Current trends in affinity-based separations of proteins/enzymes

Ipsita Roy and Munishwar Nath Gupta*
Department of Chemistry, Indian Institute of Technology, Delhi, Hauz Khas, New Delhi 110 016, India

Bioseparation steps constitute an important component in the production of enzymes/proteins. The recent trend is to use affinity-based separations which give fairly high resolution. The examples of such methods are affinity cross flow ultrafiltration, membrane affinity filtration, continuous affinity recycle extraction, two-phase affinity extractions and affinity precipitation. In all such methods, an affinity ligand has to be conjugated to a matrix. This is a costly approach and the affinity ligand is slowly released during the process. Thus, the ideal situation is when the matrix itself constitutes the macroaffinity ligand. Designer dyes, fusion proteins and products of combinatorial chemistry are examples of relatively novel affinity ligands.

MOST of the biotechnological applications involve either use or production of enzymes/proteins[1–4]. It is now fairly well-known that downstream processing costs constitute a very large percentage of overall production costs[5–7]. This percentage is usually in the range of 50–80% but can be even higher. Unfortunately, classical enzymologists are not trained to think in terms of the economics of the process. The appreciation of the inherent difficulties involved in scaling-up of a separation process is also something an enzymologist seldom bothers about. However, in view of the critical role that downstream processing plays in biotechnology, biochemists are slowly becoming aware of these aspects of protein separation. Thus, the trend is towards developing separation protocols which are less costly, more efficient and easily scaleable.

So far, biochemists tended to follow the kind of flow chart shown in Figure 1 for purification of proteins/enzymes. In fact, an oft-quoted paper[8] pointed out that ammonium sulphate was invariably the first step in purification protocols. Such protocols are quite appropriate (and will continue to be used) when the aim is to isolate a small amount of the protein for structural and mechanistic studies. However, in cases where the enzyme is to be used for bioconversion, synthesis or analysis on a large-scale, one has to worry about the cost and time involved in isolation of each milligram of the protein. One clear trend is to bring affinity-based separations right early into the downstream processing strategy. Using affinity in the chromatographic format is not viable at this stage since crude extracts with suspended particulate matter tend to choke the column. Some alternative formats for affinity-based separations which have emerged over the years are given in Figure 2 and are discussed here.

Affinity cross flow ultrafiltration

It combines the high specificity of biospecific adsorption and the scalability of cross flow ultrafiltration. Attaching the ligand (with affinity for the target protein) creates an 'affinity escort' to a polymer that shows minimum non-specific adsorption. The membrane retains the protein–ligand complex while the contaminants pass through the membrane pores. The target protein can then be released by changing the buffer. A successful application has been the purification of the lectin Concanavalin A using heat-killed yeast cells as the affinity escorts in hollow fiber ultrafiltration membrane reactor[9]. A good review that also describes some modelling work on the technique is available[10].

