AN INVESTIGATION OF THE SYNTHESES OF CELLULOSE AND AGAROSE DERIVATIVES CONTAINING SULPHATE, N-(6-AMINOHEXYL)-2-NAPHTHALENESULPHONAMIDE AND CARBOXYL GROUPS FOR THE PURIFICATION OF PROTEINS

A thesis

presented in partial fulfilment of the requirements

for the degree of

Master of Science

in

Chemistry

at

Massey University

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1988

ABSTRACT

The syntheses of three different cellulose and agarose derivatives were investigated, namely, cellulose sulphate, N-(6-aminohexyl)-2-naphthalenesulphonamide (2-ANS) cellulose and cellulose and agarose with multiple carboxyl groups.

In the case of cellulose sulphate, an attempt was made to find a sulphating reagent and conditions for a commercially convenient method of preparing a cellulose derivative with a sulphate substitution level of 3.5 meq/g. This synthesis was found to require the control of at least one of the following factors: (a) water present in the system, (b) the quantity of sulphating reagent and (c) temperature. The stability of the sulphated cellulose in 0.08M sodium hydroxide at 83°C over 28 days was also evaluated. It was found that the sulphate substitution level decreased linearly over 4 weeks at the rate of 1% per day.

Two routes of preparing 2-ANS-cellulose derivative were studied, namely, (1) the coupling of 2-ANS to epoxide activated cellulose and (2) the coupling of 2-naphthalene sulphonyl chloride (2-NSCl) to diaminohexyl (DAH)-cellulose. Both methods of synthesis were found to be equally feasible. However, the former method required the prior multi-step preparation of 2-ANS, while the latter method was carried out stepwise on the cellulose matrix. The excess reagents were readily washed away before the next step was undertaken. Also, the preparation of 2-NSCl from sodium 2-naphthalene sulphonate was quantitative. The capacity of these 2-ANS-cellulose derivatives for bovine serum albumin (BSA) was also investigated. The products prepared by method 1 showed a much lower capacity (0.05 - 0.38 gBSA/g) for BSA than those prepared by method 2 (0.49 - 0.78 gBSA/g).

The syntheses of cellulose and agarose derivatives containing alpha (A)- and beta (B)-citrylhexamethylenediamine (CM_6D), aspartic acid (Asp) and 6-aminohexylaspartate (Asp-AH) groups were investigated using both epoxide and 1,1'-carbonyldiimidazole (CDI) activation procedures. The use of these products for the purification of bovine lactoferrin (Lf) was assessed. The nature of the binding action of Lf to the CM_6D-matrices was also studied. It was found that (a) high CM_6D substitution level on the matrix, (b) high porosity of the matrix and (c) the removal of additional cationic properties from the matrix by replacing the basic nitrogen linkage resulting from the epoxide activation by a non-basic urethane linkage resulting from CDI activation, led to an increase in the strength of Lf binding to the derivative. The results also suggested that the Lf binding was predominantly ionic in nature. Finally, it was found that Lf purification on A-CM_6D-agarose gave a product of higher purity than that on Asp-agarose and Asp-AH-agarose.

ACKNOWLEDGEMENTS

I wish to thank my supervisors, Dr. J.S. Ayers and Mr. D.F. Elgar for their help and guidance throughout the course of this project. I am indebted to them for their guidance in industrial research methodology and for introducing me to the challenges of synthetic chemistry of chromatographic materials.

I am grateful to Dr. S. Ram for his constructive suggestions and comments on various parts of this thesis. I am particularly grateful to him for his helpful discussions on the biochemical aspects of the work.

Thanks are also extended to all members of the Department of Chemistry and Biochemistry for their assistance in making available various equipment and materials needed and for their generosity of time and advice.

