Chapter 4

Hydrophobic (interaction) chromatography of proteins

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Introduction and historical aspects

Hydrophobic interactions have been described as “the unusually strong attraction between non-polar molecules and surfaces in water” (Israelachvili, 1985). For two contacting methane molecules the attraction energy is ca. 6-fold higher in water than the van der Waals interaction energy in vacuum. This energy, which has been estimated to be ca. $-8.5\text{kJ mol}^{-1}$ for two methane molecules (Ben-Naim et al., 1973; Israelachvili, 1985) is due to the extrusion of ordered water on two adjacent hydrophobic surfaces into less-ordered bulk water with a concomitant increase in entropy. This entropy driven attraction between non-polar groups in water (Israelachvili, 1985; Israelachvili and Pashley, 1984; Kauzmann, 1959; Lewin, 1974; Tanford, 1973) is the basis for hydrophobic interaction chromatography as summarized in several reviews (Arakawa and Narhi, 1991; Eriksson, 1989; Halperin et al., 1981; Hjerten, 1981; Hofstee and Otillio, 1978; Hubert and Dellacherie, 1993; Jennissen, 1988; Mohr and Pommerening, 1986; Ochoa, 1978; Oscarsson, 1997; Shaltiel, 1984; Yon, 1977). For an interesting distinction between definitions of “hydrophobicity” and “hydrophobic interactions” see the technical comment by Dill (Dill, 1990). Recently the terms ultra-hydrophilic and ultra-hydrophobic have extended the above range of definitions to new applications in the area of biomaterials (Jennissen, 2001).

Reversed-phase vs hydrophobic Interaction chromatography

The chromatographic separation of proteins depends on the differential accumulation of molecules at certain sites within a chromatographic system. The term reversed-phase chromatography goes back to a paper by Howard and Martin 1950 who first separated long chain fatty acids by what they called “reversed phase partition” chromatography. The principle underlying this separation rested on the synthesis of a hydrophobic silica support (by treating it with dichlorodimethylsilane) which was capable of retaining non-polar liquid phases such as acetone, n-octane or paraffin (stationary liquid phase) when applied as the less polar phase in a solvent system. In this classical method the solutes are thus absorbed and separated (partitioned) in an apolar stationary liquid phase (i.e. a three dimensional system). Today the silica gels employed for reversed-phase HPLC of proteins and peptides are generally highly substituted with octadecyl, octyl or phenyl residues and therefore display a very high hydrophobicity capable of retaining liquid non-polar phases such as acetonitrile or methanol in water. In hydrophobic interaction chromatography the solutes applied in an aqueous phase are adsorbed and
separated directly on the apolar stationary solid phase (i.e. a two-dimensional system) in the majority of cases consisting of immobilized hydrophobic groups on a paracrystalline carbohydrate surface (e.g. agarose, cellulose see also (Jennissen, 1981, 1995)). The application of reversed phase chromatography and hydrophobic interaction chromatography is not restricted to proteins but also encompasses nucleic acids (Hjerten, 1981; Liautard, 1992).

In reverse phase chromatography of proteins there appears to be no clear theoretical boundary between partition and adsorption, since it is conceivable that a molecule the size of a protein may interact with the stationary liquid phase as well as with the stationary solid phase. As a rule the high hydrophobicity of a reverse phase matrix and the stationary organic phase make this procedure primarily suited for denatured proteins and peptides only. Thus due to the very well defined and reproducible methodological approach reversed-phase HPLC of proteins and peptides has become one of the most powerful analytical tools in protein and peptide chemistry. In hydrophobic interaction chromatography on the other hand the hydrophobicity is of a low or intermediate level obviating the organic stationary phase and (ideally) allowing the separation of proteins in their native states directly on the apolar stationary phase. The aim in this case is not so much analytical but moreover preparative and directed towards the purification of proteins in their native state. For practical purposes therefore a simple differentiation of reversed phase chromatography from hydrophobic interaction chromatography can be made on the basis that in the former an organic liquid stationary phase is present and in the latter there is not. For further details on these two chromatographic modes see also (Fausnaugh et al., 1984; Pearson et al., 1982).

The focus of this review is hydrophobic interaction chromatography, thus excluding reversed phase chromatography, although both methods indeed have a number of factors in common. Furthermore it is restricted to the separation of proteins excluding small molecular weight compounds and other classes of biomolecules such as nucleic acids. In addition a distinction between classical chromatography systems and HPLC will also not be made, since the difference between the two is essentially the bead or particle size which is mainly responsible for the higher performance.

The CNBr procedure

Hydrophobic chromatography was discovered on agarose gels containing covalently immobilized hydrophobic groups that were introduced by the CNBr procedure (for review (see Jennissen, 1995)). Therefore the CNBr method dominated all other coupling procedures for many years and is still in use today. For an understanding of the previous work in the field of hydrophobic interaction chromatography it is therefore necessary to briefly review the chemistry of the CNBr activation and coupling reaction on agaroses. The activation of polysaccharides by CNBr was described by Porath’s group in 1967, (Axen et al., 1967; Porath et al., 1967) a method which paved the way for affinity chromatography (Cuatrecasas et al., 1968) and hydrophobic chromatography. The mechanism of the CNBr coupling reaction on agarose, which is more complex than on dextrans, was clarified by Kohn and Wilchek, 1981 many years after the major affinity methods had already been established. The primary stable active intermediate on CNBr-activated agarose is a cyanate ester of which ca. 15% spontaneously converts to a cyclic imidocarbonate (see simplified scheme in Fig. 4.1). The pH of the washing solution employed after activating the agarose is decisive for the amount of charges introduced since cyanate esters are selectively hydrolyzed in alkali in contrast to the imidocarbonates.
which are hydrolyzed in acid. If a CNBr activated gel is stabilized at low pH (Joustra and Axen, 1976) and later swollen and washed with 1mM HCl as is the case with commercial CNBr-activated Sepharose 4B from Pharmacia (Pharmacia Fine Chemicals, 1979) only charged isourea coupling products are obtained e.g. when coupling primary amines or proteins. On the other hand if fresh CNBr-activated gels are washed extensively at an alkaline pH, fully uncharged imidocarbonate/carbamate coupling products may be obtained with primary amines (Kohn and Wilchek, 1981). Thus pure charged isourea gels, pure uncharged imidocarbonate/carbamate gels as shown in Table 4.1 or mixed gels containing different types of chemical bonds (see Fig. 4.1) can be obtained by the CNBr procedure. In general the CNBr activation procedure leads to more or less mixed gels (see also Section 1.4.2).

