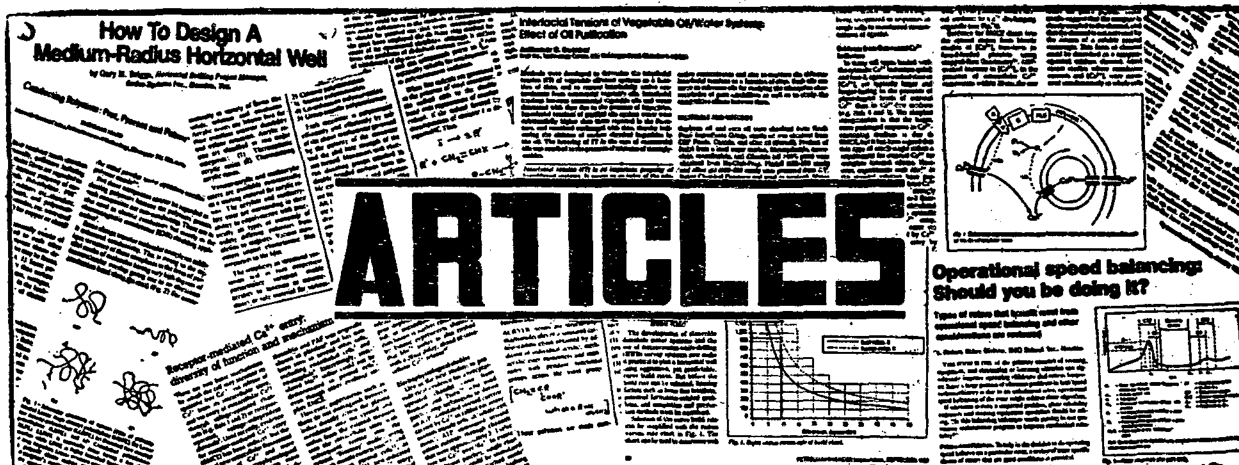

MACROAFFINITY LIGANDS IN DOWNSTREAM PROCESSING OF PROTEINS

M. N. Gupta

Different applications of enzymes/proteins demand different purity levels. Therapeutic applications require highest purity; food processing applications, on the other hand, do not generally require a very high level of purity. However, no matter what, for all applications, some purification is required before the protein or enzyme can be used. It is now well-established that the cost of this purification constitutes a major portion of the overall production cost and works out to be about 50-80% of the overall cost (Spalding, 1991).

Lately, affinity-based separation has become increasingly important in this context. The oldest and most popular kind of affinity method is affinity chromatography. In this approach, we take the affinity matrix, bind the crude extract to it and elute the desired enzyme/protein. The selectivity of the process can operate either at the binding stage (this is generally the case) or the elution stage. In lucky situations, the selectivity can operate at both the stages.

Let us backtrack a little and consider what is an affinity material. In conventional affinity chromatography, the affinity media consists of an affinity ligand covalently conjugated to an insoluble polymer. Some of the common affinity ligands are listed in Table 1.

Table 1: Classification of affinity ligands on the basis of their target enzymes/proteins

Ligand	Specificity
Triazine dyes	Nucleotide-binding proteins, kinases, Dehydrogenases
Lectins	Carbohydrates
Benzamidine	Serine proteases
Protein A	Fc antibody
Protein G	Antibodies
Chelated metal Ions	Histidine-containing residues
Histones	DNA
NAD(P)	Dehydrogenases
Poly (U)*	Poly (A)*
Poly (A)*	Poly (U)*
Lysine	Plasminogen, rRNA, dsDNA
Arginine	Prothrombin, Fibronectin
Gelatin	Fibronectin
2', 5'-ADP	NADP $^+$
Heparin	Lipoproteins, RNA, DNA
Boronate	tRNA, Plasminogen, cis-Diols
Calmodulin	Kinases
Polymyxin	Endotoxins

* A - Adenine
* U - Uridine

Figure 1 shows the conceptual transition in the design of affinity ligands. In a way, this figure, at another level also shows the nature of the biological

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affinity. Today, it is clear that an affinity material may function very well but may not have any *in vivo* relationship with the target protein. We crossed the rubicon when we started using textile dyes and metal ions. Now, the combinatorial strategies have further demystified the concept of biological affinity.

