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(54) **Affinity chromatography absorbent**

(57) A process for the preparation of an affinity chromatography absorbent consists of contacting an affinity chromatography matrix with gelatin or collagen and cross-linking the gelatin or collagen within the matrix with glutaraldehyde. The matrix is in the form of pre-formed, preferably cross-linked, beads.

Once formed the absorbents may be used in the affinity chromatographic separation of certain naturally occurring proteins in particular fibronectin.

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SPECIFICATION

Affinity chromatography adsorbents containing gelatin or collagen

The present invention relates to a process for the preparation of affinity chromatography adsorbents containing gelatin or collagen, to the adsorbents produced thereby and to the use of such adsorbents in the isolation of certain naturally occurring proteins.

The ability of gelatin and collagen to bind certain naturally-occurring proteins (e.g. fibronectin) is known. This ability has been used to good advantage in various purifications of these proteins, especially fibronectin, by affinity chromatography. Generally, the affinity chromatography adsorbents employed in these purifications have been cyanogen bromide activated matrices carrying immobilised gelatin or collagen. The use of cyanogen bromide activated matrices has a number of disadvantages. First the matrix activation step is a time consuming and hazardous operation which involves the use of toxic reagents. (Alternatively the cost of pre-activated propriatary matrices is far higher than their non-activated counterparts.) Second the leakage of gelatin/collagen from CNBr-activated matrices is considerable, which means that the adsorbent has a relatively short chromatographic life. These disadvantages render CNBr-activated matrices either too dangerous or too expensive to be used on an industrial scale.

It is one object of the present invention to provide a process for the preparation of affinity chromatography adsorbents containing gelatin or collagen that does not require the use of cyanogen bromide.

It is a further object of the present invention to provide an affinity chromatography adsorbent containing gelatin or collagen which has a long chromatographic life.

Further objects and advantages of the present invention will become apparent from the following detailed description thereof.

According to the present invention there is provided a process for the preparation of an affinity chromatography adsorbent comprising contacting an affinity chromatography matrix with gelatin or collagen and cross-linking the gelatin or collagen within the matrix with glutaraldehyde.

The affinity chromatography matrix will be in the form of pre-formed beads. The beads may be prepared from either cross-linked or non cross-linked polymeric materials, such as polysaccharides (cellulose, agarose, dextrose, dextran), polyamides (polyacrylamide) or copolymers of these materials (agarose—polyacrylamide). However, cross-linked beads, which are autoclavable, are preferred.

It is a major advantage of the present process that the gelatin or collagen is bound directly to existing affinity chromatography beads rather than, in the first instance, to an affinity chromatography gel which is then converted into beads. The present process, therefore, does not require a bead forming step, can be applied to both high and low melting matrices, can be performed at ambient temperatures and can also be performed in one step (if the gelatin/collagen and glutaraldehyde are passed through the beads simultaneously). Moreover, by using the present process, gelatin may be bound to cross-linked matrices. Such a step would be difficult, if not impossible, if gels were employed instead of pre-formed beads.

It is a further advantage of the present process that no activation of the matrix (with e.g. CNBr) is required prior to contact with the gelatin/collagen. This renders the present process both cheaper and less complex than its predecessors, and thereby more adapted for use on a large scale.

The reaction conditions under which the present process is performed will be determined primarily by the choice of matrix and the protein to be isolated on the adsorbent, once prepared. Thus when the matrix is cross-linked agarose (Sepharose CL—Trade Mark) it is preferred if a gelatin concentration of between 1%(10 mg ml^{-1}) and 2%(20 mg ml^{-1}) is used, whilst the preferred level of glutaraldehyde is between 0.25 and 1.75%, especially between 0.5 and 1.5% if the protein to be isolated is plasma fibronectin. Further, although significant cross-linking of the gelatin/collagen within the matrix may be effected at ambient temperatures (10°—30°C) and/or in short reaction times (about 2 hr.), it is preferred to conduct the reaction at a temperature within the range 30° to 70°, especially about 60°C, and for a period of between 12 and 48 hr. The optimum reaction conditions for other matrices and proteins may readily be discerned by those skilled in this art who follow the experimental techniques described in the Examples below.