For simplicity apolar gels synthesized by the CNBr procedure and containing residual charges will be called “non-charge-free” hydrophobic gels (NCF-gels). This terminology denotes that the gels are not simple ion-exchange resins. Uncharged hydrophobic gels will be called “charge-free” hydrophobic (CF-gels). An excellent and easy method for obtaining purely uncharged carbamate linked alkyl agaroses is the carbonyldiimidazole method (Bethell et al., 1979; Zumbrink et al., 1995) which is also employed below (see also Wilchek et al., 1984 for an alternative method via chloroformates). For other methods of immobilizing chemical compounds of various types for the synthesis of hydrophobic and affinity matrices see (Hermanson et al., 1992) and for novel aspects of the tresyl chloride method see (Demiroglou and Jennissen, 1990; Demiroglou et al., 1994, Zumbrink et al., 1995).

Figure 4.1 Reaction scheme for the activation of agarose by CNBr with a cyanate ester as the major stable active intermediate. The scheme is a simplified version of the one published by (Kohn and Wilchek, 1981). For further details see the text and (Kohn and Wilchek, 1981). From (Jennissen, 1995).
**Discovery and development of hydrophobic interaction chromatography**

The chromatographic separation of proteins by way of hydrophobic interactions was reported independently by two groups in 1972 (Er-el *et al.*, 1972; Yon, 1972) and by three additional groups in 1973 (Hofstee, 1973a; Hjerten, 1973; Porath *et al.*, 1973). Historically the method then evolved in two distinct branches. The first branch was developed by Shaltiel, Hofstee and Yon (Er-el *et al.*, 1972; Hofstee, 1973a; Yon, 1972) and centered on hydrophobic gels synthesized by the CNBr procedure (containing some charges) and on procedures run at low ionic strength. The second branch developed by Porath and Hjerten (Hjerten *et al.*, 1974; Porath *et al.*, 1973) focused on gels synthesized by chemical methods which did not introduce charges and which were run at high ionic strength. In the first branch the name "hydrophobic chromatography" (Shaltiel and Er-el, 1973) was coined, the dominant term for many years, whereas in the second the name "hydrophobic interaction chromatography" (Hjerten, 1973) predominated. The development of this novel separation method based on hydrophobic interactions taking place along these two branches was controversial for many years. Today these controversies have been resolved and in this review it will be attempted to depict the major highlights in the development of this still advancing field.

**Hydrophobic non-charge-free gels (NCF-gels)**

In 1972 Yon (Yon, 1972) reported that *lipophilic proteins* such as albumin or aspartate transcarbamoylase could be hydrophobically adsorbed on N-(3-carboxypropionyl) aminodecyl-agarose containing mixed hydrophobic and ionic groups ("hydrophobic affinity chromatography"). The proteins were adsorbed at low ionic strength at the isoelectric point and eluted at alkaline pH by charge repulsion (for review see Yon, 1977). In classical affinity chromatography it had been suggested by

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**Table 4.1** Synthesis charge-containing and uncharged butyl Sepharose by the CNBr method according to (Kohn and Wilchek, 1981)

<table>
<thead>
<tr>
<th>CNBr</th>
<th>Coupling of $^{14}$C-Butylamine</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>HCl wash (isourea, charged) µmol/ml packed gel</td>
</tr>
<tr>
<td>8</td>
<td>24.5 ± 2.19</td>
</tr>
<tr>
<td>15</td>
<td>30.8 ± 1.80</td>
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<tr>
<td>30</td>
<td>80.3 ± 0.12</td>
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</table>

The activation mixture contained 25g of dry Sepharose 4B, 11ml H$_2$O, 24ml (2.5M K$_3$PO$_4$ (pH 12), 18ml Dioxane, 2ml CNBr (61g/100ml). The mixture was incubated for 12min at 8°C, was then sucked dry on a Büchner funnel, washed with either 750ml 0.1M HCl or 750ml 0.1M NaOH and suspended either in 100ml HCl (0.1M) or 100ml NaOH (0.1M) and stirred slowly for 30 min. The gel was sucked dry and immediately added to 2M $^{14}$C-butylamine (Höchst AG, Frankfurt; specific radioactivity $1.5\times10^{5}$cpm/ml) pH 10 and stirred slowly for 3h at 5°C. The butylamine solution was separated from the gel by suction and then the gel was washed with 200ml each of 10mM NaOH, 10mM HCl and H$_2$O and finally suspended in 10mM sodium β-glycerophosphate, 0.01mg/ml sodium azide, pH 7.0. The pH of the butylamine solution changed insignificantly (0.5–1%) after addition of the gel. The solubilized samples (Jennissen and Heilmeyer-Jr, 1975) were counted in triplicate and are given as mean values±S.D. For further details see (Jennissen and Heilmeyer-Jr, 1975; Kohn and Wilchek, 1981).
Steers et al. (Steers-Jr et al., 1971) at that time, that a 1–2nm long spacer should be inserted between the matrix and the immobilized ligand to increase the efficiency of biorecognition. Many groups were therefore applying the new technology and it was more or less by accident that Shaltiel’s group (Er-el et al., 1972) discovered “hydrophobic chromatography”, since they had set out to purify glycogen phosphorylase by classical affinity chromatography on a glycogen-Sepharose column. The glycogen was immobilized via an alkyl spacer by the CNBr method and when the control experiment without glycogen i.e. with the alkyl spacer-Sepharose alone was run, they detected that phosphorylase was strongly adsorbed in the absence of glycogen on a hydrocarbon coated agarose alone (Er-el et al., 1972). The surprising result in Shaltiel’s experiments was that a very “normal” hydrophilic enzyme, phosphorylase b, could be purified on hydrocarbon coated agaroses to near homogeneity in one step, implicitly questioning the general doctrine of the time that all hydrophobic amino acids were buried in the interior of proteins. Phosphorylase was adsorbed at low ionic strength on immobilized butyl residues which had no resemblance to the substrates of the enzyme (thus excluding affinity chromatography) and was eluted by a “deforming buffer” reversibly unfolding the enzyme. Taken together with Shaltiel’s systematic approach of grading the hydrophobicity of the gels via an immobilized homologous hydrocarbon series (Er-el et al., 1972) (see the section on Chain-length parameter), the immediate impression was that here was a novel method applicable not only to hydrophobic or lipophilic proteins but also to hydrophilic and possibly to all proteins. A few months later Hofstee independently published a series of papers leading to similar conclusions (Hofstee, 1973a–c).

**Hydrophobic charge-free gels (CF-gels)**

Uncharged hydrophobic gels (CF-gels) were synthesized for the first time by Porath et al. (Porath et al., 1973) who reacted benzyl chloride with agarose at high temperatures. In this system the synthesis of a graded homologous series of hydrocarbon coated agaroses was not easily feasible. However Porath demonstrated the inverse salt behavior of proteins adsorbed on such gels for the first time and thus termed this method “hydrophobic salting-out chromatography”. In contrast to ion exchangers proteins were adsorbed at high salt concentrations and eluted by decreasing the ionic strength. Hjerten (Hjerten, 1973) in outlining “hydrophobic interaction chromatography” synthesized various hydrophobic supports including homologous alkylamines according to (Er-el et al., 1972) via the CNBr procedure and combined them chromatographically with the salting-out effect of Porath (Porath et al., 1973). These results demonstrated and generalized that proteins could also be adsorbed to NCF-matrices of the Shaltiel type at high salt concentrations and eluted by negative salt gradients just as on chargefree gels (see above). In 1974 Hjerten et al. (Hjerten et al., 1974) described a novel preparation of uncharged hydrophobic gels by coupling alkyl and aryl groups via the glycidyl ether method which allowed the preparation of uncharged homologous series of alkyl agaroses (see also Sections on The adsorption threshold and critical hydrophobicity of gels and on Multivalence).

