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PURIFICATION OF ENZYMES BY HEPARIN–SEPHAROSE AFFINITY CHROMATOGRAPHY

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CONTENTS

1. INTRODUCTION

In recent years the purification of enzymes has been revolutionized by the technique of affinity chromatography[1,2]. This method utilized the specific interactions found in biological systems. Most proteins undergo biological interactions with other proteins and small molecules in order to function. Thus, enzymes bind with substrates, inhibitors, coenzymes and allosteric effectors; antibodies interact with antigens; hormones bind with specific binding proteins and cellular receptors; lectins bind carbohydrate and nucleic acids and repressor proteins interact with genes[3,4]. These interactions can be used for the purification of proteins.

The general scheme of affinity chromatography involves the covalent attachment of a ligand to a solid support. A crude tissue or bacterial homogenate is then applied to the column; only the protein that specifically interacts with the ligand will be retained, whilst the other proteins, having no affinity for the immobilized ligand, will be washed out. The retained protein can be eluted specifically from the column either by adding an excess of ligand to the equilibrating buffer or by using substances that interfere with the protein–ligand binding such as salts or denaturants. The success of affinity chromatography depends largely on how closely the conditions used in the experiment mimic the native or biological interactions. Careful consideration must be given to the nature of the matrix and to the steric restriction imposed by immobilization of the ligand. The matrix should be relatively hydrophilic and should possess good chemical and mechanical stabilities. The steric effect can be overcome by using spacers. Both matrix and spacer should lack non-specific absorption sites. The other important point is the selection of ligand. There are two classes of ligands: general ligands and specific ligands. General ligands interact with many different

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TABLE 1

EFFECT OF HEPARIN ON VARIOUS ENZYMES

Enzyme	*Effect*	*Ref.*	*Enzyme*	*Effect*	*Ref.*
Acid phosphatase	Inhibition	12, 45	Ribonuclease	Inhibition	16, 17
Alkaline phosphatase	Inhibition	12	Deoxyribonuclease	Inhibition	47
N-Acetyl-β-hexosaminidase	Inhibition	11	Endonuclease	Inhibition	48
Acid lipase	Inhibition	11	Trypsin	Inhibition	20
Cathepsin Band C	Inhibition	11	Pepsin	Inhibition	21
α- and β-Fucosidase	Inhibition	11	Fumerase	Inhibition	22
α- and β-Galactosidase	Inhibition	11	Lipoprotein lipase	Activation	24, 26, 27
Hyaluronidase	Inhibition	13	Trehalose phosphate synthetase	Activation	32–34
α-Mannosidase	Inhibition	11	Tyrosine hydroxylase	Activation	35–37
Neuraminidase	Inhibition	46			
DNA polymerase	Inhibition	14			
RNA polymerase	Inhibition	15			

proteins and examples are NAD, Cibacron blue and lectins[5–7]. Ligands that interact with one or few proteins are called specific ligands, examples being antigens[5]. Thus affinity chromatography is a unique separation tool which has a major advantage over other methods of purification due to its tremendous specificity. This technique allows rapid purification of various proteins with good yield in a single step.

2. INTERACTION OF HEPARIN WITH ENZYMES

Heparin is a linear and highly sulfated glycosaminoglycan with anticoagulant properties. It exists in a wide range of polymers (mol. wt. 5000–30,000) and is composed of disaccharide units of α-L-idopyran-uronic acid 2-sulfate and 2-deoxy-2-sulfamino-α-D-glucopyranose 6-sulfate linked through positions 1 and 4[9,10]. Due to its polyanionic nature heparin interacts with many enzymes (Table 1) and produces significant changes in their activities. Thus, it inhibits almost all lysosomal hydrolases[11]. On the basis of variation of ionic strength and pH value, it was suggested that reversible electrostatic interactions between anionic groups of heparin and cationic groups of the enzyme molecule were responsible for this inhibition. Although the nature of the inhibition of all these enzymes was not determined, the inhibition of acid phosphatase[12] and hyaluronidase[13] was competitive. Heparin causes marked inhibition of enzymes associated with nucleic acid metabolism. Thus, it inhibits DNA polymerase[14], RNA polymerase[15] and ribonuclease[16,17]. It must be emphasized here that the selective binding of these enzymes to heparin is not due to ionic interactions but rather due to enzyme–inhibitor interaction. In the case of DNA-dependent RNA polymerase, the heparin interferes with the initiation of transcription[18,19]. Other enzymes such as trypsin, pepsin and fumarase are also inhibited by heparin, probably due to the formation of a heparin–enzyme complex[20–22]. Here it must be recalled that trypsin[23] contains an essential imidazole group in its active site. It is possible that heparin inhibits trypsin by blocking the positively charged imidazole group which is essential for its activity.