As I have mentioned above the present process allows the gelatin/collagen to be immobilised on the matrix in a single step. In one example of this embodiment of the invention a slurry of the matrix would be incubated with a solution of both the gelatin/collagen and glutaraldehyde. This could be done by, for example, stirring the reagents together or by passing the solution of gelatin/collagen and glutaraldehyde through a pre-packed bed of the matrix. Alternatively the immobilisation of gelatin/collagen onto the matrix may be effected in two stages. In this case the matrix would first be incubated with a solution of gelatin/collagen until a required amount of the ligand had penetrated the beads and would then be treated with glutaraldehyde to cross-link the ligand within the matrix. It is a particularly advantageous feature of the present invention that it offers the skilled addressee a process which combines simplicity with flexibility.

Once formed the present affinity chromatography adsorbents may be used with advantage in the affinity chromatographic separation of certain naturally occurring proteins.

According to this further aspect of the present invention there is provided a process for the affinity chromatographic separation of a protein from a mixture comprising contacting the mixture with an affinity chromatography adsorbent according to the present invention to cause the protein to bind to the adsorbent, washing the adsorbent free of the remainder of the mixture and eluting the protein from the adsorbent.

The protein may be any naturally-occurring protein which binds reversibly to one or both of gelatin and collagen. Particularly good levels of separation are achieved by the present process, however, when the protein is fibronectin, a glycoprotein which binds reversibly to gelatin. It follows that the present process may be employed to particularly good effect in the purification of fibronectin. Alternatively the process may form an important part of the purification of Factor VIII since fibronectin comprises 10% or more of the protein in intermediate purity Factor VIII for clinical use, but has poor solubility after freeze-drying.

When used in the above process of separation the present adsorbents have proved to be superior on a number of counts to their CNBr-activated predecessors. First, their chromatographic life is longer. The leakage of material from CNBr-activated adsorbents is considerable, Vuento and Vaheri (*Biochem. J.*, 1979, *183* 331) having reported that in the purification of fibronectin, CNBr-coupled gelatin agarose has a useful life of only ten chromatographic runs. By contrast the present inventor has found that under similar experimental conditions the present gelatin-glutaraldehyde-agarose adsorbent has a slightly diminished but still perfectly acceptable capacity for fibronectin after ten chromatographic runs. This greater stability is a function of the manner in which the ligands are bound to the matrix in the present adsorbent. Unlike CNBr-activated adsorbents in which the ligand is bound to the matrix *via* relatively weak amido carbonate or carbamate linkages, the present adsorbents have the ligand trapped within the matrix by means of relatively strong glutaraldehyde cross-linkages.

Second, the slight fall in capacity that occurs when the present adsorbents are used for a number of chromatographic separations may be recovered simply by recoupling fresh ligand to the matrix by one of the methods described above. Such a replenishment of the adsorbent, which may lead in some circumstances to an essentially indefinite chromatographic life, is not possible when CNBr-activated matrices are used.

Third, the materials employed in the preparation of the present adsorbents are neither hazardous nor toxic. This is in sharp contrast to CNBr which is both hazardous and extremely toxic. It follows that the present adsorbents are much better adapted than their CNBr-activated counterparts, for use in industrial scale purifications.

Processes according to this invention, adsorbents produced thereby and methods of protein separation employing said adsorbents will now be described by way of example only.

Preparation of gelatin containing adsorbent

Example 1

Sepharose 6BCL (Trade Mark, Pharmacia) was washed free of preservative with distilled water and sucked dry to a moist cake on a sintered funnel. 10 gm (moist weight) of Sepharose was made up to 20 ml of slurry with phosphate citrate saline (PCS) buffer (10 mM phosphate, 10 mM sodium citrate, 0.15 M sodium chloride, pH 7.5).

A gelatin solution was prepared, at a concentration of 20 mg ml^{-1} (2%), by mixing 0.4 gm, of porcine skin gelatin (Sigma Chemical) in 20 ml. of PCS buffer, and then heating the mixture until a clear solution was formed.

