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Recovery of Free Enzymes From Product Liquors by Bio-Affinity Adsorption: Trypsin Binding by Immobilised Soybean Inhibitor

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Summary. Soybean trypsin inhibitor immobilised to sub-micron ferrite particles functions as an affinity adsorbent for trypsin and the adsorbed enzyme may be recovered following elution in dilute acid. Trypsin can be adsorbed from casein solutions that is has digested. Equilibrium is reached in under 2 min with an effective dissociation constant as low as 10^{-7}M, allowing recoveries of more than 90% of added enzyme under realistic conditions.

These results suggest that bioaffinity adsorbents could be used to recover an enzyme that has converted a macromolecular substrate. The operation of such a process is discussed, and some interactions are described that could be used with suitably high affinity adsorbents for other enzymes.

The enzymic conversion of macromolecular and insoluble substrates is of considerable industrial interest. The hydrolysis of starch or proteins is often carried out enzymically, and investigations are in progress with several other such substrates, including nucleic acid and cellulose.

In some instances the cost of enzyme does not encourage recovery but in other instances, such as for papain, it is of major importance. Of potentially greater significance is the problem of inactivating the enzyme once used. For example, in protein modification by proteases the methods of inactivation can interfere with the desirable properties of the protein product.

Studies on a wide variety of enzymes immobilised to insoluble supports have usually revealed very low retention of activity against high molecular weight, and especially insoluble substrates (Melrose, 1971); better retention of activity against such substrates can be achieved with soluble immobilised enzymes (O'Neill et al., 1971; Wykes et al., 1971). However, the latter still present considerable separation problems, in either ultrafiltration reactors (O'Neill et al., 1971) or when using precipitation procedures (Charles et al., 1974; van Leemputten and Horisberger, 1976). While magnetic support materials are useful as an aid to separation when attacking small molecules in the presence of particles or fouling macromolecules, the retention of activity against

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macromolecular substrates remains rather low, even with very small, non-porous particles (Munro et al., 1977; Halling and Dunnill, 1978; and unpublished work). To overcome these various problems an alternative strategy can be suggested, in which a soluble enzyme is used in the reactor, and is subsequently recovered from the product stream by bio-affinity adsorption.

The production of a suitable affinity adsorbent presents quite a challenge. If particulate or fouling material remains in the reactor effluent, adsorption will probably have to be carried out in a stirred tank; this necessitates a very high affinity for near-quantitative adsorption. Furthermore, adsorption must occur in the presence of a high concentration of the reaction products, which themselves will often bind to the enzyme molecules in competition with the adsorbent. Indeed, products are commonly used to elute enzyme form the column of adsorbent in affinity chromatography. In this work an adsorbent has been developed based on the binding of trypsin by soybean inhibitor. This and similar inhibitors have been used on several occasions to bind proteases under the less demanding conditions of affinity chromatography (Wilchek and Jakoby, 1974). Desorption of the enzyme is readily achieved in dilute acid, where the dissociation constant becomes much higher (Laskowski and Sealock, 1972).

Experimental

Materials

Soybean trypsin inhibitor (crude) and N-benzoylarginine ethyl ester (BAEE) were obtained from Sigma (London) Ltd. Crystalline trypsin was obtained from BDH Ltd, Poole, Dorset, UK. The Mn-Zn ferrite used as support was generously given by N.V. Philips Research Laboratories, Eindhoven, The Netherlands.

Assays

Trypsin was assayed against BAEE by pH stat titration (Walsh and Wilcox, 1970). Up to 10% (v/v) of either dilute HCL or casein digest (which contained the enzyme) did not affect the assay. Trypsin samples in neutral media were assayed immediately to avoid autolysis, while those in acid media were found to lose little activity over several days at $4^{o}C$.

Immobilisation

Soybean trypsin inhibitor was immobilised to the ferrite powder via 3-aminopropyl-triethoxysilane and polyglutaraldehyde, as described previously (Halling and Dunnill, 1978). The immobilisation reaction was carried out with, for each g of ferrite, 50mg inhibitor in 20ml 0.1 M Na-borate pH 9.0. The mixture was stirred for 17 h at $20^{o}C$.

Casein Digests

These were prepared by preincubation 0.5% casein solutions in 50 mM Tris-Cl (pH 8.0) with free trypsin at $20^{o}C$. The incubation time was inversely proportional to the enzyme content, with 30 min at 0.1mg/ml. After this time digestion was essentially complete as measured by release of acid soluble peptides absorbing in the UV (1ml digest in 1.5ml 5% trichloracetic acid gave a supernatant A_{280} of about 0.9).