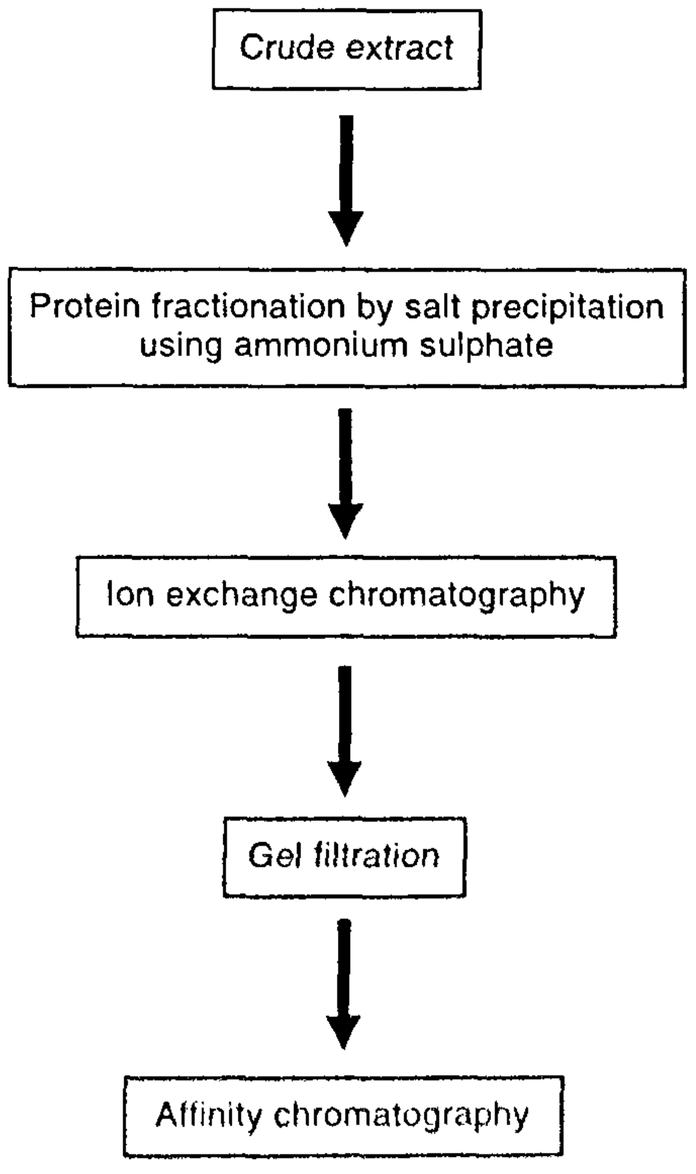

Figure 1. Flow sheet of a typical protein purification protocol.

*For correspondence. (e-mail: mngupta@chemistry.iitd.ernet.in)

Membrane affinity filtration

The overriding advantage of membranes as affinity separating matrices is their very high flow rates at low back pressure without any significant loss in performance. The faster flow rate enhances and greatly improves all the steps of the protocol, viz. washing, elution and regeneration[11,12]. Most of the membranes used are microporous and are made up of cellulose acetate, polyvinylidene fluoride, polytetrafluoroethylene, etc. The pore size distribution is mainly 3–5 μm with a 200 μm thickness. Appropriate functional groups are made through which affinity ligands available on these membranes can be conjugated. Many pre-activated membranes and affinity membranes for specific target proteins are now available. In one such application, the cytoplasmic p53 protein was the main component isolated at the maximum concentration ever reported (i.e. 5 mg ml^{-1})[13].

Continuous affinity recycle extraction

In this technique, rather than packing conventional adsorbent particles in a fixed bed (column), solid/liquid contact is carried out in well-mixed reactors[14]. Preclarification steps such as centrifugation, are thus avoided. Continuous operation is achieved by recirculation of the adsorbent particles between two or more vessels. The feed containing the crude extract is fed into the vessel containing the adsorbent. The contaminants are washed off while the target molecule-bound adsorbent is passed on to a vessel containing the eluent. After recovery of the target molecule, the regenerated adsorbent is fed back into the first vessel where it is brought in contact with fresh feed. The feasibility of this purification scheme was established with the recovery and isolation of the enzyme β-galactosidase from *E. coli*, using the affinity support PABTG (*p*-aminobenzyl 1-thio-β-D-galactopyranoside)/agarose[14].