The gelatin solution and the Sepharose slurry were then brought to 60°C, mixed and incubated, with shaking, at 60°C for 30 min. After this time, glutaraldehyde solution (1.2 ml, 25%, Sigma Chemical) was added, with mixing, to the slurry at 60°C, and cross-linking was allowed to continue for 24 hr., again at 60°C.

Excess reagent was removed from the adsorbent on a sintered funnel under suction and the adsorbent was then washed (×4) with PCS buffer at 60°C. Removal of excess gelatin from the matrix was monitored by the A230 of the washings. N.B. As the cross-linking reaction between gelatin and glutaraldehyde proceeds the solution changes from colourless to yellow. This is accompanied by an increase in the UV adsorbance of the reaction mixture at 230 nm. It is possible, therefore, to follow the progress of the cross-linking reaction has proceeded by measuring the A230 of the washings.

Results are given in Table 1, in which column A shows the adsorbance of the initial filtrate removed after 24 hr and column B shows the absorbance of the initial filtrate plus four buffer washings.

Examples 2—5

The procedure of Example 1 was repeated except that the amount of glutaraldehyde solution added was 0.16, 0.4, 1.6 or 3.2 ml. Results are given in Table 1.

Example 6 (Comparative)

The procedure of Example 1 was repeated except that no glutaraldehyde was added. Results are given in Table 1.

Table 1

Example	Amount of glutaraldehyde added (ml).	% glutaraldehyde	Column A: $^{A}230 \times Vol \times 10^{-3}$ Initial filtrate	Column B: $^{A}230 \times Vol \times 10^{-3}$ Total wash
6(comparative)	0	0	0.245	0.272
2	0.16	0.1	0.375	0.406
3	0.4	0.25	0.465	0.504
1	1.2	0.75	0.730	—
4	1.6	1.0	0.892	0.945
5	3.2	2.0	1.011	1.113

Example 7

Sepharose 6BCL was washed free of preservative with distilled water and sucked dry to a moist cake on a sintered funnel. 10 gm. (moist weight) of Sepharose was made up to 20 ml. of slurry with phosphate citrate saline (PCS) buffer (Example 1).

A gelatin solution was prepared, at a concentration of 20 mg ml^{-1} (2%), by mixing 0.4 gm, of poroine skin gelatin in 20 ml. of PCS buffer, and then heating the mixture until a clear solution was formed.

The gelatin solution and the Sepharose slurry were then brought to 35°C, mixed and incubated, with shaking, at 35°C for 30 mins. After this time 1.2 ml of glutaraldehyde solution (25%) was added, with mixing, to the slurry to give a final glutaraldehyde concentration of 0.75%. Cross linking was then allowed to continue for 24 hr. at 22°C.

Excess reagent was removed from the adsorbent on a sintered funnel under suction and the adsorbent was then washed (×4) with distilled water at 22°C.

Example 8

Sepharose 6BCL was washed free of preservative with distilled water and sucked dry to a moist cake on a sintered funnel.

A gelatin solution was prepared, at a concentration of 20 mg ml^{-1} (2%), by mixing 0.12 gm of porcine skin gelatin in 6 ml of phosphate citrate saline (PCS) buffer (Example 1), and then heating the mixture until a clear solution was formed.

4 gm (moist weight) of the Sepharose was then added to the gelatin solution and the slurry was incubated, with shaking, for 30 min. at 60°C. After this time 0.3 ml. of glutaraldehyde solution (25%) was added, with mixing, to the slurry to give a final glutaraldehyde concentration of 0.75%. Cross linking was then allowed to continue for 16 hr. at 60°C.

Finally the reaction mixture was allowed to cool, the adsorbent was collected by filtration on a sintered funnel and washed (×4) with PCS buffer.

Example 9

The procedure of Example 8 was repeated and then the adsorbent was further treated with gelatin and glutaraldehyde (using the procedure of Example 8) for a cross-linking time of 24 hr, (total time. 40 hr.). As before the reaction mixture was then allowed to cool, the adsorbent was collected by filtration on a sintered funnel and washed (×4) with phosphate citrate saline buffer.