Adsorption and Desorption

All separations were made using a V-shaped magnetic block (Cat. No. 934, Eclipse, Sheffield, UK), with gentle shaking or rotation of the flat-bottomed sample tube to give maximum compaction of the sediment at the side nearest the angle of the V. Adsorption and desorption were carried out by adding an appropriate medium to a drained sample of magnetic adsorbent, and stirring vigorously at 20°C. The inclusion of buffer washes (50 mM Tris-Cl pH 8.0) between adsorption and desorption had little effect. After desorption in acid the sediment was washed in buffer till the supernatant pH was above 7, and then stored at 20°C.

Medium Content of Magnetic Sediment

This was estimated by pre-equilibriating the adsorbent in 50 mM Tris-Cl (pH 8.0) or 0.5 M potassium phosphate (pH 6.5) and then carrying out a series of washes in H_2O. The Tris or phosphate content of each wash supernatant was determined by automatic pH titration. The volume of entrapped medium can be calculated from the ratios of these contents in successive washes, together with the volumes of the supernatants.

Results

The immobilised soybean trypsin inhibitor preparation was found to adsorb trypsin when stirred in buffers containing the enzyme. The adsorption could be followed either as a decline in enzyme activity in the supernatant, or as the activity released by subsequently washing the magnetic adsorbent in dilute HCl.

Complete desorption could be achieved by stirring in a large volume (20ml per g of adsorbent) of 10 mM HCl. Release of the adsorbed enzyme reached a maximum in less than 1 min; routinely 2 min stirring was used for desorption. Subsequent washes in HCl released only very small additional activities.

Two factors caused incomplete recovery of adsorbed activity into smaller volumes of HCl. Firstly, 10 mM HCl was unable to lower the pH to a value sufficient for complete desorption, due to the buffering power of the adsorbent and medium entrapped in it. By varying the volume and concentration of HCl added, the critical pH value was found to be approximately 3.0. Elution was therefore routinely carried out by stirring in 1ml 100 mM HCl per g of adsorbent (which brought the pH to just above 3), followed by further washes in 10 mM HCl. Secondly, even when desorption was complete, not all the activity could be recovered in the supernatant after magnetic separation, because of the substantial volume of medium entrapped in the adsorbent sludge. The measured volume (4.3ml in 3.7 g of magnetic adsorbent) was sufficient to account for the activity recovered in a series of washes in 10 mM HCl.

The general features of the adsorption of trypsin by the immobilised trypsin inhibitor are illustrated in Table 1. The ferrite-inhibitor was able to adsorb trypsin from a casein solution digested with the enzyme. The uptake under these conditions is impaired relative to that from a buffer solution, but not as much as that form a buffer containing Ca^{2+}. These ions are often included to suppress the autolysis of trypsin, and are known to change the conformation of the molecule (Lazdunski and Delaage, 1965). Table 1 shows a large loss of total activity with concentrated trypsin solutions in Ca^{2+}-free buffer, and a less severe but still important loss in casein digests. An

Table 1. *General features of trypsin adsorption by immobilised trypsin inhibitor.* Media containing trypsin were stirred with magnetic adsorbent for 30 min. After magnetic separation the supernatant activity was assayed, as was that recovered from the adsorbent in dilute HCl. Buffers were 50 mM $CaCl_2$, 100 mM KCl, 10 mM Tris-Cl pH 8 and 50 mM Tris-Cl pH 8

Medium	Adsorbent added (g/ml)	Trypsin added (U/ml)	Trypsin left in supernatant (U/ml)	Trypsin recovered in HCl washes (U/ml of original medium)
Buffer (+ Ca^{2+})	0.06	2.76	2.18	0.55
Buffer (no Ca^{2+})	0.06	2.72	0.22	1.21
Buffer (no Ca^{2+})	0.93	30.0	0.00	10.0
Casein digest	0.06	2.72	0.88	1.69
Casein digest	0.19	3.16	0.22	2.91
Casein digest	0.93	30.0	0.48	17.5

equivalent decline in activity occurred in solutions not treated with magnetic adsorbent, showing that trypsin autolysis was responsible. Finally, the bottom two lines of Table 1 show that under conditions of high adsorbent content, a high recovery of free trypsin form a casein digest can be achieved. In row 5, of 3.16 U/ml of trypsin added, 2.91 U/ml (92%) is recovered by adsorption and elution. In row 6, a substantial amount of activity is lost by autolysis. Allowing for this by just examining columns 4 and 5, (17.5 + 0.48) U/ml are not lost by auotlysis and 17.5 U/ml (97%) is recovered after adsorption.

