Journal of Chromatography, 400 (1987) 123–132
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1105

IMPROVED METHOD FOR THE IMMOBILIZATION OF HEPARIN

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SUMMARY

The optimal conditions for immobilizing heparin through its terminal formyl group were investigated. When Amino Sepharose (1 g) was suspended in 1 ml of phosphate buffer (pH 7) containing 30 mg of heparin and 3 mg of sodium cyanoborohydride, with shaking at room temperature, the maximum immobilization of heparin (10 mg of heparin per gram of wet gel) was reached within 2 days. The Heparin Sepharose thus obtained was stable: no significant loss of the heparin content was observed after storage for 4 months at 4°C. Heparin was also immobilized by the same method with Amino TSK gel G5000PW instead of Amino Sepharose 4B and was successfully applied to the high-performance liquid affinity chromatography of fibronectin and thrombin.

INTRODUCTION

Heparin is well known to interact with various substances and to have a number of biological activities in addition to its anticoagulant and lipaemia-clearing properties. Therefore, immobilized heparin has been widely used as an adsorbent in affinity chromatography for the investigation and preparation of biological substances[1–9].

The preparation of effective affinity adsorbents generally requires appropriate coupling procedures and modes of ligand attachment to the matrix. Hitherto, heparin has mainly been immobilized by derivatization through its carboxyl or amino groups. In a previous study[10], we reported a new method of immobilizing heparin by reductive amination of its terminal formyl group and amino-agarose, which was obtained by epoxy activation of Sepharose 4B with epichlorohydrin and subsequent amination. The heparin-agarose thus prepared was found to be the most efficient immobilized form for adsorption of thrombin and antithrombin III (AT-III), probably because heparin was immobilized in the most intact form. However, it required a protracted

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0021-9673/87/$03.50

coupling reaction (over 10 days)[11] to immobilize heparin by reductive amination.

In this study, to circumvent this disadvantage, we investigated precisely the optimal conditions for the coupling of heparin at the reducing end to Amino and Hydrazino Sepharose. We also studied the coupling of heparin to Toyopearl gels, which are hydrophilic vinyl polymer matrices available as carriers for high-performance liquid chromatography (HPLC), and the application of Heparin TSK gel G5000PW to the high-performance liquid affinity chromatography (HPLAC) of biological substances.

EXPERIMENTAL

Materials

The sources of materials were as follows: Sepharose 4B, Pharmacia (Uppsala, Sweden); Toyopearl HW-55 and TSK gel G5000PW, Toyo Soda (Tokyo, Japan); heparin sodium salt (porcine intestinal mucosa, 163.7 U/mg), epichlorohydrin, hydrazine hydrate, 2,4,6-trinitrobenzene sulphonate (TNBS) and adipic acid dihydrazide, Wako (Osaka, Japan); sodium cyanoborohydride ($NaCNBH_3$), Nakarai (Kyoto, Japan); bisoxirane, Aldrich (Milwaukee, WI, U.S.A.); thrombin (47 U/mg), Miles Labs. (Slough, U.K.); and t-Boc–Val–Pro–Arg–MCA (*tert.*-butoxycarbonylvalylprolylarginine 4-methyl-coumaryl-7-amide), Protein Research Foundation (Osaka, Japan). Human plasma fibronectin was purified by the method of Yamada[12]. The sample of fibronectin thus prepared gave a single band by SDS polyacrylamide gel electrophoresis, according to the method of Laemmli[13].

Preparation of amino- and hydrazino-agarose gels

Sepharose 4B was activated with epichlorohydrin, as described previously[14]. A 20-g amount of suction-dried Sepharose 4B was suspended in 30 ml of water and mixed with 13 ml of 2 *M* sodium hydroxide solution, then 3 ml of epichlorohydrin were added. The suspension was incubated at 40°C for 2 h with shaking. The gel was washed extensively with water. The epoxy-activated Sepharose obtained was subsequently converted into amino[15] and hydrazino[16] derivatives. Suction-dried epoxy-activated Sepharose 4B was treated with 1.5 volumes of concentrated ammonia solution or 1.5 volumes of hydrazine hydrate at 40°C for 1.5 h with shaking, then washed with water.

