Affinity Chromatography

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Preface

The technique of affinity chromatography has been applied to almost every area of biochemistry. The range of applications includes simple enzyme purifications, studies of enzyme mechanism, the isolation of specific receptor sites in cells and the development of complex multi-enzyme reactors. Apart from its intrinsic research value, this technique has potential uses in both industry (e.g. food and drug processing) and medicine (from routine clinical services to advances in medical technology).

As early as 1910, Starkenstein, in a preparation of amylase on insoluble starch, illustrated the inherent advantages of affinity chromatography. However, despite the early work of Lerman's and McCormick's groups, a considerable time-lapse occurred before the full potential of the technique was realized and the major advances in affinity chromatography undoubtedly stem from two papers in the late 1960's. In 1967, Porath and his group published a procedure for specific immobilization of amines onto inert polymers. The second paper, by Cuatrecasas, Wilchek and Anfinsen (1968), as well as introducing the term 'affinity chromatography', demonstrated the use of specific adsorbents in enzyme purification. The inherent advantages of this technique over classical procedures have resulted in an extremely diverse literature, the exponential growth of which must surely present a daunting task to any worker interested in this field.

Our objective in writing this book is to guide the student through the maze of literature. We have attempted to bring together some of the principles of affinity chromatography, drawing both on published work and on theoretical considerations. Some sections have been devoted to applications of affinity chromatography in molecular biology and biochemical research, while the final chapter provides details of the chemistry and methodology required to prepare affinity matrices. In writing the book we had in mind both students and research workers who are interested in using theoretical principles to help them through the design and analysis of possible experiments. The examples have been chosen, not to provide a comprehensive review of papers in the field, but rather to illustrate some of the parameters which seem to us to be important to the fundamentals of affinity separations.

Many of our ideas were developed whilst we were teaching a course in the Biochemistry Department at Liverpool University; the references used have been selected from the literature up to the middle of 1973.

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Contents

Chapter I

Introduction

Techniques for the purification of proteins have advanced considerably since the days when good fortune and an element of practical expertise were the essential qualifications of the protein chemist. Today, it is possible to devise separative procedures based on such rational parameters as the size, shape or charge of the protein, and more recently on the nature of the ligand with which the protein interacts. Indeed, the evolution of the tactics of protein purification has been such that any competent protein chemist can reasonably be expected to purify a protein to currently acceptable standards of homogeneity. Many of these advances are a direct consequence of our greater understanding of protein structure and function.

A. PROTEIN STRUCTURE

Proteins are extremely complex macromolecules which have discrete physical, chemical and biological properties. They are elaborate polypeptides containing a non-systematic but quite specific sequence of some 20 different amino acid residues.[1] Amino acids are chemical compounds which have in common an acidic carboxyl (—COOH) and a basic amino ($—NH_2$) function but differ in the nature of a group (R) attached to the same α-carbon atom.

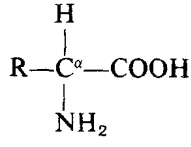
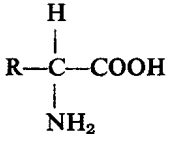
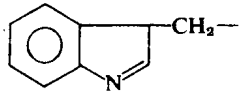
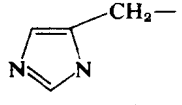
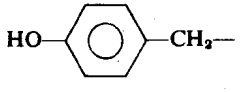
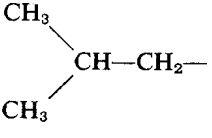


Table I.1 lists the structures of some common amino acids and emphasizes the variation in the substituent R, which may be highly reactive in some cases, like the sulphydryl group of cysteine, or chemically inert, as in leucine.

Peptide bond formation occurs when the amino group attached to the α-carbon atom of one amino acid is condensed with the carboxyl group attached to the α-carbon atom of a second amino acid, with the concomitant elimination of a water molecule (Fig. I.1). When additional amino acid residues are subsequently attached, polypeptides are formed. The fixed amino acid sequence of the polypeptide chain, the so-called primary structure, is the

basis for other levels of structural organization collectively termed the *conformation* of the protein. The polypeptide chain can be folded and bent into a secondary structure which is stabilized by the formation of linkages and bonds between adjacent amino acids. The planar character of the peptide bond and the rotational restrictions of the two single bonds (Fig. I.1) markedly limit the number of allowed conformations. If the substituents (R) on the amino acids permit, intra-chain hydrogen bonding can restrain the polypeptide chain into an α-helical form.[2] However, despite the internal hydrogen bonding, this helical structure is not sufficiently stable to exist in aqueous