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A-CM$_4$D	alpha-citrylhexamethylenediamine
AH	6-aminohexanoic acid
2-ANS	N-(6-aminohexyl)-2-naphthalenesulphonamide
AR	analytical reagent grade
B-CM$_4$D	beta-citrylhexamethylenediamine
BNS	bis-N,N'-(2-naphthalene sulphonyl)-hexamethylene-diamine
BSA	bovine serum albumin
CDI	1,1'-carbonyldiimidazole
CM$_4$D	citrylhexamethylenediamine
CM-Sephadex	Carboxymethyl-Sephadex
DAH	1,6-diaminohexane
DAH-cellulose	diaminohexyl-cellulose
DEAE-cellulose	Diethylaminoethyl-cellulose
DMF	dimethylformamide
EA-aga	epoxide activated agarose
Fe-NTA	iron (III) nitrilotriacetate
HP-cellulose	hydroxypropylated cellulose
Lf	lactoferrin
Lp	lactoperoxidase
LR	laboratory reagent grade
2-NS	2-naphthalene sulphonyl group
2-NSCl	2-naphthalene sulphonyl chloride
py-SO_3	pyridine sulphur trioxide complex

SECTION 1

GENERAL INTRODUCTION

1.1 Fractionation and purification of proteins and enzymes

The fractionation and purification of proteins and enzymes are necessary in biochemical research. They require the application of high resolution and mild separative techniques. High resolution techniques are required as these molecules are usually found in highly complex mixtures. Proteins and enzymes are sensitive to changes in their environment and the techniques used, therefore, need to be mild so as not to disturb the functional properties of these molecules [1].

The separation of these biomacromolecules is most widely achieved by chromatography [2]. Chromatographic separation is based on the different rate of movement of molecules in two different phases, namely, stationary and mobile phases. The commonly used techniques include ion-exchange, gel filtration, affinity and hydrophobic interaction chromatography.

1.2 Basic principles of ion-exchange, gel filtration, affinity and hydrophobic interaction chromatography

In ion-exchange chromatography [1,3,4], the separation is achieved on the basis of the differing polarity of molecules. The ion exchanger is an insoluble matrix containing covalently bound charged groups. The mobile counter ions associated with the matrix can be reversibly exchanged with other ions of the same charge. Basically, there are two classes of ion exchangers, namely, cationic and anionic ion exchangers. Some of the common functional groups used in ion exchangers for the separation of biomacromolecules are given in Table

1.1. Strong ion exchangers are those where the functional group is completely ionized over a wide pH range while the degree of ionization of the weak ones is more dependent on pH.