Heparin stimulates several enzymes. Korn[24,25] isolated lipoprotein lipase from rat heart and showed that the hydrolysis of chylomicron by this enzyme was activated by heparin and strongly inhibited by a polycation such as protamine. He postulated that lipoprotein lipase contained bound heparin which could be competitively displaced by protamine. Patten and Hollenberg[26] suggested that heparin stimulation of lipoprotein lipase was due to increased binding of the enzyme to chylomicrons and heparin had no effect on the stability of the enzyme or on its activity once the complex was formed. On the basis of various kinetic studies, Whayne and Felts[27] suggested that heparin may function as a specific ligand that acts as an allosteric modifier of lipoprotein lipase and alters the kinetics of substrate–enzyme interaction. Iverius *et al.*[28] also concluded that lipoprotein lipase contained small amounts of an endogenous, heparin-like material, further indicating that heparin may be part of, or bound to, the enzyme protein. Although the exact rôle of heparin in lipoprotein lipase reaction is not known, the collective evidence[29–31] indicates that intravenous injections of heparin in the rat, pig and man cause the release of two lipoprotein lipases which differ in their sensitivity to NaCl, protamine sulfate and diethyl-*p*-nitrophenyl phosphate and in their requirement for a lipoprotein polypeptide. One of these lipases is reported to originate solely in extrahepatic tissues and requires

apoLp-Glu for maximal activity. The second lipase, resistant to high NaCl and protamine sulphate, has been reported to come from the liver. *In vitro*, heparin stimulates lipoprotein lipase but the precise nature of this activation is not clear. Heparin not only stabilizes the enzyme and protects it from heat inactivation, but also plays a rôle in linking the enzyme to its lipid substrate.

Lapp *et al.*[32] were the first to report the stimulation of trehalose phosphate synthetase by heparin. They found that heparin concentrations of 0.5–1 μg/ml were optimal for activation, whereas higher concentrations (5 μg/ml) were inhibitory. Elbein and Mitchell[33,34] reinvestigated the stimulatory effect of heparin on the trehalose phosphate synthetase activity of *Mycobacterium smegmatis* and reported that in the presence of heparin (0.5 mg/ml) the trehalose phosphate synthetase shows increased stability when heated at 50°C for various time periods as compared to the enzyme in the absence of heparin. Heparin also prevents digestion of the enzyme by trypsin. In the absence of heparin, the enzyme was retained on a Sephadex G-200 column (mol.wt. 40,000–50,000). However, when heparin (0.5 mg/ml) was mixed with the enzyme the trehalose phosphate synthetase was excluded from the Sephadex G-200 column and eluted in an area suggesting a molecular weight of greater than 450,000. The data of Elbein and Mitchell[34] were consistent with the view that heparin stabilizes the trehalose phosphate synthetase and also causes an association of the enzyme to high-molecular-weight forms which have increased stability.

The enzyme tyrosine hydroxylase is also activated allosterically by heparin. Kuczenski and Mandell[35,36] reported that the addition of heparin induces a conformational change in tyrosine hydroxylase which results in a greater affinity for norepinephrine. It has also been suggested that heparin can produce a more active tyrosine hydroxylase which is responsible for modulation in the level of the end product, norepinephrine. Now it is believed that the normally soluble tyrosine hydroxylase becomes activated as it approaches the surface of the synaptic vesicle or other membrane site which contains heparin[37]. The action of heparin or heparin sulfate on tyrosine hydroxylase is not shared by the other mucopolysaccharides.

3. METHODS FOR THE COVALENT ATTACHMENT OF HEPARIN TO SEPHAROSE

Two procedures have been used for coupling heparin to Sepharose: (1) the cyanogen bromide procedure; (2) the cyanuric chloride procedure.

Iverius[38] was the first to couple heparin to Sepharose 4B using cyanogen bromide[39,40]. Sepharose 4B (settled bed volume 500 ml) was washed twice with distilled water by decantation and suspended in 1.5 l of distilled water. Cyanogen bromide (25 g) were dissolved in 50 ml of cold dimethylformamide and added to the Sepharose suspension with stirring in an ice-bath. The pH of the mixture was maintained between 10.5 and 11.5 with 5 *N* NaOH and the reaction was allowed to continue until the rate of change of the pH with time became negligible (about 1–1.5 h). The suspension was then poured over a fritted glass filter and the activated Sepharose 4B washed with 8 l of water. All of these operations were carried out in a fume cupboard. The Sepharose 4B cake was suspended in 1.5 l of 0.1 *M* $NaHCO_3$ containing 1.5 g of heparin and the suspension was stirred slowly overnight at 4°C. Triethylamine (60 ml) was then added and stirring was continued for a further 4 h.