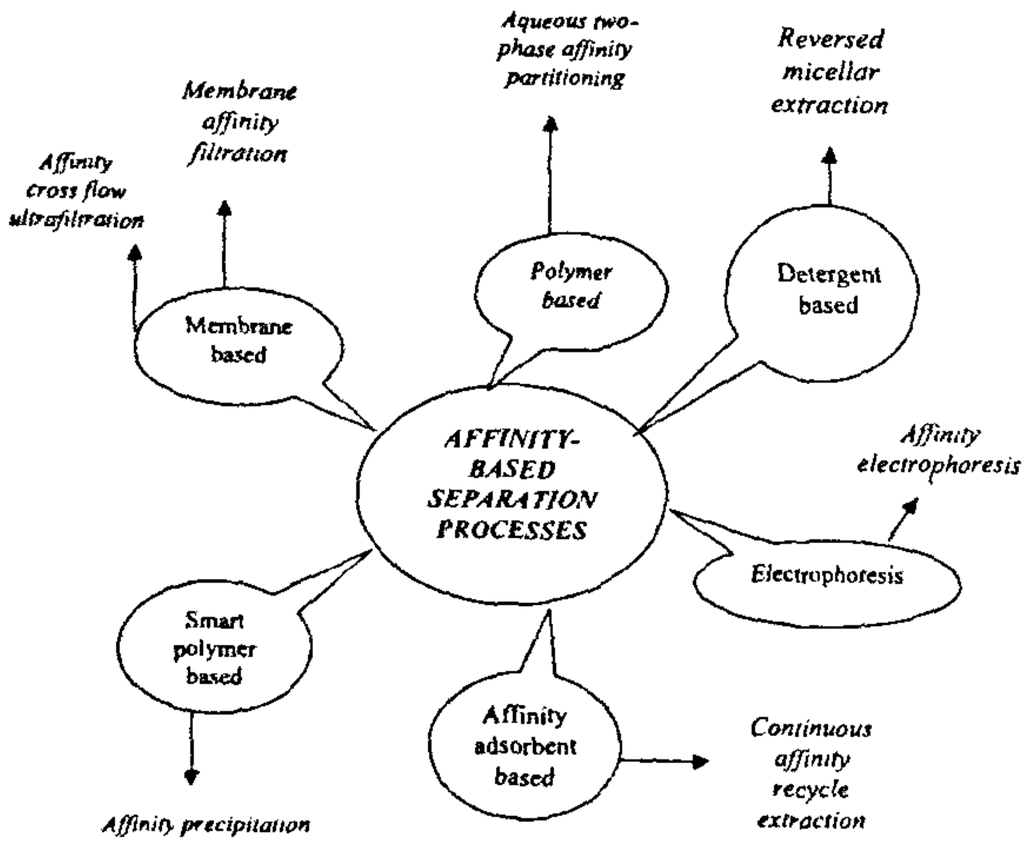

Figure 2. Some alternative affinity-based separation techniques for downstream processing of enzymes/proteins.

Aqueous two-phase affinity partitioning

Aqueous two-phase systems were first applied to the large-scale production of low molecular weight organic compounds like acetic acid. In the last twenty years however, the focus has shifted to separation of enzymes and proteins. The use of affinity ligands to phase-forming polymers offers selectivity and is known as 'affinity partitioning'. Recently, Fan and Glatz[15] have reported the partitioning of T4 lysozyme and its charge-change mutants in aqueous two phase systems (PEG-dextran) with NaCl, KCl, K_2SO_4 and potassium phosphate salts. Garg, Galaev and Mattiasson[16] have shown that addition of Cibacron Blue linked to a non-ionic detergent (Triton X-114) to the two-phase system leads to a three-phase system being formed with the target protein (lactate dehydrogenase from porcine muscle) getting concentrated in the middle layer (of detergent) from where it can be easily isolated.

Reversed micellar extraction

Reverse micelles are thermodynamically stable aggregates formed by self-aggregation of surfactant molecules in organic solvents. The partitioning of proteins between the bulk aqueous phase and the reverse micelles depends on different parameters such as ionic strength, pH of the aqueous solution, interaction potential between surfactant, protein molecules and ion, etc.[17–19]. The usefulness of this process for the large-scale recovery of proteins depends on the ease with which the protein can be 'back-extracted' into the aqueous phase. Woll and Hatton[19] were probably the first who exploited affinity interactions between the surfactant *n*-octyl-β-D-glucopyranoside and the lectin Concanavalin A. Coughlin and Baclaski[20] have extracted avidin into [*bis* (2-ethylhexyl) sodium sulphosuccinate]-*n*-laurylbiotinamide reverse micellar solution. Paradkar and Dordick[21] described a method for the separation of glycoproteins by reverse micelles; the method used lectin affinity for the glycoprotein.

Affinity precipitation

This technique exploits the affinity interactions in free solution by combining a macroaffinity ligand with a target protein. Steps involved in an affinity precipitation protocol are given in Figure 3. The key element involved here is a reversibly soluble–insoluble polymer. Such polymers are also sometimes known as 'smart polymers'[22,23]. These polymers have found a variety of applications in different areas (Table 1) and can exist both in solution and suspen-

sion (precipitate) form depending on their environment. Changing a simple parameter like pH, temperature, ionic strength, etc. or addition of a simple metal ion may bring down such a polymer from its solution to a fairly compact precipitate form. In affinity precipitation, an affinity ligand is linked to a reversibly soluble–insoluble polymer. Some of the polymers used in affinity precipitation are listed in Table 2. A large number of applications of affinity precipitation are described in the literature[7,24–31].

Affinity electrophoresis

The term 'affinity electrophoresis' was first proposed by Bog-Hansen[32]. When two substances have the same charge but different mobilities, the faster moving substance will overrun and migrate ahead of the slower one. When two substances having opposite charges are applied at different positions and electrophoresed, they will cross each other during electrophoresis. In both the cases, the electrophoretic pattern will change at the crossing or passing point, if they have an affinity for each other. The larger the affinity is, the stronger the change of the pattern. More than ten alpha-fetoprotein (AFP) glycoforms, from serum AFP samples of cord blood on full term delivery and with patients with hepatocellular carcinoma and extrahepatic malignancies including gastrointestinal tumours and yolk sac tumours, were identified using two-dimensional lectin affinity electrophoresis and also lectin affinity chromatography coupled with lectin affinity electrophoresis[33]. Like all electrophoretic methods, this method is more useful in the analytical rather than in preparative scale.