**Charge containing (NCF) versus charge-free (CF) hydrophobic gels**

In retrospect, although there is no doubt that uncharged hydrophobic gels are by virtue of displaying a single (pure) type of non-covalent interaction superior to the NCF-gels (see (Porath et al., 1973)), it
appears that all groups involved in the development of this new method observed the binding and fractionation of proteins by hydrophobic interactions. In those cases of hydrophobic NCF-gels where the CNBr activated gels had been washed by the original procedure of (Porath et al., 1967) with 0.1M NaHCO₃ (pH 7–8) or with 0.2M Na₂CO₃ (pH 9–10 (Jennissen, 1976a, b)) prior to coupling with alkyl amines a large proportion of the cyanate esters was destroyed favoring uncharged coupling products. Finally it was shown by Shaltiel in a careful study that in fact very similar results are obtained on hydrophobic CF-gels as on the original NCF-gels synthesized by the CNBr method (for review see (Halperin et al., 1981)). In addition it was shown by various groups (Hjerten, 1973; Hofstee and Otillio, 1978; Jennissen, 1976a, b) that the charges introduced by the CNBr method were effectively quenched by salt in the range of 0.3–3.0M so that the NCF-supports matched their uncharged counterparts in many of their hydrophobic properties.

Nomenclature

The two major terms that finally came to use “hydrophobic chromatography” (Er-el et al., 1972; Shaltiel, 1984) and “hydrophobic interaction chromatography” (Hjerten, 1973, 1981) have now been used interchangeably side by side for nearly 30 years. There is no reason why one of these terms should be more or less of a misnomer (Hjerten, 1976) than the generally accepted term “affinity chromatography”. New names are coined to accommodate new scientific contexts not so much for their inner logic. The term “hydrophobic interaction chromatography” is used exclusively in this review for purely editorial reasons.

Principles of hydrophobic interaction chromatography

The chain-length parameter

Shaltiel’s group (Er-el et al., 1972) introduced the principle of the variation of the alkyl chain length of homologous alkylamines on NCF-gels comprising a homologous series of hydrocarbon coated Sepharoses (Seph-Cₙ (Shaltiel, 1974, 1984)). They found that on a homologous series Seph-C₁ to Seph-C₆ the enzyme phosphorylase was excluded by Seph-C₁ and Seph-C₂, retarded by Seph-C₃ and retained by Seph-C₄ to Seph-C₆. The major conclusion of these experimental results was that an increase in the chain length by –CH₂– units concomitantly increased the strength of protein binding from retardation to reversible binding to very tight binding (“irreversible” binding). In addition to this variation in affinity with the chain length the gels also changed in specificity for the adsorbed protein as was shown in later work (see (Shaltiel, 1984)). However the enzyme could only be eluted through reversible denaturation by a deforming buffer followed by renaturation to the active form. The experiments of Shaltiel’s group demonstrated the decisive influence of a “gradable” hydrophobic interaction between the matrix and a protein. Simultaneously the optimization of the gels allowed the purification phosphorylase from a crude extract in one step. The principle applicability of these findings was confirmed on NCF-type hydrophobic alkyl gels by various groups (Hofstee, 1973c; Jakubowski and Pawelkiewicz, 1973; Jennissen and Heilmeyer, 1975; Jost et al., 1974; Raibaud et al., 1975) and also on neutral CF-type hydrophobic alkyl gels (Rosengren et al., 1975).
The surface concentration parameter

The adsorption threshold and critical hydrophobicity of gels

In 1975 however it could first be shown that a second parameter is of equal if not greater importance for the binding of proteins to alkyl substituted gels. If, instead of the chain length, the surface concentration of immobilized alkyl groups (i.e. density) is varied on Seph-C₁–Seph-C₄ protein adsorption is characteristically a sigmoidal function of the surface concentration of immobilized alkyl residues (Fig. 4.2) (surface concentration series). In such a concentration series the strength of binding also increases from retardation to very tight binding as in the homologous series of Shaltiel. Figure 4.2 illustrates the combined effect of chain elongation and surface concentration increase on the adsorption of the enzyme phosphorylase kinase to alkyl agaroses. Chain-elongation in a homologous series leads to a leftward shift of the sigmoidal curves of the concentration series and to a loss of sigmoidicity. Thus in the concentration series of alkyl agaroses a second general parameter for the variation of the gel hydrophobicity had been discovered which was equal to or more crucial for the binding of proteins than an increase in chain length alone (Jennissen and Heilmeyer, 1975). Another important finding in this work was that a threshold value of the alkyl surface concentration, a “critical hydrophobicity can be defined at which a protein is adsorbed (Jennissen and Heilmeyer, 1975). With a ratio of alkyl residues: positive charges in the gels of ca. 10:1 (Jennissen and Heilmeyer, 1975) the predominance of hydrophobic interactions as the basis for adsorption was convincing. Sigmoidal adsorption curves and critical surface concentrations were also obtained from binding data in the presence of 1.1M ammonium sulfate (see Fig. 4.3) i.e. under charge quenching (Jennissen, 1978a) and a similar behavior has also been demonstrated on charge-free hydrophobic gels at low ionic strength (Jennissen and Demiroglou, 1992). A sigmoidal binding of phosphorylase a to methyl-Sepharoses of increasing degree of substitution was also published several years later by Shaltiel’s group (Shaltiel, 1978). Also later Rosengren et al. (Rosengren et al., 1975) showed the dependence of phycocerythrin adsorption on the density of immobilized alkyl groups in a series of uncharged Seph-C₅–Seph-C₁₂ alkyl agaroses at high salt concentrations. Probably, due to the salting-out conditions and the long chain-length of the immobilized alkyl groups (≥C₅), neither a pronounced sigmoidicity of binding nor a critical hydrophobicity were described. A very similar approach to the generation of graded hydrophobicity surfaces as in the concentration series (Fig. 4.2) of alkyl agaroses has been attempted in the synthesis of so-called “hydrophobicity gradients” on glass (Elwing et al., 1988). However these latter gradients also contain a counter-gradient of charges which significantly influences the hydrophilicity/hydrophobicity balance in the adsorption of proteins so that such surfaces do not constitute true hydrophobicity gradients unless the charge gradient is effectively quenched.