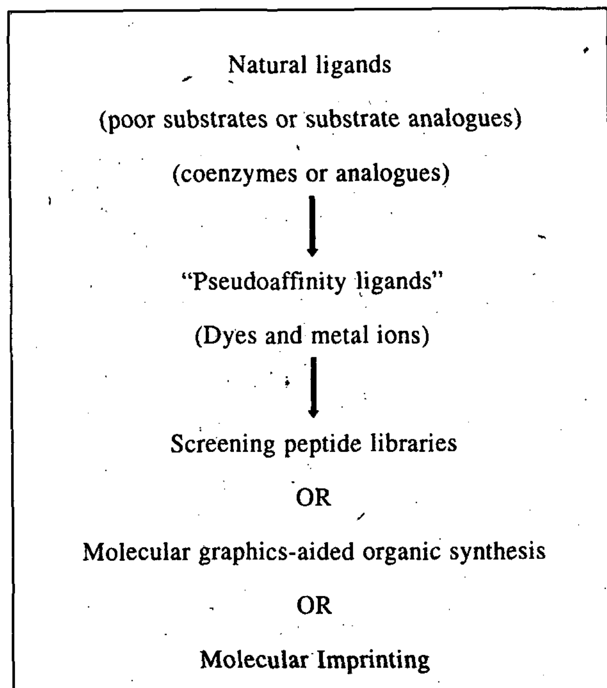

Fig. 1 : Transition in our conceptual understanding of biological affinity

It is instructive to record that nature was using combinatorial approach millions of years ago while *deciding the composition of venom of cone snails* (Olivera et al., 1995).

Coming back to the media used in affinity chromatography, it is not quite appreciated that often, some attempts failed because some additional binding forces were needed to make affinity ligand - target protein interaction adequate enough in strength. For example, yeast hexokinase does not bind to agarose C_6-acylglucosamine. It is however, retarded on a column of agarose C_8 acylglucosamine (Narayanan and Crane, 1990). Thus, non-specific interactions of the target protein with the matrix may be critically required for the successful operation of the affinity-based separation. Hence even in the context of conventional affinity chromatography, it is wise to view the affinity media as a macroaffinity ligand.

Coming to another aspect of the design of affinity media, it is very difficult to single out any method as the best one for coupling the affinity ligand to the matrix. There is a complex 'trade off' between the stability of the linkage and the non-specific binding which the coupling method brings in to the affinity matrix (Table 1).

In fact, at this point, it may be good to list the three major factors which are rate-limiting in the use of affinity chromatography at the industrial level :

- Affinity ligands, especially the biospecific ones, are costly.
- Coupling steps, generally requiring long reaction times, push the cost further upwards.
- The linkage between the affinity ligand and the matrix is seldom 'leak-proof'.

Table 2 : Summary of common methods for activation of matrices

Method	Length of spacer introduced (atoms)	Alkali lability[a]	Protonated nitrogen[a]
Cyanogen bromide	1	Yes	Yes
Epichlorohydrin	3	No	Yes
Bisoxirane	11	No	Yes
Divinyl sulphone	5	Yes	Yes
Carbonyl-diimidazole	1	Yes	No
N-hydroxy-succinimide	8	Yes	No
Tosyl/tresyl chloride	0	No	Yes

a Lability and protonation of a coupled amine - ligand

The affinity ligand leaches off with time and repeated use. The lability of the bond(s) between the ligand and the matrix is not the only reason. During a covalent coupling method, mere non-covalent force may in fact bind a significant amount of the ligand and this may slowly leach off the matrix. Williams and Blanch (1994) evaluated five methods of protein immobilization to silica while developing ENFET (enzymatic field effect transistor) sensor. It was found that about 75% of the protein in the "covalently" immobilized sample was simply adsorbed. Our own work (Table 2) although with a soluble polymer Eudragit S-100, shows that poor recovery of trypsin activity from Eudragit-PABA or Eudragit-STI, may be because of significant level of non-specific binding of the enxyme to Eudragit.