Preparation of amino- and hydrazino-polyvinyl gels

Toyopearl HW-55 or TSK gel G5000PW was activated with epichlorohydrin as described previously[17]. A 10-ml volume of 15 *M* sodium hydroxide solution and 25 ml of epichlorohydrin were added to 13 g of suction-dried gel, and the suspension was incubated at 50°C for 2 h with shaking. The gel was washed extensively with water. Amino and hydrazino gels were prepared by the same methods as the corresponding derivatives of Sepharose gel.

Preparation of long-spacer carriers

To prepare long-spacer amino and hydrazino carriers, two procedures were employed. In the first, 4 g of suction-dried Sepharose 4B were suspended in 4 ml of bisoxirane plus 4 ml of 0.6 *M* sodium hydroxide solution containing 8 mg of sodium

borohydride. The suspension was incubated with shaking at room temperature for 8 h, and the gel was extensively washed with water[18], then converted into its amino derivative. In the second method, a suspension of 1 volume of suction-dried Sepharose 4B or Toyopearl HW-55, activated with epichlorohydrin in 1.5 volumes of 0.1 *M* sodium carbonate solution, saturated with adipic acid dihydrazide (2.3 g per 20 ml), was incubated at 40°C for 1.5 h. The gel was washed extensively with water.

Coupling of heparin with amino and hydrazino carriers

A suspension of 1 g of amino or hydrazino gel in 1 ml of 0.2 *M* phosphate buffer, containing 30 mg of heparin and 3 mg of $NaCNBH_3$, was incubated at room temperature with or without shaking. The shaking was carried out vigorously using a horizontal shaker to mix well solid gel and heparin solution. To find the optimal conditions, a series of experiments were carried out with one of the parameters modified in each experiment. To remove free amino or hydrazino groups remaining in the gel, the gel was acetylated[19] before use as an affinity adsorbent. Complete acetylation was confirmed by the TNBS colour test[20].

Determination of heparin immobilized on the gel

The amount of heparin immobilized on the gel was determined by the content of glucosamine in the gel. Suction-dried heparin gel was hydrolyzed with 4 *M* hydrochloric acid at 100°C for 16 h in a sealed tube, then centrifuged. The supernatant was evaporated to dryness and the residue was dissolved in a suitable amount of water, then the concentration of glucosamine was determined by the Elson–Morgan reaction[21]. The amount of immobilized heparin is expressed as milligrams per gram of suction-dried gel (wet gel).

Measurement of thrombin activity

The thrombin activity of the eluent was measured as described by Morita *et al.*[22] using t-Boc–Val–Pro–Arg–MCA as substrate. The fluorescence of the 7-amino-4-methylcoumarin produced was measured with a Hitachi Model 650-60 fluorescence spectrophotometer with excitation at 380 nm and emission at 460 nm.

HPLAC of fibronectin and thrombin on Heparin TSK gel G5000PW

Heparin TSK gel G5000PW, equilibrated with 10 m*M* Tris–HCl buffer containing 50 m*M* sodium chloride (pH 7.6), was packed into a 10 × 6 mm I.D. column. An HPLC system with Model HLC-803D high-pressure pump (Toyo Soda) and a UV-8 Model II spectromonitor (Toyo Soda) was used. Purified fibronectin or commercial thrombin was applied to the column. After washing with the same buffer, the adsorbed substances were eluted with a 20-min linear gradient of sodium chloride from 50 m*M* to 4 *M* in 10 m*M* Tris–HCl buffer (pH 7.6) at a flow-rate of 1 ml/min.