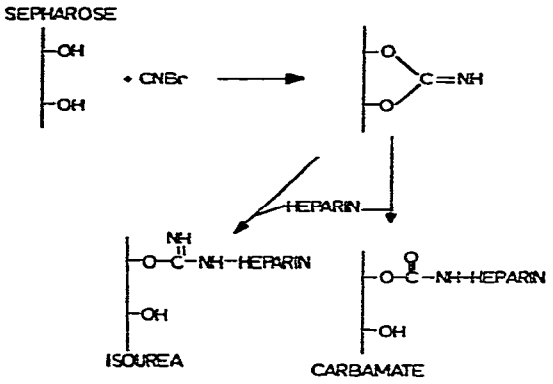

Fig. 1. Reaction sequence showing the coupling of heparin to Sepharose by cyanogen bromide procedure.

The heparin–Sepharose was collected on a fritted glass filter and washed with 3 l of water. The gel was then suspended in an equal volume of buffer containing 20 m*M* Tris–HCl buffer, pH 7.0, and 0.5 m*M* EDTA.

The chemistry of the cyanogen bromide reaction is not known but it has been suggested[41] that isourea or carbamate derivatives are formed during the reaction (Fig. 1). This procedure depends on the availability of free amino groups on the heparin molecule. The source of such groups is either an unsulfated hexosamine unit or a N-terminal amino acid retained from the native proteoglycan[38]. The preparation of heparinized aminoethyl agarose using cyanogen bromide has also been reported[42]. Srivastava and Farooqui[43] covalently attached heparin to Sepharose 4B using cyanuric chloride (2,4,6-trichloro-1,3,5-triazine). Sepharose 4B (50 ml) was washed five times with 1 *M* Na_2CO_3 and the excess of Na_2CO_3 was removed by draining. Heparin (100 mg) dissolved in 5 ml of 1 *M* Na_2CO_3 was added to Sepharose 4B with constant shaking. Cyanuric chloride (1 g) suspended in 15 ml of acetonitrile was added to the above Sepharose 4B. The mixture was maintained at pH 11 with 1 *M* Na_2CO_3 during shaking for 1 h at 65°C. The reaction mixture turned yellow during the reaction. After 1 h the mixture was filtered through a büchner funnel, and washed with 500 ml of water–ethanol–trimethylamine (2:1:1, v/v/v). Finally, the heparin–Sepharose 4B was washed with 50 m*M* Tris–HCl buffer, pH 7.0, containing 0.5 m*M* EDTA. The proposed mechanism of coupling by cyanuric chloride is shown in Fig. 2. During the activation reaction the free hydroxy group of heparin reacts with cyanuric chloride to give a heparin–cyanuric chloride complex at alkaline pH. The hydroxy group of Sepharose 4B then reacts with a second chlorine atom to form heparin–monochlorotriazinyl Sepharose 4B.

The amount of heparin covalently bound to Sepharose can be determined by measuring the hexosamine and sulfate contents of the gel. Thus the cyanogen bromide procedure resulted in coupling of about 1 mg heparin per ml of wet Sepharose 4B[38]. The cyanuric chloride procedure gave a heparin–Sepharose preparation which had 0.8 mg heparin per ml of the wet gel[43]. Recently Varshavskaya *et al.*[44] described a simple method for the determination of the heparin content in heparin–Sepharose. This method is based on the potentiometric titration of heparin with 0.5 *M* KOH. The