Table I.1. The structures of some amino acids

	Amino acid	R
	Tryptophan	
	Lysine	$NH_2—(CH_2)_3—CH_2—$
	Histidine	
	Tyrosine	
	Glutamic acid	$HOOC—CH_2—CH_2—$
	Serine	$HO—CH_2—$
	Cysteine	$HS—CH_2—$
	Leucine	

media without unfolding into a random disoriented strand. Environmental stability can often only be achieved if the helical chains are themselves folded and bent. This element of protein structure, the tertiary structure, is maintained by hydrogen bonding, electrostatic bonds, covalent linkages and hydrophobic interactions.[3] It is this level of organization of proteins that is particularly sensitive to environmental fluctuations and the one that confers on the protein the property of biological specificity. The tertiary level of organization is often augmented by a further level where polypeptide subunits are aggregated in various geometrical arrangements.[4] The resulting

multi-subunit proteins are particularly sensitive to environment and the presence or absence of small ligands termed effectors. The majority of allosteric enzymes are multi-subunit proteins.[5]

The similarity in structure of most proteins is paralleled by a marked similarity in overall physicochemical properties, although small differences in amino acid content or distribution will be reflected in a slightly altered charge, size or shape of the protein. Fig. I.2 shows the structure of a relatively simple protein.[11]

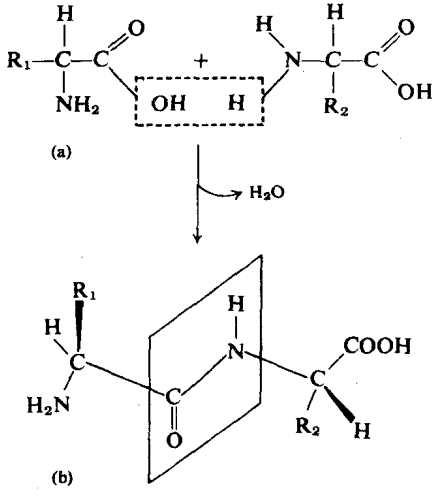


Figure I.1. The formation of a peptide bond

B. CLASSICAL PROTEIN PURIFICATION

Classical procedures of protein separation and purification are generally based on the relatively small differences in the physicochemical properties of proteins in the mixture. They are hence unselective, tedious and of poor resolution. The problems encountered by the protein chemist are thus subject to the following considerations.

The protein of interest may constitute only 0·1% or less of the dry weight of the starting material, and most of the remainder will consist of other proteins with closely allied properties. Furthermore, the classical methods adopted by the organic chemist, such as distillation and solvent extraction, are not applicable to the separation of proteins, owing to their size and instability. This instability is reflected in altered solubility properties occasioned either by changes in temperature, acidity or alkalinity or by a variety of chemical agents. The process of conversion of the 'native' protein to a