Table 2 Precipitation and recovery of trypsin from crude extract using Eudragit S-100.

Method	Precipitation		Recovery	
	Protein %	Activity %	Protein %	Activity %
Eudragit	94	100	53	65
EDC-activated Eudragit	67	71	43	54
Eudragit-PABA	64	91	41	60
Eudragit-STI	64	94	45	82

This kind of leaching affects the process in two ways. Firstly, recycling and reuse of the media is affected. Secondly, even a trace of the affinity ligand, etc. at ppm level in the product protein may preclude the process from being used when the end application is in the health sector or cosmetics.

There may not be any perfect solution to this conundrum. Let us outline some possibilities which we have explored and which we think are worth further exploration. This, in a way, is the lesson from cone snails (Olivera et al., 1995)!

Figure 2 shows that there are various ways of carrying out affinity-based separations.

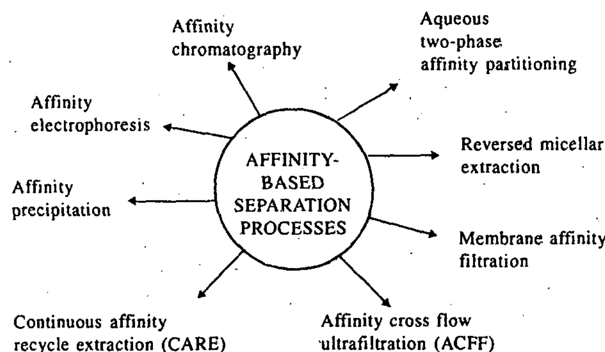

Fig. 2 Affinity-based separation processes

1. Affinity precipitation

The first one is the method of affinity-based separation (Gupta and Mattiasson, 1994) (Figure 3)

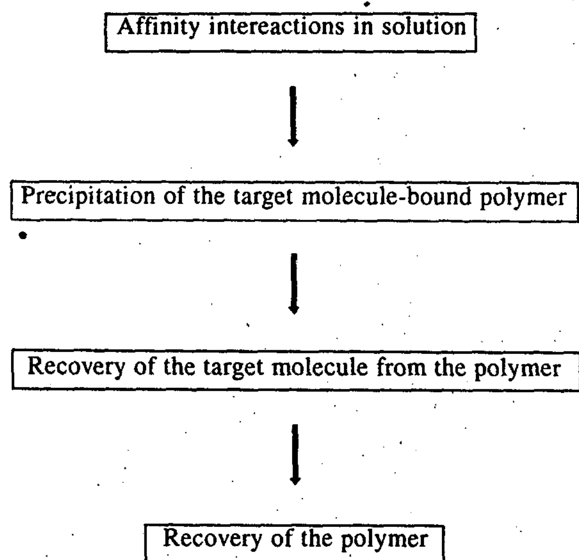

Fig. 3 Steps followed in an affinity precipitation protocol

The heart of the technique is a reversibly soluble-insoluble polymer. One may link an affinity ligand to this polymer to create a macroaffinity ligand. The rest of the steps (Fig. 3) are more or less similar to affinity chromatography. There are several advantages (and disadvantages) of this technique vis-a-vis other affinity-based methods (Gupta and Mattiasson, 1994).