When immobilised trypsin inhibitor was added during the digestion of a casein solution by trypsin, adsorption and inhibition were reflected in a rapid decline in the rate of hydrolysis. In a similar experiment, using either free or immobilised trypsin inhibitor, the inhibition of BAEE hydrolysis was much slower, the rate declining slowly over 30 min.

Measurements of A_{280} of supernatants and washes showed that non-specific adsorption of peptides from the casein digests was not a serious problem. The small amount of material adsorbed and released in HCl was mainly incompletely digested, as it was insoluble in 3% trichloracetic acid.

Figure 1 shows the kinetics of trypsin uptake from a casein digest under conditions of low enzyme and adsorbent content, when the rate is presumably slowest. Adsorption is essentially complete in 2 min. There follows a slow decline in both the supernatant and adsorbed activities, and hence in the total trypsin activity recovered. This loss is unlikely to be due to autolysis, as the enzyme activity in an untreated casein digest with 2.5U trypsin/ml did not fall measureably over 120 min incubation. The decline in total activity may indicate release of soluble trypsin inhibitor from the ferrite support at a rate of around 0.25U (trypsin equivalent)/h. This amount does not appear as part of the free enzyme activity in figure 1 since it will be present as inactive trypsin - trypsin inhibitor complexes.

Figure 2 shows how the level of trypsin adsorption reached (after 10 min) is affected by enzyme and adsorbent concentrations. The amount of trypsin bound per unit weight of adsorbent depends principally on the free enzyme concentration left in the

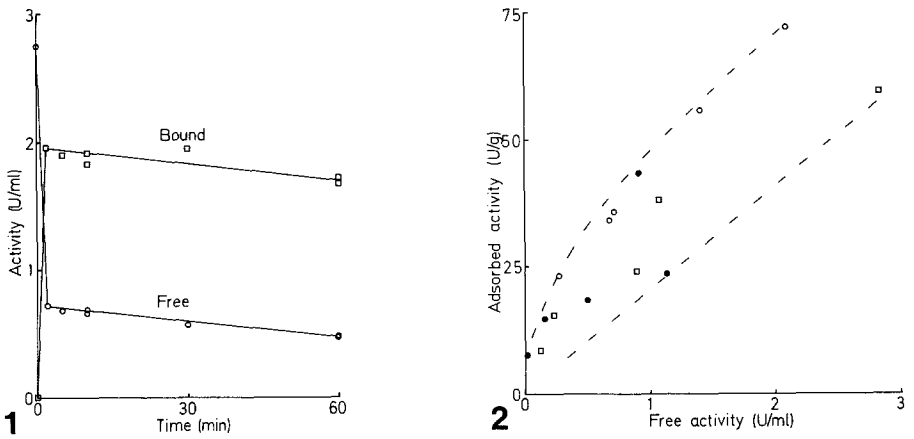

Fig. 1. *Kinetics of trypsin adsorption from a casein digest by immobilised soybean inhibitor*, 4 ml casein digest was added to 215 mg magnetic adsorbent and stirred for the time stated. After magnetic separation the supernatant activity was assayed (○), then the adsorbed activity was eluted with dilute HCl and assayed (□ ; expressed as U/ml of orginal digest). The decline in adsorbed activity does not reflect a loss of adsorbent capacity, as the incubations for different times were done in a random order

Fig. 2. *Equilibrium of trypsin adsorption from a casein digest.* Magnetic adsorbent was stirred for 10 min in casein digests of varying trypsin content. Activities left in the supernatant and bound to the absorbent were assayed in each experiment. Three sets of conditions were used: – 4 ml digest with 215 mg adsorbent (○); 20 ml digest with 3.7 g adsorbent (□); and 4 ml digest with 3.7 g adsorbent (●)

supernatant, as expected for true equilibrium adsorption. The amount bound seems to be slightly less at high adsorbent concentration, though the increased scatter in these results makes it difficult to be certain. But clearly, the effect of a 15-fold change in adsorbent concentration on the apparent equilibrium is small. Another preparation of immobilised trypsin inhibitor showed similar behaviour but only about half the trypsin capacity under any particular conditions.