Critical factors limiting the usefulness of affinity-based separations

In most of these cases, the first step is to conjugate an affinity ligand to a matrix[34]. The latter could be a water-soluble polymer[24,30] or a membrane[12,35]. This step, inadvertently, leads to some difficulties and disadvantages related to this approach. The first one is that of the cost involved. Conjugation is costly. The cost of the affinity ligands and the generally long incubation periods involved in this step make affinity-based processes expensive. The comparative prices of the components of haemoglobin–agarose are given in Table 3. This may be an oversimplified calculation but is meant to be illustrative example giving some idea of what makes affinity chromatography costly.

The second aspect is that no matter what conjugation chemistry is involved, the covalently linked affinity ligand slowly leaches off the matrix[36–39]. Some methods provide more stable linkages. In some cases, the stability of the linkage is dependent upon the matrix being used. For example, in the frequently used cyanogen bromide (CNBr) method[34], a small but constant leakage of the coupled ligand occurs because of the instability of the isourea bond formed between the activated support and an amine-containing ligand. The matrix may also influence the type of bonds formed. The hydroxyl groups of agarose form primarily cyanate esters whereas with cross-linked dextrans and celluloses, cyclic imidocarbonates are the predominant species at high pH. The issue of chemicals/

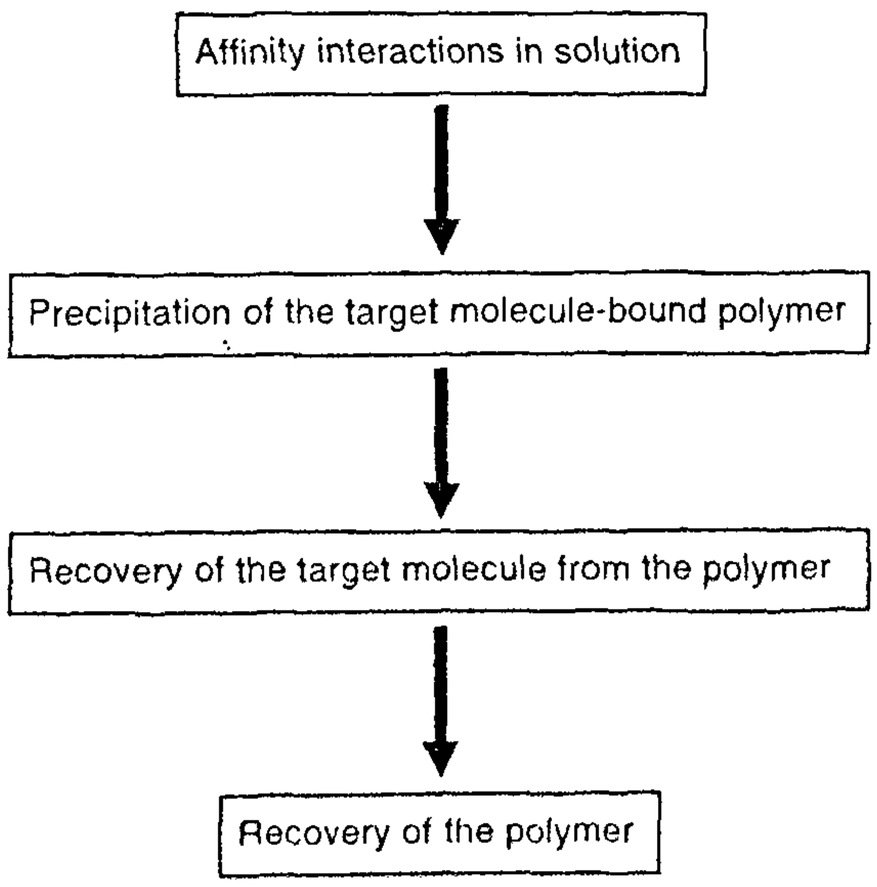

Figure 3. Steps followed in a typical affinity precipitation protocol.