Multivalence

The interpretation of the sigmoidal curves (Figs. 4.2–4.3) became possible when the concepts of multivalence and cooperativity of protein adsorption were developed (Jennissen, 1976b). This work was based on quantitative protein adsorption studies (for recent treatise (see (Hlady et al., 1999)). It became clear that the sigmoidicity and the “critical hydrophobicity” were due to the multivalence of the interaction (i.e. the necessity for a simultaneous interaction of more than one alkyl residue or more
than one separate local surface site with the protein moiety) (Jennissen, 1976b, c, 1978a; Jennissen and Botzet, 1979). The conclusion of multivalence and of protein binding on a binding-site lattice was not biased by putatively interspersed charges but could be generalized from a homologous (alkyl residues alone) and to a heterologous (alkyl residues + charges) lattice which should principally not behave differently in generating sigmoidal protein binding curves in a concentration series of alkyl agaroses. In addition, it could be expected that the heterologous lattice would become “functionally” homologous by quenching the charges in the presence of 1.1M ammonium sulfate (Jennissen, 1978a). As expected, the sigmoidal binding curves were not eliminated by high salt concentrations and on the Seph-C₄ concentration series protein binding now displayed a positive temperature coefficient as is characteristic for hydrophobic interactions (see Fig. 4.3) (Jennissen, 1976b, 1978a). Later a mathematical model was developed (Jennissen, 1976c, 1978a, 1981) allowing an estimation of the minimum number of alkyl residues (see Fig. 4.3B) interacting with the protein. The model of multivalence was also confirmed from a different perspective by equilibrium binding studies of phosphorylase b (~1mM) with ¹⁴C-hexylamine (~1mM) in solution at a salt concentration of 1.1M ammonium sulfate. In this experiment (Jennissen et al, 1982) the addition of ~1mM phosphorylase b did not alter the free bulk concentration of ¹⁴C-hexylamine indicating that phosphorylase b did not bind hexylamine under these conditions. This allowed the estimation of a maximal binding affinity for

![Dependence of the adsorption of phosphorylase kinase on the chain-length and surface concentration parameters of a homologous series of alkyl-Sepharoses at a low ionic strength.](image)

Figure 4.2 Dependence of the adsorption of phosphorylase kinase on the chain-length and surface concentration parameters of a homologous series of alkyl-Sepharoses at a low ionic strength. The amount of adsorbed enzyme activity per ml packed Sepharose was calculated from the difference between the total amount of applied units and the amount excluded from the gel. The crude extract was applied to columns containing ca. 10ml packed gel. Insert: Double logarithmic plots of adsorbed phosphorylase kinase as a function of the degree of substitution. Experiments with purified phosphorylase kinase are included. The gels were prepared by the CNBr procedure. For further details see the text. From (Jennissen and Heilmeyer, 1975). Seph-C₁: (●) crude extract; (○) purified phosphorylase kinase, Seph-C₂: (▲) crude extract; (△) purified phosphorylase kinase, Seph-C₄ (□) crude extract.
hexyl amine protein binding to $K_{0.5} \sim 10-100 \text{M}^{-1}$ (Jennissen et al., 1982). Since in binding studies with immobilized butyl residues (Seph-C$_4$ binding constants of $9-16 \times 10^4 \text{M}^{-1}$ for phosphorylase $b$ had been obtained (Jennissen, 1976b; Jennissen and Botzet, 1979) this binding experiment clearly demonstrated that not even the long C$_6$ residue, when immobilized, would be capable of adsorbing a molecule of phosphorylase in a monovalent manner (i.e. on a single immobilized residue). On a gel surface only the cooperative interaction of a critical number of such immobilized residues could lead to adsorption. Since the multivalent mechanism of protein adsorption appeared to be the common denominator of most types of adsorption chromatography (hydrophobic and ion exchange) the term “multivalent interaction chromatography” was suggested (Jennissen, 1978a) but did not come to general use. Later the concept of multivalence was also applied to reversed phase chromatography (Pearson et al., 1982). The possibility of a “multi-point” or “multiple contacts” type of binding in hydrophobic protein adsorption

Figure 4.3 Dependence of the adsorption of phosphorylase b on the surface concentration parameter of Seph-C$_4$ at $5^\circ$C and $34^\circ$C at high ionic strength. The adsorbed amount of phosphorylase in the presence of 1.1M ammonium sulfate was calculated from adsorption isotherms measured at each point at an apparent equilibrium concentration of free bulk protein of 0.07mg/ml. The adsorbed amount of enzyme ($\bar{y}$) is expressed in relation to the anhydrodisaccharide content of agarose in mol/mol anhydrodisaccharide. Similarly $C$ indicates the immobilized butyl residue concentration in relation to the anhydrodisaccharide content of agarose in mol alkyl residue/mol anhydrodisaccharide. A monomer molecular mass of $10^5$ was employed for phosphorylase b. The alkyl agaroses were synthesized by the CNBr method. A. Adsorption isotherms (“lattice site binding function” (Jennissen, 1988)) of phosphorylase b in Cartesian coordinates. Insert: Scatchard plots of the sigmoidal binding curves with extrapolation of fractional saturation of 610 ($5^\circ$) and 1220 ($34^\circ$) µmol enzyme/mol anhydrodisaccharide (corresponding to 6.2 and 13.4 mg/ml packed gel respectively). The broken lines indicate the mode of extrapolation. (●) $5^\circ$C; (○) $34^\circ$C. B. Hill plots of the sigmoidal binding curves. (Θ) the fractional saturation was calculated from the extrapolated saturation values of the Scatchard plot (A). The Hill coefficients $n_H$ are given in the graph. The apparent dissociation constants of half-maximal saturation ($K_D^{0.5}$) are 0.137 and 0.167mol butyl residue/mol anhydrodisaccharide at $5^\circ$ and $34^\circ$ respectively (which corresponds to 14.0 and 17.0µmol butyl residues/ml packed gel respectively). (●) $5^\circ$C; (○) $34^\circ$C. For further details see the text (Jennissen, 1978a, 1981, 1988; Jennissen and Botzet, 1993). From (Jennissen, 1978a).
was also envisaged in lieu of direct evidence by Hofstee and Hjerten (Hofstee, 1973b; Hjerten et al., 1974). However the concept and term of multivalence are to be preferred to terms such as “multiple contacts” since the latter term does not differentiate between the binding of a protein to separate alkyl residues (separate local surface sites) or just to different segments of one and the same alkyl residue.