Table 1.1 : Some functional groups used in ion exchangers

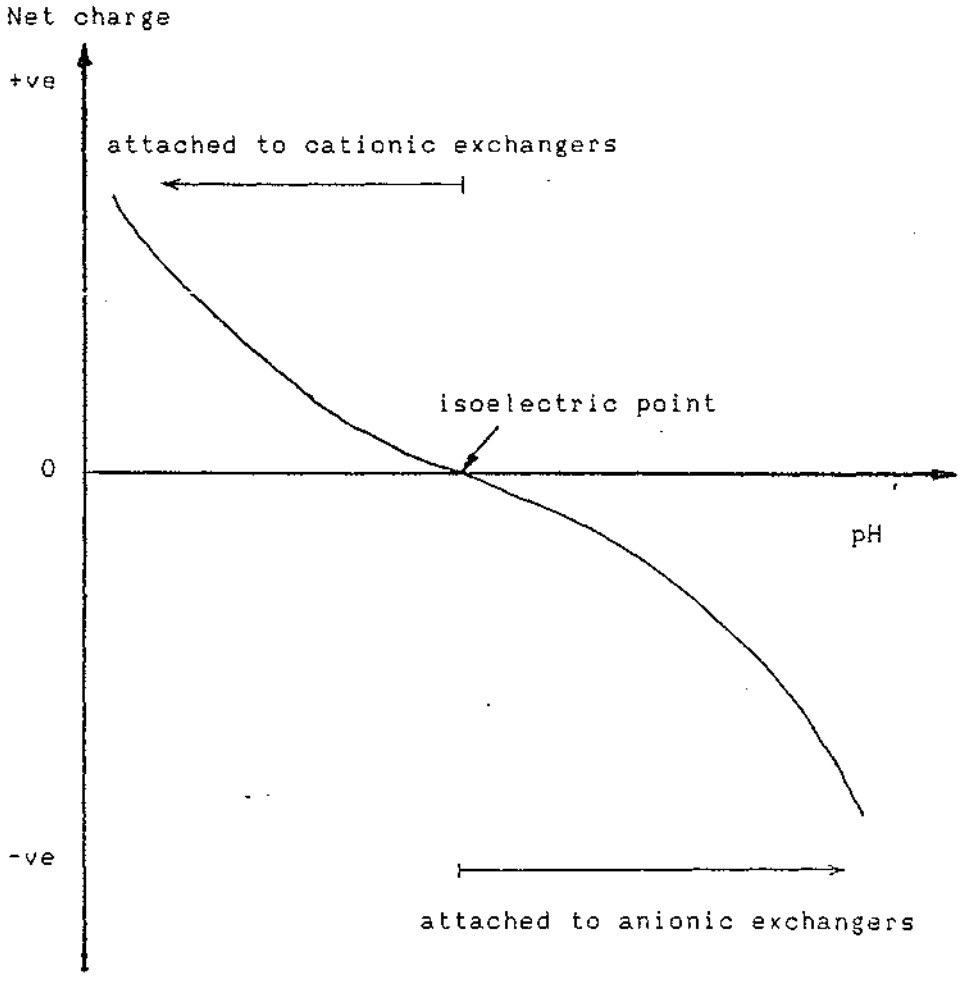
Class	Functional group	Type	Structure
cationic	sulphopropyl (SP)	strong	$-(CH_2)_3SO_3^-$
	phospho (P)	intermediate	$-OPO_3H^-$
	carboxymethyl (CM)	weak	$-CH_2COO^-$
anionic	diethylaminoethyl (DEAE)	weak	$-(CH_2)_2N^+HEt_2$
	quaternary aminoethyl (QAE)	strong	$-(CH_2)_2N^+Et_2C_3H_6OH$

Proteins and enzymes are complex molecules with differing degrees of charge depending on the number of ionic groups present in the polypeptide chain. They are amphoteric in character, that is, they can be either negatively or positively charged. The actual effective charge on them is dependent on the pH and at a particular pH, known as the isoelectric point, the net charge is zero. The class of ion-exchangers selected for the adsorption of these biomacromolecules is dependent on the pH (see Figure 1.1). The adsorption and desorption is then brought about by altering conditions such as ionic strength and pH.

In gel filtration (or size exclusion) chromatography [1,5,6], the separation of the biomacromolecules is brought about by their differing ability to distribute between the mobile phase and the liquid situated in the pores of the matrix. The volume between the

matrix particles is available to all molecules but access to the pore volume of the selected matrix is limited to molecules of smaller sizes. The very large molecules are, therefore, eluted most rapidly while the small molecules can easily enter unhindered into the available pores. They are, thus, subjected to the greatest delay. Hence, molecules are eluted in order of decreasing molecular size.

Figure 1.1 : The net charge of a protein as a function of pH [3]



The term affinity chromatography [7-9] is used to describe separations that are based on biospecific interaction between the biomacromolecules and an immobilized ligand. This ligand may be an

enzyme substrate, enzyme inhibitor, antigen or lectin. Ideally, it is covalently attached to an insoluble inert matrix via a spacer arm of usually six carbon chain length. It will then only interact with the desired biomacromolecule for which it has an affinity. When the adsorption is complete, the impurities are washed off the matrix with the starting buffer. The affinity bound material is recovered by elution with a new buffer of different pH or ionic strength. Alternatively, the elution may be carried out with a buffer containing the soluble ligand or another ligand for which the biomacromolecule has a higher affinity. The latter mode of specific elution is preferred but it is not always possible to apply in practice.