The data from Figure 2 for low adsorbent concentrations, together with additional results obtained at higher free enzyme concentrations, were replotted on a Scatchard (1949) plot (Fig. 3). This is strongly curved, indicating a small content of adsorbent sites of very high affinity, with an increasing number of progressively lower affinities.

When magnetic adsorbent with trypsin bound was washed thoroughly and then stirred in buffer, a little activity was released over a few minutes. The amount of activity released was close to that remaining in the supernatant at a similar bound trypsin content when the equilibrium was approached from the other direction.

Elution of trypsin from the adsorbent could be achieved using $NaHSO_4$ in place of HCl. This might be advantageous, since if an excess was inadvertently added, the comperatively weak dissociation of the HSO_4 ion (pK 1.9) would prevent generation of a very low pH value and possible inactivation of the enzyme or the adsorbent.

An attempt was made to recover most of the enzyme in the medium entrapped in the adsorbent sludge by dilution with the next batch of substrate solution. Enzyme

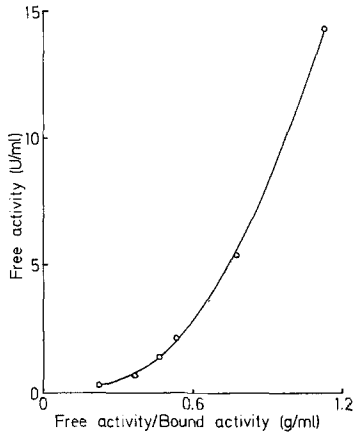

Fig. 3. *Scatchard plot for trypsin adsorption from a casein digest.* Data obtained as for Fig. 2 with 4 ml digest and 215 mg adsorbent

was eluted from 3.7 g adsorbent with 4ml 100 mM HCl, then 16ml 0.5% casein in buffer was added to the stirred suspension, followed by immediate magnetic separation. Although the addition of casein solution brought the medium pH to around 6, it was hoped that significant enzyme re-adsoption would not occur in the short time for which the adsorbent remained suspended (less than 15s). However, the supernatant contained only 39% of the trypsin activity recovered in the same total volume of HCl, showing that re-adsorption was in fact very rapid.

As it is not practically possible to saturate the adsorbent and directly measure a maximum capacity, quantitative results cannot be presented for the storage or operational stability of the immobilised trypsin inhibitor. One preparation was taken through a total of 27 cycles of adsorption and elution, distributed over 3 weeks, with no noticeable fall in capacity. The early washes in acid on a new preparation produced supernatants with a pale yellow colour, and a small adsorption peak at 400 nm. The identity and source of the material responsible is unknown, but it did not appear essential to the functioning of the adsorbent.

Discussion

The Trypsin Adsorbent

Three distinct factors tend to oppose enzyme adsorption under the conditions of these experiments, as compared with conventional affinity chromatography. Firstly, adsorption is carried out in a well mixed tank, so that adsorption behaviour is very much worse than in the near plug-flow conditions of an affinity column, where a continuous cascade separation operates. Secondly, substrates, products or other materials in the medium will bind to the enzyme (and possibly the adsorbent as well), shifting the equilibrium towards desorption. Thirdly, the binding of these substances to the enzyme may cause an unacceptable decrease in the rate of adsorption. Only free enzyme molecules (unbound to adsorbent *or* ligand) can actually bind to the adsorbent.

In view of these considerations, the fact that substantial enzyme adsorption occurs form a casein digest is a reflection of the high affinity of trypsin for the soybean inhib-

itor ($K_i < 10^{-9}$M; (Laskowski and Sealock, 1972). For quantitative interpretation of the results, weights and hence moles of trypsin must be obtained from the measured activities. The trypsin sample used had a specific activity of 30.8 U/mg, but like all commercial preparations it presumably contained a substantial portion of inactive material. Walsh and Wilcox (1970) report maximal activities of 70 U/mg for highly purified enzyme and this figure has been used for calculations, since the adsorbent should interact exclusively or at least preferentially with native, active enzyme molecules.

From the intercepts of tangents to the curve of Figure 3, there appear to be a few adsorbent sites with effective dissociation constants less than 10^{-7}M, while others have values ranging up to 10^{-5}M. Probably these values indicate a genuine spread of affinities due to inhibitor molecules with varying degrees of accessibility and modification as a result of immobilisation. From the tangent gradients in Figure 3, adsorbent capacities of up to 450U of trypsin/g may be calculated (the maximal observed value is 236U/g. The crude trypsin inhibitor preparation used had a quoted inhibitory power equivalent to 39U of trypsin/mg. Hence we can calculate a figure of 11.5mg of inhibitor per g of support, which compares well with estimates of protein immobilisation capacity for this same ferrite support and the similarly sized protein chymotrypsin (Halling and Dunnill, 1978). This suggests that few inhibitor molecules are totally inaccessible to trypsin, and that the major effect of immobilisation is just a decrease in trypsin affinity.