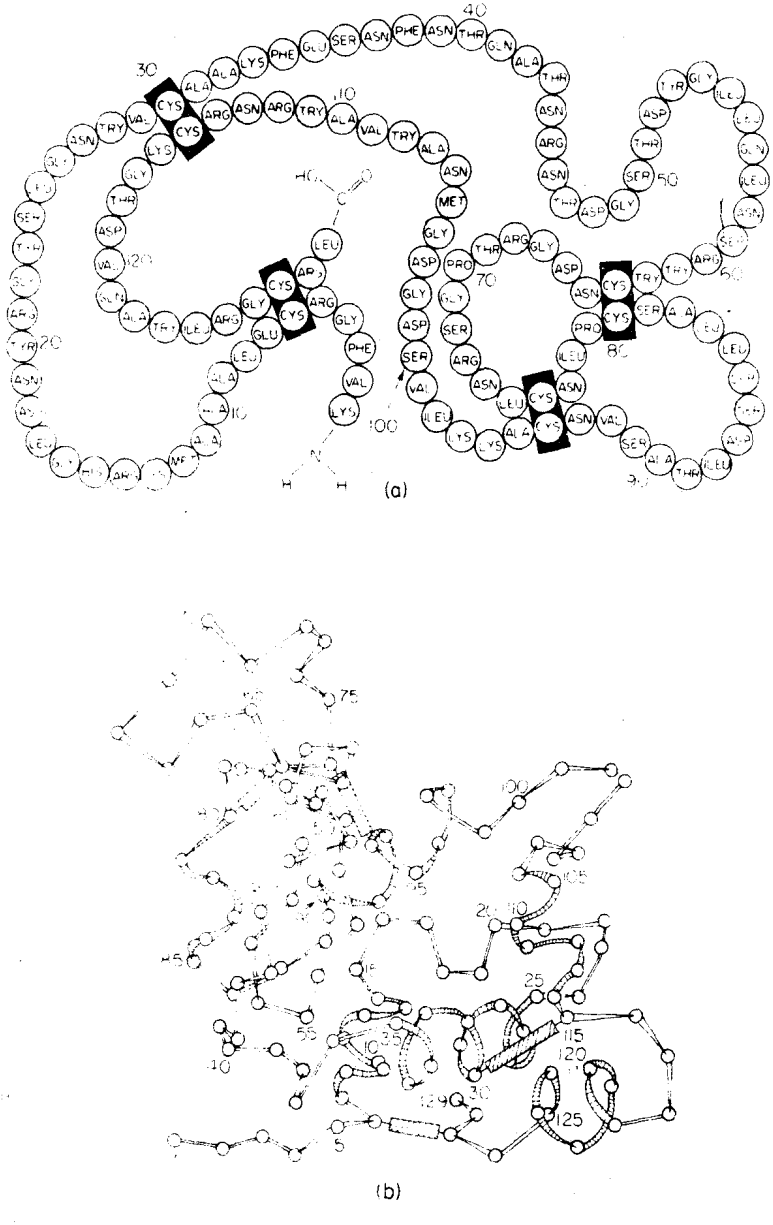


Figure I.2. The structure of egg white lysozyme. (a) The primary structure depicting the sequence of amino acid residues and the positions of the four disulphide bridges. (b) Schematic drawing of the main chain conformation. (a) Reproduced with permission from R. E. Canfield and A. K. Liu, *J. Biol. Chem.*, **240**, 1997 (1965). (b) Reproduced with permission from C. C. F. Blake *et al.*, *Nature* (*London*), **206**, 757 (1965)

product with altered properties is termed 'denaturation' and the product is a 'denatured' protein.

The first consideration in the isolation of proteins from natural materials is to find a source that is rich in the desired protein. This may frequently involve the assay of the discrete biological activity of the protein in a variety of potential sources to select the one that is the most favourable. Where the biological source is a micro-organism the amount of the desired enzyme or protein may be increased by genetic and environmental manipulation by harnessing such control mechanisms as induction, end product repression and catabolite repression. Since each control mechanism is influenced by environmental conditions, parameters such as temperature, pH, medium composition, aeration and stage of development are critical and optima must be determined empirically for the desired protein. The specific activity of enzymes can be altered several hundredfold by these methods.

Once the preliminary screening of natural sources has been performed, the next stage is to free the protein from the tissue. This is usually done by disrupting the cellular organization of the tissue by exposure either to a grinding or shearing action, to ultrasonic irradiation or to solutions of low ionic strength. If the desired protein is bound to a particulate portion of the cell, additional treatment with detergents or lipid solvents may be necessary to release it.

Since proteins are fragile molecules, several important precautions should be observed during the procedures necessary for extraction of the protein from the tissue and during the subsequent fractionation steps.

(i) The temperature should be maintained as near to the freezing point of the solvent as possible since exposure even to physiological temperatures can elicit slow denaturation *in vitro*.

(ii) The pH must be carefully controlled since the susceptibility of proteins to alterations in pH varies greatly.

(iii) The protein concentration should be kept as high as possible to prevent denaturation by dilution.

Many fractionation methods have been devised for the purification of proteins that depend on the small differences in stability, charge, size or shape of individual proteins. These will be considered briefly in turn.

(*a*) *Stability*. If the protein of interest happens to be unusually stable to heat or to extremes of pH, a brief heat treatment or exposure to high or low pH may serve to precipitate the bulk of contaminating material. This could effect a substantial purification. The solubility of proteins varies greatly with pH, and for each protein there is a pH value of minimum solubility, the *isoelectric point*.