Example 10

The procedure of Example 9 was repeated and then the adsorbent was further treated with gelatin and glutaraldehyde (using the procedure of Example 8) for a cross linking time of 24 hr., (total time 64 hr.). After this further coupling the reaction mixture was allowed to cool, the adsorbent was collected by filtration on a sintered funnel and washed (×4) with phosphate citrate saline buffer.

Example 11

The procedure of Example 8 was repeated except that the cross linking was conducted at 45°C.

Example 12

The procedure of Example 8 was repeated except that the cross-linking was conducted at 75°C.

Example 13

The procedure of Exmaple 8 was repeated except that Sephacryl 8300 (Trade Mark, Pharmacia) replaced Sepharose 6BCL as the matrix.

Example 14

The procedure of Example 8 was repeated except that Sepharose 4BCL (Trade Mark, Pharmacia) replaced Sepharose 6BCL as the matrix.

Example 15

The procedure of Example 8 was repeated except that Sepharose 2BCL (Trade Mark, Pharmacia) replaced Separose 6BCL as the matrix.

Example 16

Sepharose 6BCL was washed free of preservative with distilled water and sucked dry to a moist cake on a sintered funnel. A chromatographic column was packed with the matrix (60 ml.) and phosphate citrate saline (PCS) buffer was pumped through the matrix bed using a Pharmacia P3 (Trade Mark), pump. The matrix was kept at 60°C throughout by means of a water jacket surrounding the column.

A gelatin solution was prepared, at a concentration of 10 mg ml^{-1}, by mixing 0.4 gm of porcine skin gelatin in 40 ml of PCS buffer, and then heating the mixture until a clear solution was formed. At the same time a 0.2% glutaraldehyde solution was prepared by mixing 0.32 ml of glutaraldehyde (25%) in 40 ml. of PCS buffer. Both the gelatin and the glutaraldehyde solutions were then heated to 60°C and immediately pumped onto the column. In this way a gelatin/glutaraldehyde mixture was passed over the matrix at a flow rate of about 20 ml hr^{-1}. After 4 hr., the adsorbent material that had been formed on the column was washed with PCS buffer (4×40 ml.) and distilled water (2×40 ml.).

Preparation of collagen containing adsorbent

Example 17

Sepharose 6BCL was washed free of preservative with distilled water and sucked dry to a moist cake on a sintered funnel.

Pepsin soluble collagen was then prepared from Bovine Achilles Tendon, insoluble collagen, Type I (Sigma Chemical), by digesting insoluble collagen with pepsin (Enzyme:Substrate—1:10) for 24 hr. at 22°C in 0.2 M acetic acid. Removal of the insoluble residue by centrifugation gave a solution of pepsin soluble collagen, the concentration of which was estimated, by its viscosity, to be 2—2.5 mg ml^{-1}.

After raising the pH of the collagen solution to 7.2 (by NaOH addition), 10 ml. of the solution was mixed with 6 gm (moist weight) of the Sepharose 6BCL. Glutaraldehyde solution (0.3 ml., 25%) was then added, with mixing, to the slurry to give a final glutaraldehyde concentration of 0.75%. Cross linking was then allowed to continue for 64 hr. at 22°C.

After this time the reaction mixture was centrifuged at low speed to settle the adsorbent. Gelled collagen, which was suspended in the solution, was then removed by decanting the solution from the adsorbent. Finally the adsorbent was washed consecutively with 0.2 M acetic acid, phosphate citrate saline buffer and distilled water.

Flow characteristics of the adsorbents

i. A chromatographic column (1.6 cm diameter×2.5 cm length) was packed with an adsorbent (5 ml.) prepared according to Example 8, and phosphate citrate saline buffer (Example 1) was pumped through the adsorbent bed using a Pharmacia P3 (Trade Mark) pump, set at 3. Flow rates were measured over 10 ml. or more of effluent once they had been allowed to stabilise. Results are given in Table 2.

ii. The procedure of Example (i) was repeated except that the adsorbent prepared according to Example 8 was replaced by adsorbents prepared according to Examples 13, 14 and 15. Results are given in Table 2.