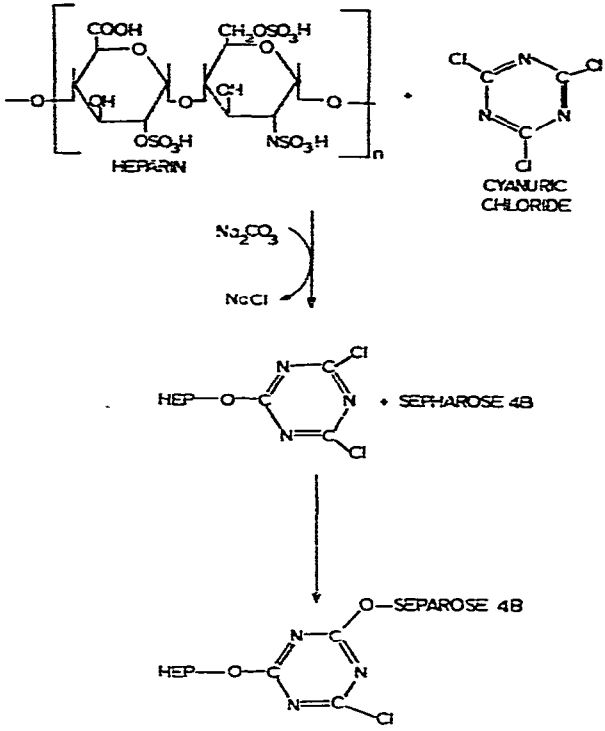

Fig. 2. The mechanism of coupling of heparin to Sepharose by cyanuric chloride procedure[43].

values obtained for the coupled heparin by the potentiometric method and by the determination of hexosamine and sulfate content were similar.

4. PURIFICATION OF ENZYMES BY HEPARIN–SEPHAROSE CHROMATOGRAPHY

The list of enzymes purified by heparin–Sepharose chromatography is shown in Table 2. The heparin–Sepharose was washed with the desired buffer and packed into a small column. The crude enzyme preparation, dialysed against the column

TABLE 2

ENZYMES PURIFIED BY HEPARIN–SEPHAROSE AFFINITY CHROMATOGRAPHY

Enzyme	*Purification achieved (Fold)*	*Ref.*
Lipoprotein lipase	200–600	49–53
DNA polymerase	107–125	14
RNA polymerase	10–80	54–58
Restriction endonuclease	10	48
Collegenase	7	59
Iduronidase	18	60
Heparin N-sulphatase	4	61
Hyaluronidase	4–10	43, 62
Threalose phosphate synthetase	10–38	34

equilibrating buffer, was applied to the heparin–Sepharose 4B column at a flow-rate of 10–12 ml/h. The enzymes having affinity or interaction with heparin were retained and the rest of the proteins were washed out. The enzymes appeared as a sharp peak when the column was eluted with a gradient of NaCl or heparin or some other compound which interferes with the binding of heparin–Sepharose with the retained enzymes. The heparin–Sepharose can be regenerated for rechromatography by washing the gel alternately with fifteen to twenty column bed volumes of buffers of pH 8.0 and pH 5.0 containing 0.5 *M* NaCl.

It is obvious from Table 1 that heparin–Sepharose can be used for the purification of lysosomal hydrolases, and enzymes of nucleic acid and lipid metabolism. According to Brennessel *et al.*[14], heparin–Sepharose may be useful in evaluating the subunit structure and mechanism of action of eukaryotic DNA polymerases, particularly since the inhibition of at least one cellular DNA polymerase enzyme by heparin can be correlated with a dissociation of the polymeric form of the enzyme into smaller active species[63]. The differences in the molecular weights of DNA polymerase isolated by heparin–Sepharose chromatography and by conventional methods may help in determining the structure and function of these important cellular enzymes. Lipases having similar physicochemical properties can also be separated by heparin–Sepharose chromatography. Thus, Boberg *et al.*[51] separated lipoprotein lipase and hepatic triglyceride lipase by this procedure. Heparin–Sepharose chromatography has been successfully used for the separation of two forms of hyaluronidase[43] and heparin N-sulphatase[61].

It must be noted here that it was not possible to separate these forms either by ion-exchange chromatography or polyacrylamide gel electrophoresis. During the purification of hyaluronidase[43,62] by heparin–Sepharose chromatography it was clearly shown that the binding of this enzyme to heparin–Sepharose was due to enzyme–inhibitor interaction. As heparin–Sepharose is a polyanionic gel, it may interact with many proteins such as lysozyme[64] and acrosin[65].

These proteins have isoelectric points of 10.5 and 10.2 respectively. It is interesting that both these proteins are retained on the heparin–Sepharose column and are readily eluted with a linear gradient of 0–1 *M* NaCl but not with heparin or hyaluronic acid. This strongly suggests that, unlike hyaluronidase, the binding of lysozyme and acrosin to the heparin–Sepharose was due to charge interactions.

5. OTHER USES OF HEPARIN–SEPHAROSE CHROMATOGRAPHY

Heparin binds selectively and firmly to many blood proteins such as fibrinogen, thrombin and plasmin[66]. It also interacts with low and high density serum lipoproteins[67,68]. Heparin–Sepharose was successfully used for the purification of thrombin[69], antithrombin III[70], arginine-rich apoprotein[71] and low and high density lipoproteins[72,73]. On the basis of acetylation of low density lipoproteins, Iverius[72] suggested that lysine residues of these proteins were involved in binding with heparin. In the case of arginine-rich apoprotein[71], arginine residues have been shown to be important during the interaction of heparin with protein. The fact that the heparin–protein complexes can be disrupted by high salt concentration indicates the ionic nature of these interactions.