The separation of biomacromolecules by hydrophobic interaction chromatography [9-11] relies on their tendency to associate in aqueous solution. Generally, proteins and enzymes are folded in such a manner as to bury most of the hydrophobic sidechains in the interior of the molecule while most of the polar sidechains are exposed on the surface. Some hydrophobic groups remain exposed at the surface forming hydrophobic patches or pockets. These patches can interact with hydrophobic ligands immobilized onto a suitable matrix to form "hydrophobic bonds". Adsorption of the biomacromolecules by hydrophobic interaction is enhanced with increasing ionic strength and a pH which is close to the isoelectric point. Desorption of the desired protein or enzyme can be achieved by varying ionic strength, pH and the polarity of the eluent. A decrease in the polarity of the eluent, for example, by the addition of ethylene glycol weakens the hydrophobic interaction. Hofstee et al. [12] have suggested the use of an increasing hydrophobicity gradient for binding proteins so that the subsequent elution would be carried out under mild conditions without the danger of denaturation of the bound proteins.

1.3 Characteristics of matrix

The characteristics desirable in a good matrix or support for the chromatographic methods are described below [3,6-8].

1. The matrix should be insoluble under operating conditions. Generally, the isolation and purification of proteins and enzymes is carried out in an aqueous medium. Hence, the matrix should be insoluble in aqueous solution of varying ionic strength and pH.

2. The matrix should be hydrophilic. Hydrophobic interactions should be minimized as they tend to complicate the adsorption of the biomacromolecules and to cause their denaturation. It is best that the matrix is swellable or, at least, highly wettable with water.

3. The matrix should be stable to mechanical, physical, chemical and microbial degradation. This stability should extend over long periods of time and under a range of pH, temperature and ionic strength so as to ensure it can be used repeatedly. Mechanical stability ensures that it is not deformed by the forces exerted by the flow of eluent.

4. The matrix should be inert and neutral. These characteristics are desirable for they eliminate non-specific ionic interaction.

5. The matrix should consist of particles which are rigid, uniform and spherical with good flow properties. The use of irregularly shaped particles results in band broadening because of the unequal paths taken by the biomacromolecules to be separated.

6. The matrix should be porous. This permits unimpaired diffusion of the biomacromolecules and consequently, maximum interaction with the matrix surfaces. In gel filtration chromatography, the pore size of the matrix determines the fractionation range. In affinity chromatography, large pores matrices are particularly important for maximum ligand-biomacromolecule interaction.

7. The matrix should have an ample supply of chemical groups available for covalent coupling with a variety of ligands where a modified matrix is required.

8. The matrix should contain a low density of modifier groups so as to allow the elution of the biomacromolecules under relatively mild conditions and thereby, prevents their denaturation.

It is obvious that no matrix will have all these desired characteristics. There are various types of matrices in use (see Table 1.2) and a discussion on them is found elsewhere [4,6,9]. The matrices used in this project are cellulose and agarose. Their advantages and limitations are briefly discussed below.

Table 1.2 : Some chromatographic matrices in use

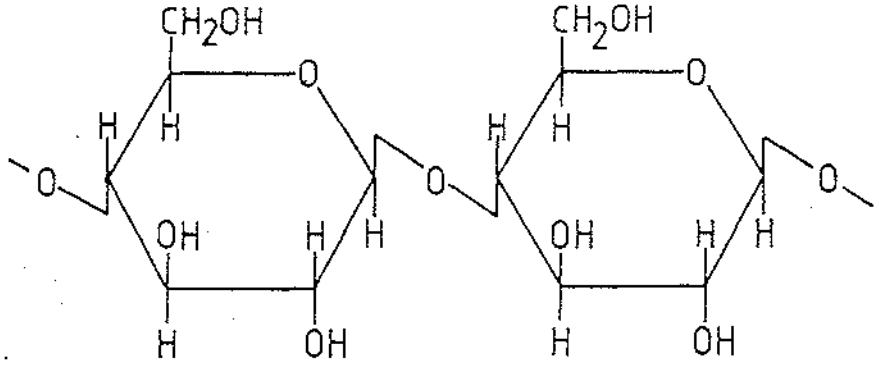
Type	Example
Biopolymers (polysaccharides)	agarose, cellulose, dextran
Synthetic polymers	polyacrylamide, polystyrene
Inorganic materials	silica
Biopolymers/synthetic copolymers	agarose polyacrylamide
Inorganic materials/organic polymers	silica/hydrophilic copolymers