Several years ago, Senstad and Mattiasson (1998) published purification of the wheat germ ligand by using chitosan as the macroaffinity ligand. Lw pH was used to dissociate the lectin-chitosan complex. More recently, we have purified lectins from rice, tomato and potato (Tyagi et al., 1996). All these four lectins are specific for N-acetylglucosamine. Thus, it is not very surprising that chitosan showed affinity towards these lectins. In fact, lysozyme could also be purified but the "elution" had to be done with 2.5 M $MgCl_2$.

A few years ago, we found that the water-soluble polymer Eudragit S-100 bound to xylanase from *Trichoderma viride* (Gupta et al., 1994). The enzyme could be purified to a level where it showed a single band on SDS-PAGE. Now, structurally speaking, Eudragit does not resemble the substrate of xylanase. We just thought it was one of those lucky happenings.

Around the same time, we also purified pectinase using alginate as the macroaffinity ligand (Gupta et al., 1993). The only similarity is that pectin, the substrate of pectinase and alginate, are both polysaccharides!

Recently, we found that α-amylases from various sources bind to alginate (Sharma et al., 2000a). The α-amylases from whole wheat and wheat germ, in fact, could be purified with this simple approach. The fold purification was 68 for wheat germ enzyme and 54 for the enzyme from whole wheat. The purified preparations showed single bands on SDS-PAGE.

We also found that peanut phospholipase D bound rather selectively to alginate (Sharma et al., 200b). The crude extract processed by affinity precipitation with alginate could be purified 34-fold and the purified preparation showed a single band on SDS-PAGE. Phospholipase D is a rather interesting enzyme in phospholipid metabolism and availability of this simple purification protocol is not unwelcome.

2. Sequential precipitation

Sometime using polymers in sequence is required for achieving the desired purification. A commercial preparation of cellulase was used for purification of β-glucosidase activity. Precipitation with chitosan left the enzyme purified 3.8-fold in the supernatant. Cellulase activity was removed as it bound to chitosan. The enzyme could be further purified by precipitating with Eudragit S-100.

3. Alternative modes of precipitation of Eudragit S-100

One can even vary the 'affinity' of the polymer towards a protein by changing the mode of precipitation of the polymer. Thus, Eudragit S-100, which normally precipitates on lowering the pH, can also be precipitated by addition of an organic solvent like acetonitrile. This gives quite different data on the binding of enzymes to Eudragit S-100. Thus we have yet another handle to modulate the 'affinity' in such cases.

4. Expanded bed chromatography

Stable fluidized beds or expanded beds, as they are called, can also be operated in the affinity mode.

Calcium-alginate beads, used in the batch mode, showed that a-amylase could be selectively picked up from crude extracts (Sardar et al., 1998).

Thus, calcium-alginate beads, operating in the fluidized bed mode, could purify α-amylases from bacterial, mammalian and plant sources. Some α-amylases bind to alginate with high mannuronic acid content but few prefer alginate beads containing higher guluronic acid content (Roy et al., 2000).

Yet another system was the purification of cellulase on chitosan beads (Roy et al., 1999).

A more predictable result was the purification of cellulase on cellulose beads in the expanded bed affinity mode (Roy et al., 2000)

5. Magnetic supports

One can also, of course, use magnetic supports for affinity-based separations. Our results with alginate-magnetite beads show that purification of α-amylases from various sources can also be carried out in this mode as well and again exploits the 'affinity' of alginate towards α-amylases (Teotia and Gupta, 2000).

Conclusion

Materials may exhibit unusual affinity for usual enzymes. We have only tried chitosan, alginate and Eudragit. Two of these are naturally occurring polysaccharides and one is a synthetic methacrylate. Thus, combinatorial chemistry happens both by nature as well as in an organic synthesis Laboratory.

It may not mean that one will always be able to get a macroaffinity ligand for every protein or enzyme. What is certain is that there are numerous materials, may be other polysaccharides, may be other commercially available polymers, which may be ideal macroffinity ligands for your target enzymes/proteins.

Acknowledgments

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Abbreviations used

PABA	P-Aminobenzamidine
STI	Soybean trypsin inhibitor
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

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