RESULTS

Optimal conditions for the immobilization of heparin on amino carriers

Effect of heparin concentration. Samples of amino gels were mixed with 0.2 *M* phosphate buffer containing various concentrations of heparin and shaken for 3 days, then the heparin contents of the gels were determined. The amounts of heparin im-

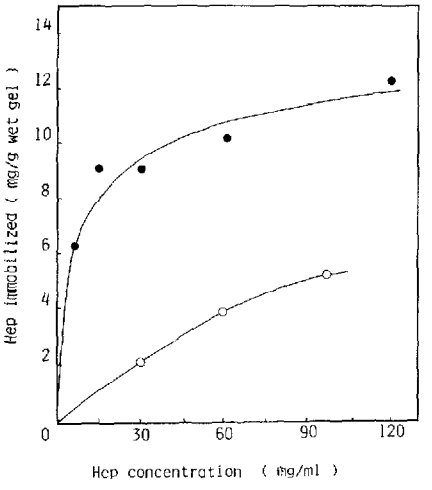

Fig. 1. Effect of heparin (Hep) concentration in the reaction mixture on the immobilization of heparin on the carriers. ● = Amino Sepharose 4B; ○ = Hydrazino Sepharose 4B.

mobilized increased with increasing initial concentrations of heparin in the reaction medium. Initial concentrations of up to 30 mg/ml of heparin gave more efficient results than higher concentrations (Fig. 1).

Effect of shaking. Heparin was immobilized on Amino Sepharose 4B or Amino Toyopearl HW-55 with or without shaking. With both carriers, shaking resulted in much greater amounts of heparin being introduced in shorter coupling-reaction times, as shown in Table I.

Effect of reaction time. The time course of immobilization of heparin with shaking are shown in Fig. 2. A reaction time of 2 days was sufficient for both Amino Sepharose and Amino Toyopearl.

Effect of pH. The amount of heparin introduced with phosphate buffer of pH 7 was higher (8.5 mg/g gel) than that at pH 9 (6.5 mg/g gel). From this result and the stability of heparin, pH 7.0 was used in subsequent experiments.

Under optimal conditions, the incubation of 1 g each of Amino Sepharose 4B, Amino Toyopearl HW-55 or Amino TSK gel G5000PW in 1 ml of 0.2 *M* phosphate buffer (pH 7), containing 30 mg of heparin and 3 mg of $NaCNBH_3$, at room tem-

TABLE I

EFFECT OF SHAKING ON IMMOBILIZATION OF HEPARIN

Conditions	*Amount of heparin immobilized (mg/g wet gel)*	
	Amino Sepharose	*Amino Toyopearl*
With shaking	10.0 (4 days)	4.9 (4 days)
Without shaking	3.0 (20 days)	0.9 (18 days)

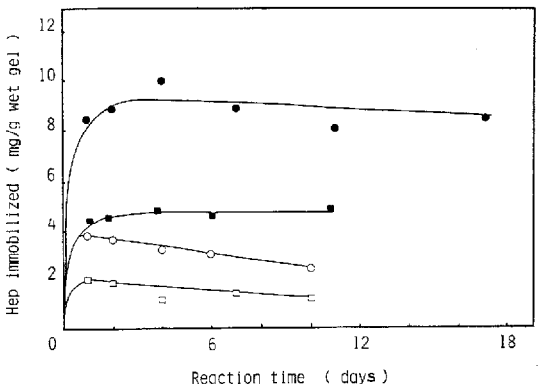

Fig. 2. Effect of reaction time on the immobilization of heparin on the carriers. ● = Amino Sepharose 4B; ■ = Amino Toyopearl HW-55; ○ = Hydrazino Sepharose 4B; □ = Hydrazino Toyopearl HW-55.

perature with shaking for 2 days resulted in immobilization of 10, 5 and 4 mg of heparin, respectively. Long-spacer amino carrier, prepared by amination of bisoxirane-activated Sepharose 4B, bound less heparin than short-spacer Amino Sepharose, prepared by amination of epichlorohydrin-activated Sepharose 4B (3 mg/g for long-spacer Sepharose *versus* 10 mg/g for short-spacer Sepharose).

