolve thrombi. The proenzyme to plasmin is plasminogen, a glycoprotein which is present in plasma in a concentration of 0.2 g/l. The best method for purifying plasminogen is to use affinity chromatography on lysine–Sepharose[45–47], from which the plasminogen can be eluted with 0.2 *M* ε-aminocaproic acid. A 200-fold purification is obtained in one step with good yield. Plasmin can also be purified on lysine–Sepharose. The basis for the interaction between lysine and plasminogen is that ε-aminocaproic acid, which can be visualized as being a part of lysine, is strongly bound to plasminogen. This binding of ε-aminocaproic acid to plasminogen is probably related to the presence of a site with a similar structure in fibrin having affinity for plasminogen. ε-Aminocaproic acid is used clinically to inhibit fibrinolysis. Affinity chromatography has also been used to establish[48,49] which parts of plasminogen participate in the binding of ε-aminocaproic acid.

Activators

The two main physiological fibrinolysis activators are urokinase and tissue activator. The vascular activator is probably identical with tissue activator. The proteases urokinase and tissue activator transform the one-peptide-chain plasminogen to the two-chain plasmin by cleavage of a few sensitive peptide bonds.

Urokinase has been purified in a number of ways using both conventional methods and affinity chromatography. The affinity chromatographic procedures de-

veloped use benzamidine–Sepharose[50] for adsorption and elution with pH 4.0 buffer containing 0.4 *M* sodium chloride or use agmatin [(1-amino-4-guanidinobutane)–Sepharose][51] and elution with buffer containing 0.4 *M* sodium chloride. Good separations were obtained with both methods. The agmatin was coupled to Sepharose using an ε-aminocaproic acid spacer. Both benzamidine and agmatin competitively inhibit trypsin-like enzymes and could then be expected to interact with urokinase. Urokinase is used clinically for thrombolysis and there has been a considerable interest in purifying urokinase from urine. This has caused special problems as very large amounts of urine have to be processed. Affinity chromatography could have been expected to give a solution, but so far it has not been possible to use it in order to solve this problem.

In the purification of tissue or vascular activator a number of affinity chromatographic methods have been used. One special problem with this protein is that it is easily adsorbed to surfaces and, to prevent this and aggregation, one must work with solutions containing a detergent or a high concentrations of salt. Other difficulties, such as low concentrations and extraction problems, will also have to be solved. Tissue activator has been purified to homogeneity from pig heart and human uteri by Wallén *et al.*[52] and Rijken *et al.*[53].

Wallén *et al.*[52] used adsorption to fibrin and affinity chromatography on arginine–Sepharose as the main separation steps, and Rijken *et al.*[53] used the sequence ammonium sulphate precipitation, zinc chelate–agarose chromatography, butyl-agarose chromatography, concanavalin A–agarose chromatography and gel filtration on Sephadex G-150. The use of adsorption to fibrin is based on the fact that the physiological action of this activator is to bind to fibrin and activate the fibrinolysis there and dissolve the fibrin or thrombus. This is probably an essential mechanism for protection against thrombosis. The use of hydrophobic affinity chromatography can be related to the strong tendency of the protein to adsorb to various surfaces and to aggregate. Zinc ions have previously been used to precipitate tissue activator and the application of zinc chelate chromatography can be seen as an improvement of that procedure.

Vascular activator has been purified by similar methods. Starting with extracts from blood vessels of cadavers, Binder *et al.*[54] used ammonium sulphate precipitation, ammonium sulphate solubilization and octyl-Sepharose affinity chromatography for the purification. Aasted[55] used adsorption to fibrin–Sepharose and affinity chromatography on phenyl-Sepharose as the essential purification steps.

In the former process the final yields were low (between 3 and 5%). In that process, elution from octyl-Sepharose was accomplished using a gradient of 0–50% ethylene glycol in buffers of fairly high ionic strength.

One important way of preventing activity losses which was used in the purification procedure of Aasted was to connect the fibrin–Sepharose column to the phenyl-Sepharose column and allow the 2 *M* potassium thiocyanate eluate from fibrin–Sepharose to be adsorbed directly to the phenyl-Sepharose. The phenyl-Sepharose was then eluted with a gradient of 1–2 *M* phenylethylamine at pH 7.0. Completely pure vascular activator was not obtained with this procedure.

INHIBITORS

Antithrombin III

This glycoprotein is the most important coagulation inhibitor in blood. It inhibits coagulation through complex formation and inactivation of most coagulation enzymes. Further, it is identical with heparin cofactor, *i.e.*, the factor in blood which is necessary for the anticoagulant activity of heparin. Heparin binds to antithrombin III and makes it a more rapidly acting inhibitor.

Antithrombin III was first prepared in trace amounts by conventional techniques. When affinity chromatography on heparin–Sepharose was introduced[56] much larger amounts of antithrombin III could be prepared fairly easily and made available for studies. Antithrombin III is strongly bound to heparin–Sepharose, but can be desorbed using salt gradient elution. A number of other proteins including lipoproteins are also bound to heparin–Sepharose but are desorbed at lower salt concentrations than antithrombin III.