Protein adsorption hysteresis

Cooperative multivalent protein binding on alkyl substituted surfaces can lead to protein adsorption hysteresis (Jennissen, 1978b, 1985, 1988; Jennissen and Botzet, 1979). In protein adsorption hysteresis, the adsorption isotherm is not retraced by the desorption isotherm, due to an increase in binding affinity after the protein is adsorbed. This indicates that protein adsorption to multivalent surfaces is thermodynamically irreversible ($\Delta S > 0$) and that a true equilibrium has not been reached. The binding affinity increase can be attributed to an increase in multivalence (Jennissen and Botzet, 1979) which is either due to a reorientation of the protein on the surface or to a conformational change in which buried hydrophobic contact sites (valences) are exposed by the binding strain on the adsorbed protein (Jennissen, 1985; Jennissen and Botzet, 1979). In the case of the adsorption of phosphorylase $b$ to butyl agarose there was no evidence that an irreversible conformational change was taking place (Jennissen and Botzet, 1979), since the fully active enzyme can be desorbed from such gels by “deforming buffer” (Er-el et al., 1972) (see above). The danger of a protein undergoing an irreversible conformational change and even a denaturation after adsorption has however been shown for other proteins binding to hydrophobic matrices (Ingraham et al., 1985; Wu et al., 1986) as well as to non-hydrophobic affinity supports (Jost et al., 1974).

Adsorption hysteresis has decisive effects on the chromatographic behavior of proteins during hydrophobic interaction chromatography leading to non-linearity and skewed elution peaks in zonal chromatography and to false “irreversibility”, i.e. extremely high affinity, in adsorption chromatography (Jennissen, 1981, 1986). Hysteresis can be reduced by a decrease in the surface concentration of immobilized residues which reduces multivalence (Jennissen, 1981).

The salt-parameter

Salting-out on unsubstituted hydrophilic gels

Reversible salting-out adsorption of proteins by neutral salts below their precipitation points on solid supports (silica gel, filterpaper) goes back to Arne Tiselius (Tiselius, 1948). In a column chromatography system this technique was reported by Porath (Porath, 1962) as a “zone precipitation” of serum proteins on cross-linked dextran (Sephadex G-100). Although in that paper no distinction is made between an actual precipitation of the proteins on the gel and an adsorptive mechanism, both factors were probably involved. The same phenomenon has also been observed on other crosslinked hydrogels such as Sephacryl (Ashton and Anderson, 1981) and Sepharose CL-6B (Sawatzki et al., 1981). From adsorption studies of cyclohexane and 1-pentanol on highly crosslinked dextran (Sephadex G-15), which show positive temperature coefficients, anhydroglucose itself has been implicated as exhibiting nonpolarity (Mardsen, 1977). However since the nonpolar adsorption of the organic solutes also correlated with the crosslinking density of the dextran (Mardsen, 1977), which
could not be differentiated from the effect attributed to glucose, the matter remains unclear. On the other hand it has been reported (Janado and Nishada, 1981) that the sugars glucose and mannose possess negative as well as positive cosolvent effects in respect to hydrophobic solutes e.g. octanol. The positive cosolvent effect is enhanced by oligomerization of the sugar (maltose, dextran) and by increasing the temperature or the salt concentration (2M NaCl), which strongly indicates nonpolar interactions of the sugar moiety. In the case of galactose a significant positive cosolvent effect was not shown (Janado and Nishada, 1981).

The salting-out of proteins on non-crosslinked and non-substituted agarose (Sepharose 4B) was reported independently by two groups (Mevarech et al., 1976; Von der Haar, 1976). At high ammonium sulfate concentrations (2–3M), but below the precipitation point of proteins, agarose is capable of adsorbing large amounts of proteins (for review see (Von der Haar, 1979)). The proteins can be eluted by a negative ammonium sulfate concentration gradient and purified. Characteristically this adsorption is only elicited by strongly salting-out ions like sulfate, phosphate and citrate (Von der Haar, 1979) at high concentrations. No examples with other salts like NaCl have been reported. Non-crosslinked cellulose also appears to exhibit a similar behavior as an adsorbent for salted-out proteins (Ashton and Anderson, 1981; Tiselius, 1948).

The mechanism of this latter type of salting-out chromatography remains unclear, since no thermodynamic studies have been made. It may very well be that protein adsorption in these cases displays a negative temperature coefficient similar to the adsorption of phosphorylase b in the presence of 1.1M ammonium sulfate on Seph-C1 (see also Fig. 4.6) (Jennissen, 1976b, 1978a). Since this behavior suggests that the free energy of binding is primarily enthalpic and not hydrophobic it has been termed “exothermic” salting-out chromatography (Jennissen, 1978a). This interpretation is supported by results obtained with nucleic acids. Nucleic acids (tRNA) can also be salted-out on Sepharose 4B and various other neutral polysaccharides in the presence of 2M ammonium sulfate as shown by Morris (Morris, 1978). Morris also found a negative temperature coefficient for the retention volumes of individual zones (fractions) of tRNA and the distance between them and concluded an enthalpic, non-hydrophobic adsorption mechanism. Surprisingly deviations in the expected temperature dependence were also observed in reversed-phase liquid chromatography with acetonitrile/water phases indicating non-hydrophobic interactions (Cole et al., 1992) even on these very hydrophobic surfaces. Therefore, as a rule the temperature dependence of the adsorption reaction can be taken to be a more sensitive parameter than a salt effect per se (e.g. salting-out) in gaining information on the actual adsorption mechanism involved (see Jennissen, 1978a).

As to the predominant non-covalent interaction responsible for the binding of proteins and nucleic acids to neutral carbohydrates under salting-out conditions a likely candidate is hydrogen bonding (C.J.O.R.Morris, personal communication, 1977). However more basic work in this area is required before final conclusions can be reached (Morris, 1978).

Another major conclusion from the above observations is that in all salting-out experiments with proteins on chemically modified hydrophilic gels (especially with high concentrations of ammonium sulfate) it is essential to run controls on the corresponding unsubstituted matrices.
Porath (Porath et al., 1973) showed that the hydrophobic adsorption of trypsin inhibitor can be enhanced by high concentrations of salts on uncharged benzyl ether agarose. Trypsin inhibitor could be purified 25-fold after being adsorbed at 3M NaCl followed by elution in buffer without salt. These results were confirmed by Hjerten (Hjerten, 1973) on NCF-gels containing aliphatic residues by the adsorption of serum proteins at 4M NaCl and elution by lowering the ionic strength 400-fold. A similar chromatographic technique was described by Rimerman and Hatfield (Rimerman and Hatfield, 1973) who salted out *Escherichia coli* proteins on an alanine-Sepharose with 1M potassium phosphate and eluted with a decreasing salt concentration gradient. Application of a cell-free *E. coli* extract to the column and development with a negative concentration gradient led to the elution of *E. coli* proteins in relation to their solubility in concentrated ammonium sulfate (Rimerman and Hatfield, 1973). Similarly alkaline phosphatase could be adsorbed on phenylalanine-Sepharose in the presence of 1.25M ammonium sulfate and eluted by a negative salt gradient (Doellgast and Fishmann, 1974).