As demonstrated in Figure 1, there are no problems with the rate of trypsin adsorption from a casein digest. In contrast, the slow inhibition of BAEE hydrolysis shows that the kinetics of adsorption can be a limitation. From the rate of possible inhibitor desorption derived from Figure 1, of approximately 1U(trypsin equivalent)/h/g adsorbent, estimated time for 50% desorption is about 310h. This is similar to that observed for desorption of chymotrypsin immobilised to ferrite powder by the same method as used for trypsin inhibitor (Halling and Dunnill, 1978). Because of the rapid rate of trypsin adsorption and desorption, this time could accommodate many thousands of cycles, if no other inactivation process is occurring.

Loss of trypsin activity by autolysis, as observed at high concentrations, could be an important problem in cyclic re-use of trypsin and other proteases. If high enzme concentrations are used it might be worthwhile slowing autolysis by Ca^{2+} addition, by enzyme modification (Rice et al., 1977) or immobilisation to a soluble support (O'Neill et al., 1971).

In addition to enzyme recovery, the ferrite immobilised trypsin inhibitor could prove useful for the isolation of trypsin and related proteases from crude cell extracts. The advantages of magnetic supports in this respect have been discussed and demonstrated (Dunnill and Lilly, 1974). Furthermore, it could be used to remove unwanted protease activity as a first step in processing such extracts; this would prevent the degradation of other materials that it was desired to isolate, e.g., insulin isolation from pancreas.

Process Considerations

Figure 4 is a basic flow-chart that illustrates how affinity recovery of a soluble enzyme would be applied. A primary requirement for efficient enzyme adsorption is an adsorbent with a suitably low dissociation constant (K_d). The exact value necessary will depend on the level of recovery required, the enzyme concentration used, and the seriousness of competition from other ligands in the product liquor. It will be necessary to make a trade-off between the operational capacity of the adsorbent and the concentration of enzyme left unrecovered, as clearly demonstrated by the curves of Figure 2. As with any adsorption process, there would be a great incentive to introduce plug-flow and countercurrent behaviour, in multiple stirred tanks.

The adsorbent capacity at a suitably high affinity is another important consideration. Higher capacities can be exploited to run the reaction at higher enzyme concentrations, giving faster reaction rates and hence lower reactor volumes for a fixed conversion rate. In the adsorption step, higher concentration of both enzyme and adsorbent sites will give faster uptake rates. About the same amount of enzyme will be lost by incomplete recovery on each cycle, though it will be a smaller fraction of the total in use. Alternatively, higher capacities could permit a reduction in the amount of adsorbent used per unit volume of product liquor, lessening the problems due to carry-over of entrapped medium from both adsorption and desorption steps.

The conditions for enzyme elution are more restrictive than those commonly employed in affinity chromatography. Elution by a specific high affinity ligand will be costly; and it will be returned to the enzyme reactor, interfering with subsequent adsorption and contaminating the product. The same applies to high concentrations of salt which have been sometimes used. Fortunately a pH change will often be effective.

The extent of recovery in the enzyme desorption step is not as critical as the extent of enzyme adsorption. Enzyme not desorbed on any one cycle is not lost, but simply causes a decline in effective capacity the next time the adsorbent is used.

The entrapment of medium in the separated magnetic sludge is a major problem. In the separation following the adsorption step it will cause loss of some product, though this will eventually be returned to the enzyme reactor with the recovered enzyme. More seriously, after desorption it will prevent the recovery of the enzyme in

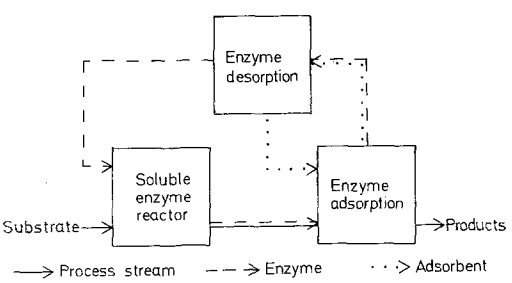

Fig. 4. *Basic flowsheet for process including affinity recovery of free enzyme catalyst.* Each of the three basic operations can involve any of several reactor types. In addition, both enzyme adsorption and desorption may involve magnetic separation as a distinct step

a small volume of concentrated solution, the most desirable form for return to the enzyme reactor. The entrapped volume may be different after use of large-scale magnetic separation equipment. There should be proportionally less medium retained with the sludge as surface droplets etc., though from the volume of the ferrite bed after compaction on the magnetic block, 0.7 - 0.8ml medium per g adsorbent must be truly entrapped. Depending on the field strength and geometry of large-scale equipment, compaction might be better or worse.