1.4 Cellulose

Cellulose is a polysaccharide containing beta-1,4-linked D-glucose units with occasional 1,6-bonds [13] (Figure 1.2). Native cellulose contains microcrystalline regions interspersed with amorphous regions. The former regions result from extensive hydrogen bonding between the adjacent linear polysaccharide chains. While, in the latter regions, there is less hydrogen bonding. The early commercial native cellulose used was a dense fibrous powder of

irregular shape [3,14,15].

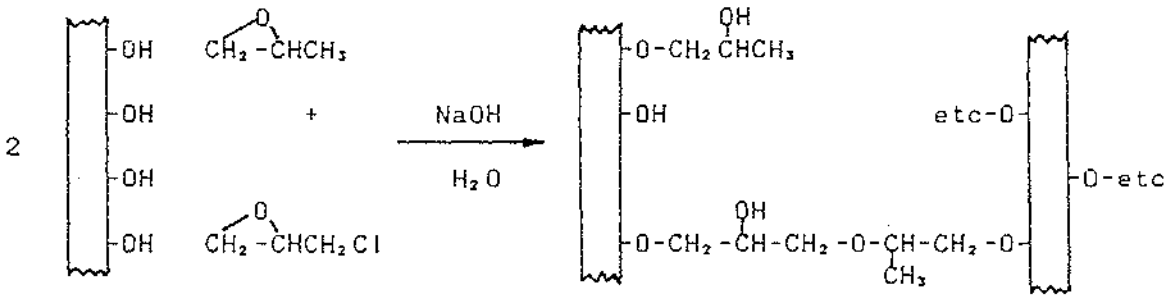
Figure 1.2 : Partial chemical structure of cellulose



Sober and Peterson [16] were the first to use cellulose for the ion-exchange chromatographic separation of proteins. Since then, other forms viz native fibrous [17], microgranular [17], microcrystalline [3] or regenerated [18,19] have become available for various chromatographic applications.

The cellulose matrix used in this project was a cross-linked hydroxypropylated cellulose (HP-cellulose) prepared from regenerated cellulose by reaction with propylene oxide and epichlorohydrin in the presence of sodium hydroxide [20,21] (Figure 1.3). Regenerated cellulose is a particularly robust, resilient and long-lasting form of cellulose capable of withstanding high operating flow rates [19]. The one which was used in this project was made via the xanthate derivative [15,21].

Figure 1.3 : Typical reactions for preparation of cross-linked-HP-cellulose [21]



Cellulose

CL-HP-Cellulose
insoluble in water

The HP-cellulose has previously been used to prepare several derivatives including DEAE-cellulose and CM-cellulose [19,22]. The advantages of using HP-cellulose are [21]:

1. It is hydrophilic and can be easily modified chemically via the OH groups.

2. It is mechanically, chemically and physically stable. It is free of noticeable compression in column applications. It is resistant to dilute aqueous sodium hydroxide and is stable to repeated handling and mechanical stirring.

3. The hydroxypropyl cross-linking of the cellulose gives it gel-like properties, enhanced porosity and stability to microbial degradation compared to the starting regenerated cellulose.