Subsequently it could be shown that the effect of salts on the adsorption and elution of proteins on alkyl agaroses indeed followed the Hofmeister series of salts (Jennissen and Heilmeyer, 1975; Raibaud et al., 1975). We could show that phosphorylase kinase is eluted (“salted-in”) from a Seph-C2-column by increasing salt gradients (Jennissen and Heilmeyer Jr, 1975). In a series of experiments the ionic strength of the peak fractions eluted, was inversely related to the salting-in power of the anions ions in the gradient in agreement with the Hofmeister series of salts (see Fig. 4.4). Raibaud et al. (Raibaud et al., 1975) reported that the concentration of salts necessary for the inhibition of β-galactosidase adsorption on a Seph-C3-column shows a similar inverse relationship to the salting-in power of the anions employed. These experiments clearly indicated that the action of the ions was not due to a pure electrostatic but to a lyotropic effect. It could be argued that this effect is again due to the interspersed charges in the NCF-gels. However, proteins could also be eluted (salted-in) from uncharged CF-gels (octyl-Sepharose) by an increasing salt gradient as shown by (Raymond et al., 1981): after adsorption of proteins at high ionic strength a first major fraction of proteins was eluted by a decreasing salt gradient followed by the elution of a second fraction by an increasing MgCl₂ gradient. In other experiments it could be shown that haemoglobin, serum albumin (Memoli and Doellgast, 1975) and phosphorylase b (Jennissen, 1976a, b) were salted-out by ammonium sulfate on NCF-alkyl Sepahroses, in column (Jennissen, 1976a) (see Fig. 4.5) and in batch experiments (Jennissen, 1976b) under conditions, where unsubstituted Sepharose showed no adsorption. Finally Pahlman et al. (Pahlman et al., 1977) showed that the salting-out power of anions, for the adsorption of HSA on uncharged Seph-C₅, followed the order of the Hofmeister series of salts. In sum, these experiments again demonstrate the similar properties of the hydrophobic matrices containing some residual charges (NCF-gels) and the uncharged hydrophobic gels (CF-gels) in respect to the lyotropic action of salts on protein adsorption.

The mechanism of protein binding was further analyzed at different temperatures under equilibrium binding conditions on NCF-gels (Jennissen, 1976b; Jennissen and Botzet, 1979). At very low ionic strength (10mM Tris/HCl, pH 7.0) the adsorption of phosphorylase b exhibited a negative temperature coefficient on Seph-C1 as well as on Seph-C₄ (Jennissen, 1976b). At high ionic strength (1.1M ammonium sulfate) Seph-C₁ retained its negative coefficient but Seph-C₄ now exhibited a positive temperature (see Fig. 4.3) coefficient (Jennissen, 1976b; Jennissen and Botzet, 1979) demonstrating that thermodynamically two forms of salting-out adsorption on hydrophobic gels can be distinguished.
as exothermic and endothermic salting-out chromatography respectively (see above) (Jennissen, 1978a). It may be argued that one may not be observing the temperature dependence of adsorption but that of a temperature dependent conformational change of the protein on the gel surface as reported for a-lactalbumin on a C2-ether silica column (Wu et al., 1986). However, besides having to explain two thermodynamically different conformational changes of phosphorylase on Seph-C1 and Seph-C4 under otherwise identical conditions, the corresponding temperature-dependent conformational changes of the protein have not been observed in solution. In fact the opposite has been shown in the hydrophobic interaction chromatography of myoglobin and cytochrome c. In this case it was documented that a temperature increase enhances binding without a major change in protein conformation (Ingraham et al., 1985). The salting-in action of salts can be counteracted by salting-out salts in the same system (Hanstein, 1979). Thus various binary and ternary salt systems (el-Rassi et al., 1990) and combinations with detergents (Buckley and Wetlaufer, 1990) have been devised.

Figure 4.4 Influence of the salt parameter on the desorption of purified phosphorylase kinase from Seph-C2 (25µmol/ml packed gel) with salt gradients of different ionic composition. Each column with 5ml of the above gel (CNBr method) was loaded with ca. 11mg of the enzyme. The gradients were produced from 100 ml low ionic strength adsorption buffer and 100ml salt containing buffer. The number at the maximum of the elution profiles indicates the ionic strength of the peak fraction. For further details see the text and legend to Fig. 4.1 and (Jennissen and Heilmeyer, 1975). (From Jennissen and Heilmeyer, 1975.)

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Theories of salt actions on proteins

An early approach on the salt effects exerted on macromolecules is based on the action of chaotropic ions (Hamaguchi and Geiduschek, 1962) on the solubility of proteins (Hatefi and Hanstein, 1969). The action of the chaotropic anions (SCN\(^{-}\), ClO\(_4\)^{-}, I^{-}, NO\(_3\)^{-}, Br^{-}), which can be arranged in a series similar to the Hofmeister (lyotropic) series (Hofmeister, 1887, 1888), is regarded to involve a structure-breaking effect on water since the ionic entropies of hydration are positive. The original chaotropic series was later supplemented by the chaotropic haloacetates (Hanstein et al., 1971). Thus one can observe a decreasing increment of the salt series on the surface tension of water and an increase in surface potential (Hatefi and Hanstein, 1969). The authors conclude that “by making the water more

Figure 4.5 Application of the salt parameter by utilizing the inverse salt dependence for the chromatography of purified phosphorylase b on Seph-C\(_1\) (30µmol/ml packed gel). In this form of salting-out chromatography the equilibration buffer contained 10mM sodium \(\beta\)-glycerophosphate, 20mM mercaptoethanol, 2mM EDTA, 20% sucrose, 0.5µM PMSF, pH 7.0 (buffer A) to which either 1.1M ammonium sulfate or NaCl was added. 6mg/3ml phosphorylase b was added to 20ml Seph-C\(_1\) in a 2cm i.d.\(\times\)17cm column. Fractions of 6.5ml were collected. The gel was prepared by the CNBr procedure. A. Application of enzyme to a column equilibrated with buffer without (NH\(_4\))\(_2\)SO\(_4\) or NaCl: (1) Application of phosphorylase \(b\) in buffer A; (2) Elution with buffer A+1M NaCl. B. Application of enzyme to a column equilibrated with buffer with (NH\(_4\))\(_2\)SO\(_4\) (1) Application of phosphorylase \(b\) in buffer A+1.1M ammonium sulfate; (2) Elution with buffer A; (3) Elution with buffer A+NaCl. For further details see the text and (Jennissen, 1976a). From (Jennissen, 1976a).