Using heparin–Sepharose adsorption–elution it has been possible to devise large-scale procedures for the preparation of antithrombin III[57,58]. This has then provided the possibility of preparing antithrombin III in amounts sufficient for clinical studies on the possible use of antithrombin III[59]. There are cases of hereditary deficiency of antithrombin III. These patients have a high frequency of thrombosis and can probably benefit from treatment with antithrombin in connection with situations of increased risk, *i.e.*, surgery or childbirth. There are also other clinical conditions where treatment with antithrombin III may be of value[59,60].

Heparin and heparan sulphate

Heparin has been the most commonly used agent for immediate anticoagulant treatment for several decades. Heparin is present in man as in most vertebrates. It is doubtful, however, whether it really has a physiological anticoagulant function in man, as it cannot be found in the blood.

A closely similar type of sulphated glucose-aminoglucan, heparan sulphate, is found in the vascular system and there are indications that this component does contribute to the non-thrombogenic properties of the vessel walls where it is present on the surface of the endothelial cells.

The anticoagulant action of heparin is dependent on the presence of antithrombin III. Heparin has been fractionated on matrix-bound antithrombin III and it was found that two thirds of the material passed through the adsorbent whereas one third was bound[61,62]. The material passing through had very low anticoagulant activity. In contrast, the adsorbed material that could be eluted with buffers containing high concentrations of salt showed very high activity values. The overall compositions of bound and non-bound material did not show any significant differences. Recent studies have shown that the high-activity heparin contains a special eight-residue sequence which is responsible for the binding to antithrombin III and anticoagulant activity[63]. The affinity chromatographic separation of heparin was given better possibilities of studying other biological activities of heparin that are not related to the anticoagulant activity.

Antiplasmin

The main inhibitor of the fibrinolytic enzyme plasmin is the glycoprotein antiplasmin. It rapidly inhibits plasmin by forming inactive complexes. Antiplasmin was discovered fairly recently and has been purified by affinity chromatography[64-66].

The adsorbent used is plasminogen–agarose and elution is accomplished by desorption with ε-aminocaproic acid. Before the affinity chromatography, plasminogen has to be removed using, for instance, lysine–Sepharose adsorption. Approximately 100-fold purification is obtained in one step. The preparation is not pure, however, and further separation steps are necessary. Better purification is obtained[67] if the affinity chromatography is performed on matrix-bound elastase-degraded plasmin A-chain containing the three N-terminal triple-loop structures. Immunosorption can also be used to purify[68] antiplasmin. Elution was performed with a gradient of potassium thiocyanate at low temperature and rapid dilution of the eluate to decrease the denaturing effect of the high concentration of potassium thiocyanate.

The affinity between antiplasmin and plasminogen is related to the fact that plasminogen contains some of the structures in plasmin that are involved in the plasmin–antiplasmin interaction.

DISCUSSION

Affinity chromatographic procedures can be used for a number of different purposes. In most applications purification has been the main goal, but affinity chromatography has also been used successfully to study biological interactions and mechanisms of reaction. The haemostatic system has a number of features that make it attractive to try to apply affinity chromatography both for separation and for other purposes. The system involves a large number of interactions where affinity chromatographic techniques potentially can be used. Many of the components involved are present at very low concentrations in blood and some of them are also very similar to each other, and therefore very powerful and specific separation methods are needed for their purification. Further, many of the components are labile and sensitive to trace amounts of proteases. Affinity chromatographic separations are well suited to meet these problems and it is not surprising to find that affinity chromatography has been applied for the purification of most of the components in the coagulation–fibrinolysis system. A number of different types of affinity chromatographic separations, including inhibitor interaction chromatography, effector interaction chromatography, immunosorption, hydrophobic interaction chromatography, metal chelate chromatography and covalent chromatography, have been used. In most instances very good separations have been obtained.

Elution from the affinity adsorbents is often important both for the purification and for the recovery of active protein. Salt gradient elution has been used frequently and has the advantage of being mild and easy to perform. From a theoretical point of view, elution with the affinity ligand is advantageous and there are some good examples of this, as in the purification of plasminogen by lysine–Sepharose chromatography.

Desorption from immunosorbent columns with good recoveries of biologically active protein is often difficult but was sucessfully performed with prothrombin[5] and antiplasmin[68] using high concentrations of sodium bromide and potassium

thiocyanate, respectively. In the future, the use of monoclonal antibodies can be expected to make immunosorption affinity chromatography even more attractive, as one then would have the possibility of choosing an antibody which binds the antigen with a suitable strength and of performing the desorption under mild conditions.

Affinity chromatographic techniques have also been used successfully to clarify which parts of fibrinogen and fibrin interact during fibrin polymerization, which parts of the plasminogen molecule interact with lysine and the nature of the Factor VIII/Von Willebrand Factor complex. Also important is the discovery of the heterogeneity of heparin with respect to anticoagulant activity and binding to antithrombin III.

It can be expected that affinity chromatographic techniques will be of great value in further studies on the interactions and mechanisms involved in the haemostatic system. In particular, surface-dependent reactions will probably be studied using this type of technique. Various models of the different steps in the coagulation cascade could, for instance, be constructed by various gel-bound systems.

Finally, as regards practical applications there is one example where affinity chromatography has been the essential step for the purification of a component of clinical importance, *viz.*, antithrombin III. Without applying affinity chromatography it has not been possible to prepare it on a large scale at reasonable cost. Antithrombin III is now used for the treatment of hereditary deficiency cases in connection with surgery, childbirth and thrombosis and further clinical studies on possible other uses are in progress.

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