4. It is relatively cheap because cellulose is such an abundant natural material. It has good flow properties.

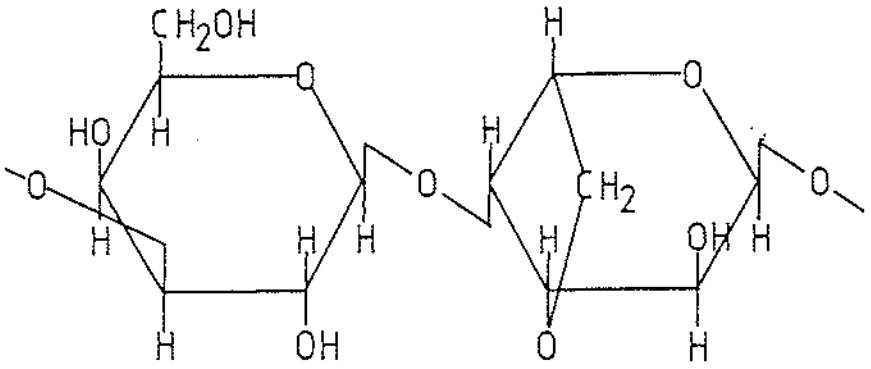
Hence, it is a good choice for industrial scale application.

However, the porosity of the HP-cellulose is much lower than that of agarose [21] which has limited its applications mainly to ion-exchange chromatography.

1.5 Agarose

Native agarose is isolated from a complex mixture of charged and neutral polysaccharides referred to as agar. It is a linear water-soluble polysaccharide consisting of alternating residues of 1,3-linked beta-D-galactose and 1,4-linked-3,6-anhydro-L-galactose [9,13] (Figure 1.4).

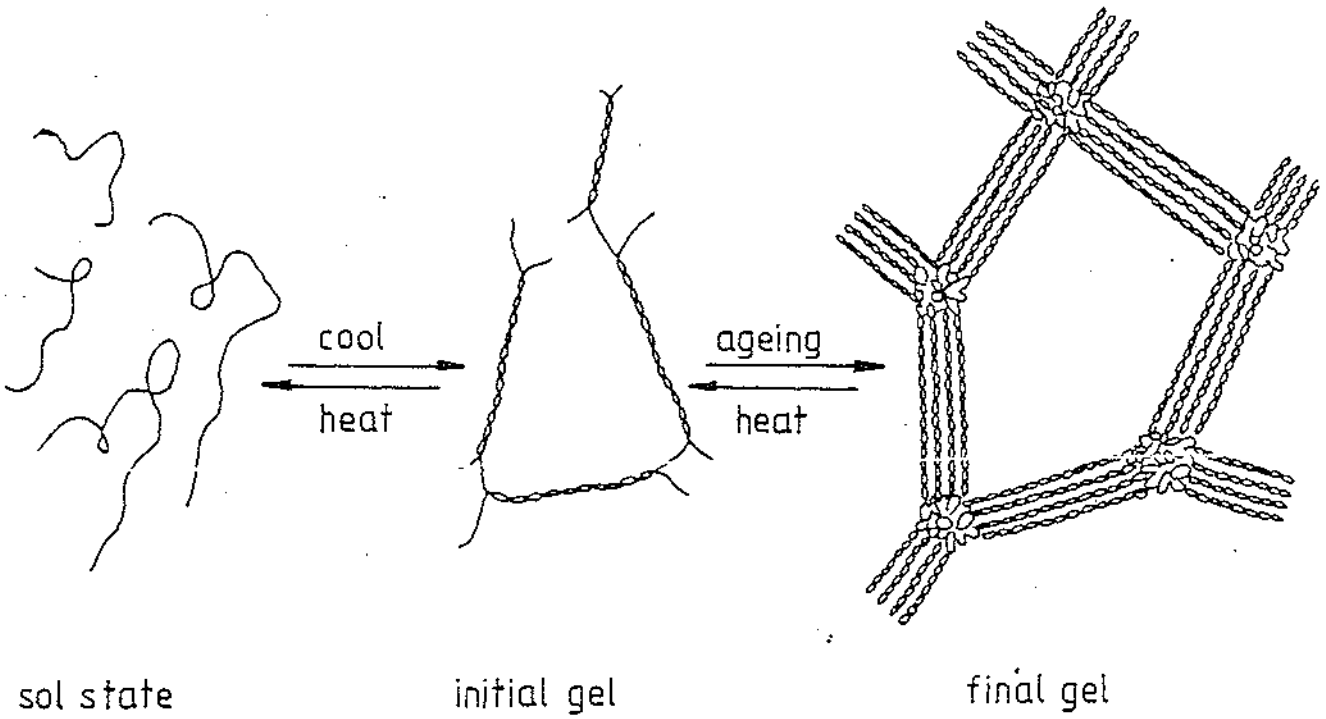
Figure 1.4 : Partial chemical structure of agarose