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disordered and lipophilic in the presence of the appropriate ions, it should be possible to weaken the hydrophobic bonds of membranes and multicomponent enzymes and increase the water solubility (salting-in) of particulated proteins and nonelectrolytes” (Hatefi and Hanstein, 1969). This interpretation is supported by the solvent isotope effects observed in D₂O vs H₂O (Hanseit et al., 1974). The opposite effect can be observed with so-called antichaotropic salts (SO₄²⁻, HPO₄²⁻, F⁻) which reverse the resolution of hydrophobic interactions by stabilizing the water structure (salting-out) (Davis and Hatefi, 1972) and which generally have negative values of the ionic entropy of hydration (Hanstein et al., 1971). For a quantitative description of the effect of salt concentrations on the solubility of the model solute 2-methylnaphthoquinone an extended Setschenow equation has been utilized (Hanstein, 1979; Hanstein et al., 1971). The general conclusion of this work, with consideration of electrostatic and dispersion forces, is that chaotropes interact indirectly with solutes (e.g. proteins) mainly through their effect on water structure (Hanstein, 1979).

The solvophobic theory of Melander and Horvath, 1977 allows the description of reversed-phase retention for a wide variety of conditions including organic solvents and neutral salts. Salting-in and salting-out is explained on the basis of the respective surfacetension-decreasing-/increasing effect of the salt which is applicable to reversed-phase chromatography as well as to hydrophobic interaction chromatography (Melander and Horvath, 1977; Melander et al., 1984). Specifically in respect to retention in hydrophobic interaction chromatography it has been suggested that water plays a central role as displacing agent (Geng et al., 1990). In addition to the dependence of retention on the non-polar molecular area of the protein and the interfacial tension in the aqueous salt solution there also appear to be specific salt effects (divalent cations) resulting from a direct binding to protein e.g. of MgCl₂ (Arakawa and Timasheff, 1984; Szepesy and Horvath, 1988). In the paper of Arakawa and Narhi (Arakawa and Narhi, 1991) this approach, that salts do not act by influencing the surface tension but by directly binding to the protein, is extended. The action of thiocyanates is therefore interpreted as due to a preferential binding to proteins followed by a destabilization of the hydrophobic interactions.

Irrespective of the mechanism, the applicability of the Hofmeister (lyotropic) series of salts, expanded by the chaotropic series, to hydrophobic interaction chromatography has been verified by many groups and these salts are important tools in controlling the adsorption and elution of proteins on these resins. The individuality of each protein in its quantitative interactions in such a system especially when the native state should be conserved, however, remains a very important factor in determining the behavior of each individual chromatographic system.

Optimization of alkyl agaroses for hydrophobic interaction chromatography

Introduction

Even now nearly 30 years after the introduction of hydrophobic interaction chromatography the method has failed to gain the same foothold in the methodological repertoire of protein chemistry as has affinity chromatography, although a large number of proteins has been successfully purified by this method (Eriksson, 1989; Hjerten, 1981; Hofstee and Otillio, 1978; Mohr and Pommerening, 1986; Ochoa, 1978; Shaltiel, 1984; Yon, 1977). In fact, a recent paper comes to the conclusion that certain “classical” commercial hydrophobic adsorbents are inadequate for an ideal downstream processing because of their high hydrophobicity (Oscarsson et al., 1995). The criticism of these authors is essentially
correct. The major problem encountered on such hydrophobic gels is that proteins can be very effectively adsorbed but an elution in a native state is often practically impossible. The problem therefore is to find a hydrophobic matrix with such a low hydrophobicity as to allow a binding and an elution of the intact protein. This problem is not new. The possibility of synthesizing low hydrophobicity gels has been present from the very beginning of hydrophobic interaction chromatography (Jennissen, 1976a). In fact, such a “weak-hydrophobic chromatography system” in which the protein (phosphorylase \( b \)) eluted as a retarded peak on Seph-C4 at a high salt concentration was demonstrated many years ago (Jennissen, 1981). The question is therefore: Can a generally applicable method be devised for the synthesis of gels with an optimal low hydrophobicity for each protein? As will be shown below we think that such a method is conceivable.

**The homologous series procedure**

The synthesis of controlled hydrophobicity gels via the *homologous series* of hydrocarbon-coated Sepharoses (variation of alkyl chain length (Er-el *et al.*, 1972; Halperin *et al.*, 1981; Shaltiel, 1984)) was soon commercialized by introduction of the so-called *exploratory kit* for choosing the most appropriate column and for optimizing resolution (Shaltiel, 1974). This analytical kit, which was commercially available for some years, contained a homologous series of small columns from Seph-C1 to Seph-C10 with two control columns (Shaltiel, 1974). The principle was to determine—at low ionic strength—the lowest member of the homologous series capable of retaining the desired enzyme or protein. This column was then selected for the purification of the desired protein. In a second step it was attempted to increase resolution by optimizing the elution procedure which ranged from mild salting-in procedures to reversible denaturation steps.

It had been shown by Hjertén's group (Hjertén, 1973; Hjertén *et al.*, 1974; Rosengren *et al.*, 1975) that proteins could also be adsorbed to homologous series of hydrophobic agaroses at high salt concentrations to be subsequently eluted by an inverse salt gradient. This was shown for the uncharged and the charge-containing gels. The optimization strategy was similar to that of Shaltiel only that elution was facilitated by an inverse salt gradient. Uncharged alkyl agaroses optimized and eluted according to the procedure outlined by Hjertén were also widely utilized and commercialized. However similar to the Shaltiel type of gels the main problem encountered in the high salt concentration approach of Hjertén was that again the proteins could often not be eluted (Oscarsson *et al.*, 1995) in the native form by the inverse salt gradient. Nevertheless this procedure or variants thereof are still the method of choice for most groups today.

**The critical-hydrophobicity procedure**

The importance of the second of the two procedures, i.e. optimization via the surface concentration series, has been underestimated. Although the decisive importance of the immobilized alkyl residue concentration for the hydrophobic adsorption of proteins (critical hydrophobicity) was stressed for many years (Jennissen and Heilmeyer, 1975; Jennissen, 1976b, 1978a, 1979 Jennissen and Demiroglou, 1992) no hydrophobicity gradient gel series has ever been produced commercially. Against the above background of the problems reported in hydrophobic interaction chromatography a novel rational basis for the design of a hydrophobic chromatography systems was recently introduced.
It has been called critical hydrophobicity hydrophobic interaction chromatography (critical hydrophobicity HIC).

This concept is based on a very simple idea. High yields in hydrophobic interaction chromatography can only be obtained if the protein to be purified is fully excluded from the gel under as physiological elution conditions as possible i.e. at low ionic strength. This means that the gel should be fully non-adsorbing under these conditions. On the other hand, since a purification is only possible if the protein is adsorbed to the gel, the matrix should be constructed in a way, that adsorption can be easily induced by other means without denaturing the protein. According to this concept, working at or near (juxtacritical) the critical hydrophobicity point should solve both problems. At critical or juxtacritical hydrophobicity of the matrix no protein is adsorbed at low ionic strength but adsorption can be induced by a finite high salt concentration with the possibility of utilizing either (a) elution chromatography by a negative salt gradient (“irreversible” adsorption conditions) or (b) a linear zonal chromatography (reversible adsorption conditions) (Jennissen, 1981). If the selected gel is significantly below the critical hydrophobicity protein adsorption may not be inducible by salt and if the gel is significantly above the critical hydrophobicity a complete elution of the protein will not be possible.