Table 2

Matrix	*Flow rate (ml cm^{-2} hr^{-1})*
Sepharose 6BCL	30
Sephacryl S300	25
Sepharose 4BCL	26
Sepharose 2BCL	15

Fibronectin purification on a gelatin containing adsorbent

Example 18

Plasma cryoprecipitate was resuspended in 0.02 M Tris (pH 6.6) buffer. The pH of this suspension was then increased to 7.3 at 7°C and the precipitate that formed was collected by centrifugation. This precipitate was then resuspended in citrate saline buffer (0.02 M sodium citrate, 0.15 M sodium chloride, pH 7.0) at 37°C and stored at —20°C, in the form of a cold insoluble extract (CIE), until required.

A chromatographic column (1.6x2.5) was packed with an adsorbent (5 ml.) prepared according to Example 1 and equilibrated with phosphate citrate saline (PCS) buffer (10 mM phosphate, 10 mM sodium citrate, 0.15 M sodium chloride, pH 7.5).

The CIE was warmed to 37°C, filtered and pumped onto the column (linear flow rate of 100 to 150 cm hr^{-1}) until the adsorbent was saturated with fibronectin. The column was washed with PCS buffer to remove unbound protein and then the bound fibronectin was eluted by the consecutive addition of 1 M arginine and 3 M urea, each dissolved in PCS buffer (pH 7.5). The capacity of the adsorbent for fibronectin was estimated by measuring the A280 of the eluted protein.

Material in the arginine eluted fraction was finally dialysed against the PCS buffer to remove eluant and concentrated on a stirred cell (Amicon) using a PM10 (Trade Mark) ultrafiltration membrane.

The %(by wt) of fibronectin in the CIE and in the eluted arginine fraction was estimated by electrophoresis on agarose gel. The gels were fixed in 20% sulphosalicylic acid (5 min.), stained for 20 min. in 0.25% aqueous coomassie brilliant blue, destained in water and scanned at 580 n.m.

In the present case the composition of the CIE was 13% fibronectin, 65% fibrinogen, 13% globulins and 5% albumin, whilst the eluted arginine fraction contained more than 99% fibronectin. The capacity of the adsorbent for fibronectin is given in Table 3.

Examples 19—22 and Example 23 (Comparative)

The procedure of Example 18 was repeated except that the adsorbent prepared according to Example 1 was replaced by adsorbents prepared according to Examples 2—5 and Example 6. The capacities of the adsorbents for fibronectin are given in Table 3.

Table 3

In the Table the "usable" fibronectin capacity is that eluted by 1 M arginine, whilst the "total" capacity is that eluted by the combined 1 M arginine and 3 M urea elutions.

Example	*Adsorbent preparation*	*Glutaraldehyde concentration (%)*	*Fibronectin capacity (mg per ml of adsorbent)*	
			Usable	*Total*
23	6	0	0.192	0.250
19	2	0.1	0.335	0.335
20	3	0.25	0.540	0.588
18	1	0.75	0.565	0.615
21	4	1.0	0.574	0.628
22	5	2.0	0.247	0.347

Example 24

A bed of adsorbent prepared according to Example 1, 12.5 ml. settled volume, was put through 10 cycles of fibronectin purification using the procedure of Example 18. In runs 4 to 10 the column was saturated with respect to fibronectin. In each case, the protein was eluted, first with 1 M arginine and second with 3 M urea. The amount of fibronectin in each fraction was measured by means of its A_{280}. This measure assumes that purified (i.e. 100% pure) fibronectin was eluted from the column. This appears to be a valid assumption since, in every case, after dialysis and concentration of the eluant, only one band was observed by agarose gel electrophoresis.

The usable and total fibronectin capacities for runs 4—10 are given in Table 4.

Table 4

Run no.	*Fibronectin capacity (mg per ml of adsorbent)*	
	Usable	*Total*
4	1.43	1.56
5	1.20	1.44
6	1.05	1.17
7	0.83	1.04
8	1.02	1.27
9	0.98	1.20
10	0.93	1.08

Examples 25—27

The procedure of Example 18 was repeated except that the adsorbent prepared according to Example 1 was replaced by adsorbents prepared according to Examples 8, 9 and 10. The usable and total fibronectin capacities are given in Table 5.