Heparin also interacts with cytosol oestrogen receptor of lactating mammary

glands and uterus[74]. The treatment of cytosol with heparin dissociates the oestrogen-binding activity from the aggregate into a receptor. Auricchio *et al.*[75] purified this receptor by heparin–Sepharose chromatography and suggested that the heparin-induced dissociation of the receptor could be due either to the removal of substances associated with the receptor or to the direct interaction of the receptor with heparin. Heparin–Sepharose chromatography was efficiently used for the purification of androgen receptors from rat prostate[76]. It must be noted here that the nature of the interaction between these receptor proteins and heparin was not determined. It is hoped that some explanation of receptor–heparin interaction will be forthcoming.

The binding of heparin to other proteins also includes insulin, oxytocin, corticotropin and vasopressin[77]. Aborg and Uvnas[78] reported a protamine–heparin complex which has the ability to bind histamine and monoamines by ionic interactions.

Several studies[79–82] have indicated that heparin–Sepharose chromatography was efficiently used for the isolation of protein synthesis factors and ribosomes. The polyribosomes retained on heparin–Sepharose are readily dissociated into subunits by a mere increase in the K^+/Mg^{2+} ratio. The use of heparin–Sepharose chromatography for the separation of various protein synthesis factors will result in further characterization of initiation, elongation and protein synthesis factors and will result in considerable knowledge regarding the protein synthesis.

It has recently been reported[83,84] that heparin–Sepharose can be used for the removal of cholesterol from the blood and can be important for the treatment of homozygous familial hypercholesterolemia.

6. ADVANTAGES AND DISADVANTAGES OF HEPARIN–SEPHAROSE CHROMATOGRAPHY

Heparin–Sepharose chromatography offers many advantages over ion-exchange chromatography. Heparin has a stable structure and once coupled the heparin–Sepharose is stable for at least 1 year. This gel has excellent flow properties and packs well into the column. The chemical stability of heparin–Sepharose appears to be quite good. Due to the polymeric and polyanionic nature of heparin, the gel has a very high capacity allowing the use of small columns. As a result the enzymes elute from the column in small volumes with very high activity. The proteins retained on heparin–Sepharose are very sensitive to variation of pH and ionic strength. Furthermore, the peaks of enzymes eluted by heparin are sharper than those eluted by a salt gradient.

Heparin is a general ligand which interacts with many enzyme proteins. Some of these interactions are specific and others are ionic. The major disadvantage of heparin–Sepharose chromatography is its broad specificity. Thus it is not possible to obtain homogeneous enzyme preparations in a single step. If homogeneous preparations of enzymes are required, the heparin–Sepharose chromatography must be combined with other methods of purifications. The heparin–Sepharose cannot be used below pH 3.0 and above pH 9.0 because many proteins which bind to heparin–Sepharose between pH 5 to 7 are either not retained in the column or are irreversibly bound to the heparin–Sepharose matrix.

7. CONCLUSION

From the above discussion it is clear that heparin interacts with many enzymes and proteins. These interactions may result in activation or inhibition of enzyme activity. The fact that heparin can bind to or alter enzyme activities is perhaps not surprising since it is highly charged at physiological pH values. The crucial questions, whether these interactions occur *in vivo* and play some rôle in regulation of enzyme activities, cannot be answered at this time because our knowledge of heparin–protein interactions is still incomplete. However, there is a reasonable body of evidence to indicate that heparin does interact with cells or protein in connective tissue[85] and circulating system[86,87]. The "discovery" of heparin–Sepharose has been one of the most important developments in analytical and preparative techniques used for the separation and isolation of various proteins. For the last 3–4 years, heparin has been highly exploited as a ligand for affinity chromatography. Yet, only a small percentage of the total number of enzymes which interact with heparin have been chromatographed on heparin–Sepharose. It is hoped that in the future heparin–Sepharose chromatography will be widely used for protein fractionation.

8. SUMMARY

Heparin selectively interacts with many enzymes and brings about significant changes in their activities. Due to its polyanionic nature, heparin also binds to many cationic proteins. Both these interactions may be used as a basis for the purification of proteins by heparin–Sepharose affinity chromatography. Recently heparin–Sepharose has been extensively used for the purification of enzymes, coagulation proteins, steroid receptors and protein synthesis factors.

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