Immobilization of heparin to hydrazino carriers

The amount of heparin immobilized on Hydrazino Sepharose 4B increased with increasing initial concentration of heparin in the reaction medium (Fig. 1). As Fig. 2 shows, with both Hydrazino Sepharose 4B and Hydrazino Toyopearl HW-55, heparin coupling reached a maximum within 15 h, after which it tended to decrease. The maximum amounts of heparin introduced were 4 and 2 mg/g gel, respectively. With the long-spacer hydrazino carriers, prepared with adipic acid dihydrazide, the amounts of heparin introduced were 0.1 mg/g gel for the long-spacer Sepharose 4B derivative and less than 0.1 mg/g gel for the Toyopearl HW-55 derivative.

HPLAC of fibronectin and thrombin on Heparin TSK gel G5000PW

The results of the HPLAC of fibronectin and thrombin on a column of Heparin TSK gel G5000PW are shown in Fig. 3A and B, respectively. In the latter instance, unbound and bound fractions were separately pooled and assayed for thrombin activity. No activity was detected in the unbound fractions. Over 80% of the total activity was recovered in the bound fractions and the specific activity was increased by 3.5-fold per $A_{280\ nm}$.

DISCUSSION

Heparin, immobilized through its reducing end to Amino Sepharose by reductive amination[10], has several advantages: (1) the linkage between heparin and the matrix is a stable covalent bond; (2) all the active sites of heparin remain available to interact with biological substances, such as thrombin, AT-III and chicken liver lectin; (3) the procedure is simple; and (4) after acetylation of remaining amino

TABLE II

COMPARISON OF IMMOBILIZATION OF HEPARIN BY VARIOUS METHODS

EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. CDAP = 1-cyano-4-dimethylaminopyridinium tetrafluoroborate.

Method of heparin attachment	*Functional group used for linkage*		*Mode of heparin attachment**	*Heparin bound (mg/g wet gel)*	*Adsorption capacity*** *for AT-III*	*Ref.*
	On heparin	*On carrier*				
Reductive amination of Amino Sepharose	CHO	NH_2		10	+ + + +	This work
Reductive amination of Aminohexyl Sepharose	CHO	NH_2		0.71	+ +	24
Reductive amination of nitrous acid-degraded heparin, Aminohexyl Sepharose	CHO	NH_2		20	+ +	23
EDC condensation with Aminohexyl Sepharose	COOH	NH_2		0.6	+ + +	25
Diazo coupling of amino-phenethylheparin with aminophenethylagarose	COOH	NH_2		5.6	+ + +	26
EDC condensation with Amino Sepharose	COOH	NH_2		18.6	+ + +	10
CNBr activation of heparin, Aminohexyl Sepharose	OH	NH_2		7.1	+ +	24
CNBr activation of heparin, Sepharose 4B	OH	OH		20	+	3

CNBr activation of Sepharose 4B	NH_2	OH		3.2	+++	25
CL-4B	NH_2	OH		0.44	24	
CL-6B	NH_2	OH		–	++	27
CNBr activation of Sepharose 4B, N-desulphated heparin	NH_2	OH		2.9	++	25
CDAP activation of Sepharose CL-4B	NH_2	OH		1.5	++	24
Glutaraldehyde cross-linked gelatin–heparin	NH_2	NH_2		–	+	24
Acid hydrolysis of Sepharose CL-4B + reductive amination	NH_2	CHO		–	+	24
EDC condensation with Carboxyl Sepharose	NH_2	COOH		1.0	+	10