Table 5

Example	Adsorbent preparation	Total cross-linking time(hr)	Fibronectin capacity (mg per gm of adsorbent) Usable	Total
25	8	16	1.20	2.17
26	9	40	2.44	3.64
27	10	64	2.04	3.00

Examples 28 and 29

The procedure of Example 25 was repeated except that the adsorbent prepared according to Example 8 was replaced by adsorbents prepared according to Examples 11 and 12. The usable and total fibronectin capacities are given in Table 6.

Table 6

Example	Adsorbent preparation	Cross-linking temperature (°C)	Fibronectin capacity (mg per mg of adsorbent) Usable	Total
28	11	45	1.00	1.91
25	8	60	1.20	2.17
29	12	75	1.18	1.98

Examples 30—32

The procedure of Example 25 was repeated except that the adsorbent prepared according to Example 8 was replaced by adsorbents prepared according to Examples 13, 14 and 15. The usable and total fibronectin capacities are given in Table 7.

Table 7

Example	Adsorbent preparation matrix	Molecular weight exclusion limit for globular proteins	Fibronectin capacity (mg per gm of adsorbent) Usable	Total
30	13 Sephacryl S300	1.5×10^6	1.17	2.00
25	8 Sepharose 6BCL	4×10^6	1.30	2.50
31	14 Sepharose 4BCL	20×10^6	1.60	2.51
32	15 Sepharose 2BCL	40×10^6	2.31	3.44

There seems to be a direct correlation between the usable, arginine eluted, fibronectin capacity of an adsorbent and the molecular exclusion cut off (i.e. pore size) of its matrix. This suggests that, below a nominal exclusion limit of about 20×10^6 (Sepharose 4BCL) the matrix tends to interfere with the gelatin fibronectin interaction. As a consequence of this, the adsorbent prepared from Sepharose 2BCL has a disproportionately high capacity for fibronectin. The disadvantage of using Sepharose 2BCL, however, is the flow rate observed when the adsorbent is packed in the column. As we have shown above, (Table 2) this is about half that of adsorbents prepared from Sepharose 6BCL.

Claims (Filed on 4 Jan 1984)

1. A process for the preparation of an affinity chromatography absorbent comprising contacting an affinity chromatography matrix which is in the form of pre-formed beads with gelatin or collagen and cross-linking the gelatin or collagen within the matrix with gluteraldehyde.

2. A process according to claim 1 wherein the matrix is in the form of a pre-formed, cross-linked beads.

3. A process according to either claim 1 or claim 2 wherein the matrix comprises a polymeric material selected from a polysaccharide, a polyamide and a copolymer of polysaccharide and polyamide.

4. A process according to claim 3 wherein the matrix comprises a polymeric material selected from agarose, polyacrylamide and a copolymer of agarose and polyacrylamide.

5. A process according to any one of claims 1 to 4 wherein the matrix is contacted with a solution containing glutaraldehyde and either gelatin or collagen.

6. A process according to any one of claims 1 to 5 comprising contacting cross-linked agarose beads with a solution containing gelatin at a concentration of between 1% and 2% (w/v), either simultaneously or subsequently contacting the beads with a solution containing glutaraldehyde at a concentration of between 0.5 and 1.5% (v/v) and cross-linking the gelatin within the beads by heating to about 60°C for between 12 and 48 hr.

7. A process for the preparation of an affinity chromatography absorbent according to claim 1 substantially as hereinbefore described with particular reference to any one of Examples 1 to 5 and 7 to 17.

8. A process for the affinity chromatographic separation of a protein from a mixture comprising contacting the mixture with an affinity chromatography absorbent prepared in accordance with the process of claim 1 to cause the protein to bind to the absorbent, washing the absorbent free of the remainder of the mixture and eluting the protein from the absorbent.

9. A process according to claim 8 wherein the protein is fibronectin.

10. A process for the affinity chromatographic separation of a protein according to claim 8 substantially as hereinbefore described with particular reference to anyone of Examples 18 to 22 and 24 to 32.

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