The aim of this new procedure is, therefore, to synthesize uncharged alkyl-Sepharose supports very close to the “critical hydrophobicity” of the protein under physiological conditions. Although essentially any type of molecule displaying hydrophobic properties could be coupled to the matrix, even proteins (Jennissen and Botzet, 1993), the immobilized residues should be restricted to alkane derivatives, to insure a “purity” of hydrophobic interactions. This restriction, in the first instance, also excludes pi- or d-electron containing molecules e.g. phenyl residues or sulfur atoms which should be avoided, because they may introduce additional charge-transfer interactions (Porath, 1978, 1989, Jennissen, 1995). Strongly salting-out ions should also be avoided, since they may induce nonspecific protein adsorption on the carbohydrate support itself (see discussion above). NaCl centrally located in the Hofmeister series appears to be an ideal salt to employ from the biochemical as well as the physical chemical view point. The critical hydrophobicity procedure thus involves 3 basic steps under standard conditions:

1 Selection of an appropriate alkyl chain length from a homologous series which is capable of adsorbing a selected protein under semiphysiological low ionic strength conditions.
2 Determination of the critical hydrophobicity at the predefined low ionic strength on the gel selected from the homologous series under point 1.
3 Determination of the minimal salt concentration (NaCl) necessary for a complete adsorption of a specified amount of the selected protein (e.g. 0.5mg/ml packed gel) on the critical hydrophobicity support (point 2).

The three parameters can be determined by a simplified version of quantitative hydrophobic interaction chromatography (Jennissen and Demiroglou, 1992) aimed primarily at the high affinity adsorption sites. In distinction to the previously employed quantitative chromatographic method (Jennissen and Heilmeyer, 1975) the selected protein in this case is not applied to the column until equilibration is complete from an identical concentration of protein or catalytic activity in the run-through and sample. Instead, a constant amount of enzyme activity or a protein (in this case fibrinogen) at an intermediate bed load is applied, followed by a wash with protein-free buffer until no
more protein (enzyme activity) is detected in the run-through (see Methods). In single protein systems the adsorbed protein can then be eluted by urea or SDS to gain additional information on the adsorption state.

**SELECTION OF THE APPROPRIATE ALKYL CHAIN LENGTH**

The appropriate alkyl chain length is selected from test runs on Seph-C₄–Seph-C₆ similar to the homologous series method of Shaltiel at low ionic strength (see Jennissen, 2000). The surface concentration is set to ca. 20µmol/ml packed gel. In general a constant amount of protein (ca. 0.5mg/ml packed gel, which can be 100% adsorbed on the column of highest hydrophobicity) is applied at low or physiological salt concentration (0.15M NaCl) to each column (1–2ml packed gel). Then the gel in the homologous series is determined which adsorbs ca. 50% of the applied protein at a medium surface alkyl concentration of ca. 20–30µmol/ml packed gel. In the case of the example below, ca. 50% of the applied fibrinogen was adsorbed on an uncharged Seph-C₅ gel containing 22µmol/ml packed gel, so that Seph-C₅ was chosen for an expanded concentration series to determine the exact critical hydrophobicity point. For more information on the various methods of measuring protein adsorption in batch, column and surface spectroscopy systems (see Hlady et al., 1999).

**DETERMINATION OF THE CRITICAL HYDROPHOBICITY**

As previously defined the critical hydrophobicity is that degree of substitution where the adsorption of a protein begins (Jennissen and Heilmeyer, 1975). As shown in Fig. 4.6 a strongly sigmoidal adsorption curve is obtained on the uncharged Seph-C₅ at a physiological NaCl concentration for the adsorption of fibrinogen as a function of the degree of substitution. The aim is to get as close as possible to the critical hydrophobicity without a significant adsorption of the protein. Since there was no measurable adsorption of fibrinogen at 12µmol/ml packed gel and only ca. 2% was adsorbed at 13.6µmol/ml packed gel (critical hydrophobicity, Fig. 4.6), the ideal juxtacritical hydrophobicity range was taken as 12–14µmol/ml packed gel.

**THE MINIMAL SALT CONCENTRATION FOR ADSORPTION**

The non-adsorbing critical hydrophobicity gel above (13.6µmol/ml packed gel) can now be transformed into an adsorbing gel by the addition of salt. In the case of fibrinogen this can be achieved by adding 1.5M NaCl to the adsorbing buffer (see Jennissen, 2000)). At lower salt concentrations the full load of fibrinogen is not adsorbed. Since the amount of fibrinogen adsorbed to the critical hydrophobicity pentyl Sepharose (13.6µmol/ml packed gel) at low ionic strength (i.e. 0.15M) was negligible (i.e. only 2% of applied amount), it is clear that a complete recovery of fibrinogen adsorbed at high ionic strength (1.5M NaCl) should now be possible by simply decreasing the salt concentration to the original concentration of 0.15M. This is indeed the case.

**One-step purification of native fibrinogen from human blood plasma**

Employing Seph-C₅ of critical hydrophobicity it is now possible to purify fibrinogen from human plasma in a single step (Fig. 4.7). The hydrophobicity of the column even suffices to purify fibrinogen
from plasma not equilibrated with 1.5M NaCl, allowing a direct application of human plasma without any preparatory steps (Fig. 4.7A). This leads to a temporary decrease in NaCl concentration (fractions 5–9), however, without any significant loss of fibrinogen. After extensive washing with 1.5M NaCl pure fibrinogen (clottability 93–99%, Fig. 4.7B) can be eluted at a NaCl concentration of 150mM. In this case the total yield was 25%, which is not due to residual fibrinogen on the column but to errors inherent in the use of clottability for the calculations and losses in the run-through. Maximal yields of fibrinogen of 60% have been obtained. If blood plasma equilibrated with 1.5M NaCl is applied to the gel and eluted by a negative salt gradient a maximal clottability of 80% is obtained (not shown).

Conclusions

Hydrophobic interaction chromatography is one of the very basic separation methods in classical biochemistry. However a simple optimization method for the purification of proteins on low hydrophobicity gels is still lacking. In the chapter it has been outlined that the successful hydrophobic interaction chromatography of proteins on alkyl-matrices under standard conditions depends on three basic parameters: (i) the chainlength parameter, (ii) the surface-concentration parameter and (iii) the salt parameter. Only if these three parameters are optimized in an integrated manner can a successful implementation of hydrophobic interaction chromatography in protein purification be expected. The presented data strongly suggest that the critical hydrophobicity method for the optimization of hydrophobic supports poses a general, successful and rational approach to the purification of proteins by hydrophobic interaction chromatography. The only drawback is that hydrophobic concentration-series gel kits are not commercially available so that the application of the critical hydrophobicity method necessitates experience in the synthesis of alkyl agaroses and the quantification of the immobilized residues.
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References


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