(*b*) *Exploitation of charge proteins*. As already noted, proteins are large molecules with many positively and negatively charged groups. These can interact with each other, with small ions of opposite charge in the solvent, or

with water. The force, F, between two charges of opposite sign (q_1 and q_2) is given by the expression:

$$F = \frac{q_1 q_2}{Dr^2}$$

where r is the distance between them and D is the dielectric constant of the medium. The latter, which is a measure of the influence of the medium of the interaction, can be altered by the addition of inorganic salts or organic solvents to the medium. The counter-ion concentration is increased until aggregation occurs and the protein is said to be '*salted out*'. Neutral salts such as ammonium sulphate, potassium sulphate or sodium sulphate are commonly used although the concentration and nature of the salt used are generally determined empirically. In general, selection of a pH value near the isoelectric point of the desired protein will lead to sharper precipitation.

The addition of organic solvents to the medium lowers the dielectric constant, reduces the activity of water, and hence favours protein–protein interaction and precipitation. Ethanol, methanol, acetone and 1,2-pentanediol are commonly used although the method suffers the disadvantage that enzyme activity can be lost if the temperature is not well regulated. The advantages of the method arise from the lower density of the resultant solution and hence lower centrifugation times. Neutral salts in general increase the solubility of proteins in organic solvents and hence careful optimization of temperature, pH, protein concentration, ionic strength and the dielectric constant are necessary in order to precipitate the desired protein. Under these conditions, individual proteins may be precipitated by neutral salts or by organic solvents within a comparatively narrow concentration range of the precipitants.

The selective adsorption and subsequent desorption of proteins on certain inert materials has been used as a purification procedure. Such adsorbents include calcium phosphate, starch and alumina gels, hydroxylapatite or diatomaceous earth (celite), and the process can be effected either on a column or in slurries of the materials.

A more universal approach to the purification of proteins employs column chromatography on ion-exchange resins. Resins with hydrophilic polysaccharide backbones that allow ready access of the protein to the ion-exchange group are most satisfactory for this purpose. The most commonly employed materials are an anion-exchange resin, diethylaminoethyl-cellulose (DEAE-cellulose) and a cation-exchange resin, carboxy-methyl-cellulose (CM-cellulose). The structures of these exchangers are shown in Fig. 1.3. Such columns are developed by increasing the ionic strength or by changing the pH, temperature or dielectric constant of the eluting buffer. Providing that the correct methodology is employed, excellent resolution of complex mixtures of proteins and hence substantial purifications can be achieved.

Preparative electrophoretic techniques such as starch block, agarose elec-

trophoresis or isoelectric focusing, may effect excellent resolution of some complex mixtures with almost quantitative recovery of the proteins. However, the low capacity of these procedures limits their general applicability.

(*c*) *Size and shape.* The use of molecular sieves, which fractionate proteins according to their size, has found considerable application in protein isolation. The most commonly employed sieves consist of cross-linked polysaccharide matrices that are available commercially in a variety of pore sizes. The exclusion limit is determined by the degree of cross-linking and can be employed for proteins with molecular weights ranging from less than 20,000 to several millions. Similar sieves based on cross-linked polyacrylamide have been utilized. More recently, DEAE- and CM-functions have been linked to these gels, such that simultaneous fractionation on the basis of size and charge may be effected.

A typical purification scheme will consist of a combination of these techniques to encompass the differences in stability, charge and molecular weight

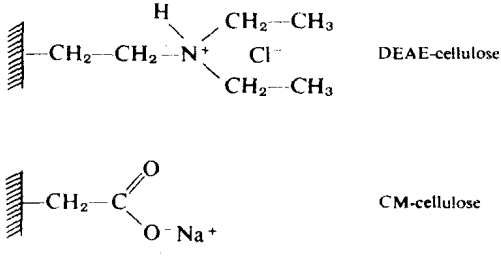


Figure I.3. The structures of two common cellulosic ion-exchange adsorbents

of the desired protein over the contaminating materials. Provided that the use of these procedures has yielded a reasonably homogeneous protein preparation, crystallization may sometimes be achieved. Repeated recrystallizations can often significantly increase the purity of the final product.

The evaluation of a proposed step in an overall purification scheme rests on two criteria: the recovery and the degree of purification. These parameters are based on the specific activity of the protein. This is a convenient measure of the purity of the desired protein based on the enzymatic activity or some spectral property and related to the total amount of protein present. The yield represents the percentage of the total activity in the original extract that is recovered after each step, and the degree of purification the increase in specific activity at each stage. A typical purification scheme is presented in Table I.2, which demonstrates the relationship between, and usefulness of, the various quantities used to define the scheme.