The agarose gels are available in bead, pellet or spherical forms which are made from the spontaneous gelation of aqueous agarose solutions cooled to below 50°C [9]. The gel structure is thought to be composed of highly ordered fibres or bundles resulting from the aggregation, through hydrogen bonding and hydrophobic interaction, of

the double helix formed by the polysaccharide chains (Figure 1.5). The resulting structure is macroporous and hence, allows the relative free diffusion of biomacromolecules [15,23].

Figure 1.5 : Schematic representation of gelation of agarose



The agarose matrices used in this project were Sepharose 6B and Sepharose 6B-CL. Sepharose 6B contains about 6% agarose [5]. Sepharose 6B-CL is the cross-linked product of Sepharose 6B. It is made from the latter by reaction with 2,3-dibromopropanol in the presence of alkali to produce cross-linking. It is then subjected to alkaline hydrolysis under reducing conditions to remove sulphate groups and give a product with an extremely low content of ionizable groups [3,5].

Like the celluloses, these matrices are hydrophilic and contain readily modifiable OH groups. They are mechanically, chemically and

physically stable (Table 1.3). Because of their macroporous structure, their porosity is much higher than that of cellulose [9]. Their molecular weight exclusion limit is of the order of 4 x 10^6 for proteins [5]. Generally, they do not exhibit non-specific binding of proteins. However, any weak non-specific interactions noted could be due either to the residual sulphate and carboxyl groups via the ester in position 6 or to hydrophobic interactions, probably resulting from the ether bridge of the anhydro-galactose unit [9]. They exhibit good flow properties. Generally, they are resistant to microbial attack due to the unusual sugar 3,6-anhydro-L-galactose. However, an antimicrobial agent is usually added in prolonged experiments and during storage in the swollen state [5].

Table 1.3 : Stabilities of Sepharose 6B and 6B-CL [5,9]

	Sepharose 6B	Sepharose 6B-CL
pH	4 - 9	3 - 14
Temperature range (°C)	below 40	below 70
Solvents	stable in aqueous solutions with high ionic strength, urea, guanidine.HCl, detergents; DMF-H_2O (1:1), Ethylene glycol-H_2O (1:1)	stable in aqueous solutions with high ionic strength, urea, guanidine.HCl, detergents over pH 3-11; organic solvents.
Sterilization	not autoclavable	autoclavable at pH 7, 120°C
Chaotropic ions (eg. KSCN)	low stability	high stability

Sepharose 6B is sensitive to extremes of pH, temperature and solvents and unstable to continuous mechanical disruption. A leakage of the covalently attached ligand may also result on prolonged washing [24]. The cross-linked product is more stable towards extremes of pH, temperature, solvents and mechanical disruption [5]. Generally, they may be hydrolyzed under oxidizing conditions [3,5]. Compared to the celluloses, they are very much more expensive and less robust to mechanical disruption. Hence, they are less suitable for industrial usage.

1.6 Nature of project

This project was an investigation of the synthesis of three different types of matrix derivatives, namely,

1. cellulose derivative containing sulphate groups (section 2),
2. cellulose derivative containing N-(6-aminohexyl)-2-naphthalene-sulphonamide groups (section 3) and
3. cellulose and agarose derivatives containing carboxyl groups (section 4).

It was the intention of the work in sections 2 and 3 to establish the optimal conditions for preparing the derivatives stated. This work was carried out with the ultimate aim of the products being useful for industrial applications. This orientation influenced the direction of the project. Whenever possible, the attempt at synthesis and the choice of starting materials were directed to devising methods of synthesis that were simple and cost effective.

The work reported in Section 4 involved not only the preparation of special carboxylic acid derivatives of various matrices but also an investigation of their use for purifying bovine lactoferrin.