* ⊛ = Carrier; ▬ = heparin; ▬ = heparin fragment

** ++++ = >500; +++ = 100–500; ++ = 5–100; + = <5 AT-III U/g wet gel.

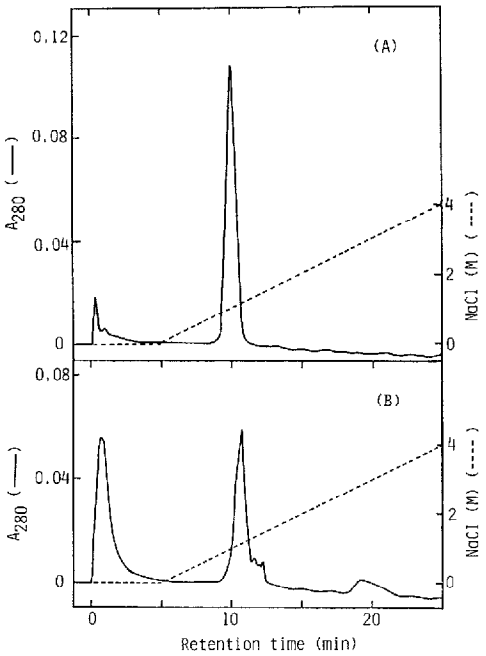

Fig. 3. High-performance affinity chromatography of heparin-binding substances on Heparin TSK gel G5000PW. A 100-μg amount of (A) purified fibronectin or (B) commercial thrombin was applied to the column.

groups, the spacer–carrier portions of the gel have no functional groups that might cause non-specific interactions. However, this method also has disadvantages: a low recovery of heparin (and therefore a low concentration of heparin in the gel) and the requirement of a prolonged reaction time, as described previously[11].

By use of hydrazino carriers, which are more reactive than amino carriers, the reaction time for coupling was shortened, as expected. However, the amount of heparin immobilized decreased slowly after reaching a maximum, and the immobilized preparations were not sufficiently stable, even after acetylation of the remaining hydrazino groups; the heparin content decreased to only 30% of the initial content after storage for 4 months at 4°C as packed gel. Also, less heparin was immobilized on hydrazino carriers than on amino carriers, perhaps because of the lower stabilities of the immobilized hydrazino preparations.

To study the effect of spacers, two carriers with long spacers were used for the coupling with heparin: long-spacer amino carrier, prepared by amination of bisoxirane-activated carrier, and long-spacer hydrazino carrier, prepared with epichlorohydrin-activated carrier and adipic acid dihydrazide. The concentrations of heparin immobilized to these carriers were lower than with the corresponding short-spacer carriers. This may be because of their low content of amino or hydrazino groups, which resulted from the formation of cross-linking to some extent and the low solubility of adipic acid dihydrazide.

Immobilization of heparin on short-spacer Amino Sepharose was therefore investigated further, and it was found that shaking the reaction mixture markedly

promoted the coupling reaction. Under the optimal conditions used here, the highest amount of heparin was immobilized on Amino Sepharose 4B at neutral pH within 2 days. Heparin, immobilized on amino carriers, was very stable on storage as packed gels at 4°C, with over 90% of the heparin remaining in the gels after 4 months.

Heparin Sepharoses prepared by various methods are listed in Table II. Hoffman *et al.*[23] reported the immobilization of heparin by reductive amination between Aminohexyl Sepharose and the terminal formyl group, obtained by nitrous acid treatment of heparin. This method has the advantage of introducing more heparin than the method in which intact heparin is used, but it inevitably causes depolymerization of the heparin molecule.

Recently, Mitra *et al.*[24] reported the affinity chromatographic purification of human AT-III with immobilized heparins prepared by six different methods. Among the adsorbents used, that obtained by coupling cyanogen bromide-activated heparin to Aminohexyl Sepharose had a low AT-III capacity per heparin molecule, despite having the highest heparin content. This is probably the result of modification of the heparin molecule. They also immobilized heparin on Aminohexyl Sepharose by reductive amination and obtained an adsorbent with a low heparin content (0.71 mg/g of dry gel). However, using the improved procedure described in this paper, an adsorbent with an extremely high content (10 mg/g of wet gel) was obtained by reductive amination. The adsorbent showed the highest adsorption capacities for antithrombin, as high as 6000 U of AT-III per gram of suction-dried gel (measured by the method described previously[10]), among the adsorbents listed in Table II.

The hydrophilic vinyl polymers Toyopearl HW-55 and TSK gel G5000PW were also converted into amino and hydrazino derivatives and coupled with heparin. The heparin contents of these gels, however, were lower than those of the agarose adsorbents prepared by the same methods. Nevertheless, because of its mechanical stability, the Heparin TSK gel G5000PW thus prepared could be used successfully for the HPLAC of various heparin-binding substances.

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