Table 1. Some major applications of reversibly soluble–insoluble polymers in different areas

Area	References
Bioseparation/downstream processing	24–29
Immobilization/biocatalysis	60–66
Diagnostics	67–69
Biosensors (transducers)	70

Table 2. Some of the polymers used in affinity precipitation

Polymer	Enzyme/protein purified	References
Eudragit S-100	Xylanase	49
	IgG-type monoclonal antibody	71
Alginate	Pectinase	44, 45
	α-amylase	28, 50, 51
	Phospholipase D	29
Chitosan	Wheat germ lectin	43
	Lysozyme	72
Copolymer of 1-vinylimidazole and *N*-vinylcaprolactum	Kuntz soybean trypsin inhibitor	73
Cu (II) loaded onto a copolymer of 1-vinylimidazole and *N*-isopropylacylamide	α-amylase inhibitor (from wheat meal)	74
Poly (*N*-isopropyl acrylamide)	Alkaline protease	27
Dextran	Lactate dehydrogenase	75
Galactomannan	Protein A	76

Table 3. Cost of cross-linking (prices are as per Sigma catalogue *for biochemicals and reagents, 1999*)

Description	Amount	Price (US $)
Bovine haemoglobin	1 g	16.30
6% cross-linked agarose (1.5% gel)	0.75 g (for 50 ml of agarose beads)	6.53
Haemoglobin–agarose (ligand concentration: 10–20 mg ml^{-1})	50 ml	241.30
Cost of cross-linking		218.50

affinity ligand leaching off the column is not so critical when affinity chromatography is used in a small scale and the affinity media is used for a couple of times. Also, in most of the cases, traces of affinity ligands present do not affect the results while characterizing the purified enzyme/protein. However, currently a number of pharmaceutically important proteins are being produced by conventional as well as recombinant methods[40–42]. In such cases, an affinity-based separation is generally preferred for its high resolution and the mere suspicion of the possibility of the presence of even traces of chemicals off the column is enough for regulatory agencies like FDA to disapprove the product. Wherever enzymes are used in other health-related products, cosmetic industry and in food processing, similar considerations apply. Thus, stringent and expensive quality control methods have to be applied by industries.

Matrixless affinity-based separations

A solution may be to look for a design such that the conjugation step is abolished while using affinity-based separations. The concept of 'matrixless affinity'-based separation has evolved out of the strategy of affinity precipitation. If one looks at the examples given in Table 2, there are a few cases where the 'macroaffinity ligand' is simply the polymer itself. Thus no conjugation step is involved.

Perhaps the first case of this kind was the use of the polymer chitosan for obtaining gram quantities of wheat germ lectin[43]. Chitosan is the partially decaetylated form of chitin. The latter is poly (*N*-acetylglucosamine) and is the major constituent of the exoskeleton of arthropods. The binding of wheat germ lectin (with its known specificity for *N*-acetylglucosamine) to chitosan is not surprising. The surprising part was the elegant way in which the separation could be carried out. The lectin-bound chitosan was precipitated by a simple shift in the pH from 5.5 to 8.5. The precipitate was dissolved by lowering the pH again to 5.5. A simple gel filtration separated the polymer (which could be recycled) and the target protein.

Over the years, similar examples have been encountered. The affinity of alginate towards pectinase is now well-documented[44,45]. Pectinase is widely used in food processing for cloud stability of fruit and vegetable nectars and the clarification of fruit juices and wines[46]. In this case, it is debatable whether we are dealing with a true affinity interaction or a result of some unintentional combinatorial chemistry carried out by nature. In our laboratory, we have recently observed that alginate is also fairly selective towards yet another and totally unrelated enzyme called phospholipase D (from various plant sources)[29]. The enzymology of phospholipase D has lately attracted considerable attention because of the growing awareness about its biochemical and physiological importance[47,48]. Similar has been the case of a synthetic enteric polymer Eudragit S-100. The polymer showed considerable selectivity in picking xylanase out of the impure preparations[49].

An exciting example is that of alginate with high mannuronic acid content binding very selectively to α-amylase from wheat germ[50]. In this case, alginate beads have also been used in an expanded bed format for obtaining homogeneous α-amylase by a single-step protocol. In this case, we are dealing with 'true affinity' since the enzyme can only be dissociated from the polymer by elution with maltose[50,51] which is a competitive inhibitor of the enzyme[52]. In another case, expanded beds of cellulose or chitosan beads could be successfully used for the purification of cellulases[50,53].

Conclusion

There is a lot which is happening in the design of affinity ligands. Designer dyes[54,55], fusion proteins[56,57] and peptide ligands generated by combinatorial chemistry[58,59] are some such developments. It is hoped that judicious use of some of the approaches described here would ultimately lower the production costs of some key proteins/enzymes. This, ultimately, will lower the cost of many biotechnological processes.

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