The volume needed to recover the bound activity could be decreased by moving towards a plug-flow system. Alternatively, if desorption was carried out by pH shift, operation of the enzmye reactor could be adjusted to receive the enzyme in a large volume of dilute solution. The substrate could then be added as a solid or in a small volume of liquid, and the mixture adjusted to the reaction pH.

A non-porous magnetic support has been used to investigate affinity recovery of an enzyme, because of its advantages in the presence of suspended particles or fouling liquors (Munro et al., 1977; Halling and Dunnill, 1978). In some cases it may be possible to use a conventional porous support for the adsorbent molecule. However it is doubtful if there would be any advantage in doing so. The effective maximum capacity of the magnetic adsorbent, about 5mg enzyme per ml of reactor volume, may be difficult to surpass with conventional supports because of their very low solids content per total volume. Furthermore the higher content of entrapped medium and the likely diffusional limitations on the rates of adsorption and desorption will both cause problems.

In some cases, affinity recovery might be considered as a modification of an exisiting free enzyme process. If enzyme cost is of major importance, it could be viewed as a supplement to enzyme isolation, when as little as 50% recovery might be worthwhile. Alternatively, it might replace a specific enzyme inactivation step.

More often, affinity recovery would be compared with enzyme immobilisation. If enzyme cost is the dominant factor, the low retention of activity of an immobilised enzyme against macromolecular substrates, typically around 5%, will favour affinity recovery. Twenty re-uses of the immobilised enzyme will be needed to obtain the same total conversion per unit weight of enzyme as with a single use of the free species. So a recovery of 99.5% for the immobilised enzyme will be equivalent to 90% for the free enzyme, giving the same total conversion over the period for which the enzyme is successfully retained.

Other Interaction as the Basis for Adsorbents

As explained, an effective adsorbent for affinity recovery must have a very low K_d value. Interactions betweeen free molecules with dissociation constants greater than 10^{-7}M or so are unlikely to be suitable as the basis for simple adsorbents, and often much smaller values will be desirable. Such high affinities require a number of specific non-covalent atomic interactions, and are most usually associated with molecules specifically designed to interact, particularly in the control of biological processes.

It should be noted that the kinetics of adsorption are unlikely to be a major determinant in the selection of a suitable interaction. The association rates are generally similar for most protein-ligand reactions while differences in affinity are reflected in the rates of dissociation (Weber, 1977).

Protein and peptide inhibitors for proteases have been widely searched for and studied, and could form the basis for adsorbents analogous to that for trypsin. Potentially suitable inhibitors are known for the industrially important proteases, for example, papain (Fossum and Whitaker, 1968), rennin/pepsin (Umezawa, 1976) and subtilisin (Inouye et al., 1977). Though much less work has been done on protein inhibitors specific for other types of enzyme, it seems probable that they are commonly produced to control the activity of a variety of hydrolytic and degradative enzymes that might damage components of the living cell. For example, such inhibitors have been demonstrated for nucleases (Uozumi et al., 1976) and amylases (Shainkin and Birk, 1970).

To extend the scope of affinity recovery, it would be possible to modify enzymes by attachment of a group specifically as the site for adsorption. The modification would add an extra cost, and would need to be made without destroying the enzyme activity; but would provide a general recovery method independent of the enzyme being used. For example, the egg white protein avidin might be used to recover enzymes modified by attachment of biotin (Green, 1975) or possibly iminobiotin, which would be easier to dissociate from the adsorbent at acid pH (Greeen, 1966).

Alternatively, affinity recovery might be applied to soluble immobilised enzymes, when adsorption at multiple sites would permit the use of interactions of rather higher dissociation constants. The adsorbent could be specific for the enzyme moeities, for some other immobilised ligand, or for the support itself. With the usual polysaccharide supports either plant lectins (Lis and Sharon, 1973) or bacterial binding proteins (Oxender, 1974) might prove useful.

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