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Review

Trypsin and affinity chromatography

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ABSTRACT

Affinity adsorbents for trypsin which were prepared by immobilizing product-type ligands, that is, peptides having C-terminal arginine, proved to be effective not only for preparative purposes but also for basic research on molecular recognition. The properties of the binding site of trypsin were revealed by chromatographic experiments. Quantitative analysis based on the theory of frontal affinity chromatography proved to be extremely effective. As an extension of the product-type ligands, peptide argininals were also used and information on the mechanism of action of these inhibitors was obtained. Anhydrotrypsin, which lost the hydroxyl group of Ser183, was found to gain increased binding ability for product-type compounds. This inactivated enzyme was also used as an immobilized ligand and the unique affinity adsorbent thus prepared proved to be extremely effective for the separation of peptides and recombinant proteins based on their C-terminal structures. High-performance affinity chromatography of trypsin and related enzymes using a polymer-based support was also developed.

CONTENTS

1. INTRODUCTION

One of the target proteins described in the paper published by Cuatrecasas *et al.* [1] in 1968, which is now widely recognized as a landmark in affinity chromatography, was chymotrypsin. Chymotrypsin was specifically adsorbed to an agarose derivative which had D-tryptophan methyl ester, an enantiomer of the substrate of chymotrypsin, as immobilized ligand. The idea of using substrate ana-

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logues which are resistant to enzyme action stimulated us to develop affinity adsorbents for trypsin, on which we had been carrying out extensive studies.

Although trypsin itself is only a digestive enzyme, a number of related proteolytic enzymes, *e.g.*, thrombin, plasmin, kallikrein and acrosin, are very important regulatory enzymes. Hence studies of trypsin as a model were expected to contribute greatly to the understanding of the functions of these proteins. The development of affinity adsorbents for trypsin should provide efficient purification procedures and investigative tools from the viewpoints of not only basic studies on trypsin-family proteases but also their application.

Specific ligands used for affinity adsorbents for trypsin-family enzymes are roughly classified into four categories. The first category includes trypsin inhibitors of a protein nature such as soybean trypsin inhibitor (STI), pancreatic inhibitor and ovomucoid [2–4]. The second includes inhibitors of low molecular mass such as leupeptin and chymostatin, produced mainly by *Streptomyces*. They had not been utilized at the time when we began studies of the affinity chromatography of proteases. The third category is synthetic competitive inhibitors such as benzamidine [5,6] and the last are products of trypsin action. Our first choice of product-type ligands strongly favoured the subsequent development of the affinity chromatography of trypsin-family proteases.

In general, substrates do not seem suitable as ligands for affinity adsorbents because they are converted into products immediately after contact with the target enzyme, except under very limited conditions such as at very low temperatures and extreme pH. On the other hand, products may be more advantageous because they will not be easily transformed into substrates, especially in the case of proteolytic enzymes. However, the binding ability of products will generally be inferior to that of substrates in order to ensure turnover at the active site. This may be a serious disadvantage of using product-type compounds as immobilized ligands for affinity adsorbents.

However, with trypsin, it was found that, under certain circumstances, product-type compounds interact strongly with the binding site with an affinity comparable to that of substrates [7]. They proved to be very efficient immobilized ligands. Affinity adsorbents of this type became applicable not only for preparative purposes but also for basic studies on the mechanism of biochemical recognition because the ligands were very similar to the natural substrates. Starting from this type of adsorbent, various aspects of affinity chromatography have been revealed and thus accumulated experience and knowledge have contributed greatly to the general development of this new technique. This paper reviews the development of affinity chromatography in which trypsin always had a crucial role.

2. PREPARATION AND PROPERTIES OF AP-AGAROSE (ARGININE-TERMINATED PEPTIDE-AGAROSE)

Fig. 1 shows the general structure of affinity adsorbents for trypsin which have peptides having arginine at the C-terminus (AP-agarose). A ligand having such a structure could be easily prepared from natural sources. Protamines contained in sperm of fish are mixture of extremely basic peptides. Fig. 2 shows the primary structure of one component of salmine, which is a protamine of salmon [8]. As about one third of their component amino acids are arginine, digestion with trypsin gave various small peptides terminated with arginine. They had favourable properties as ligands for affinity adsorbents for trypsin. The C-terminal arginine should interact with the binding site of trypsin. They have free amino groups available for immobilization reaction with activated agarose. Residues prior to the C-terminal arginine will play the role of a spacer. A free carboxyl group at the C-termini seemed to weaken the specific interactions with the binding site, and therefore at first it was planned to block them with an appropriate chemical group which would not be removed by trypsin action, *e.g.*, a secondary amine or proline.

Salmon sperm salmine was digested with trypsin and the fraction composed mainly of di- and tripep-

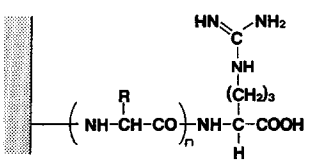

Fig. 1. General structure of AP-agarose.

Pro-(Arg)$_4$(Ser)$_3$-Arg-Pro-Val-(Arg)$_5$-Pro-Arg-Val-Ser-(Arg)$_6$-(Gly)$_2$-(Arg)$_4$

Fig. 2. Primary structure of a component of salmine.

tides was prepared. These mixed arginine-terminated peptides (AP) were immobilized to agarose gel by the cyanogen bromide method. The amount of immobilized AP could be easily determined by amino acids analysis of the resulting adsorbents. Usually, 1–5 μmol of peptides were immobilized to 1 ml of agarose gel. When a solution of commercial bovine trypsin was applied to an AP-agarose column, AP-agarose caused retardation of trypsin (Fig. 3a).

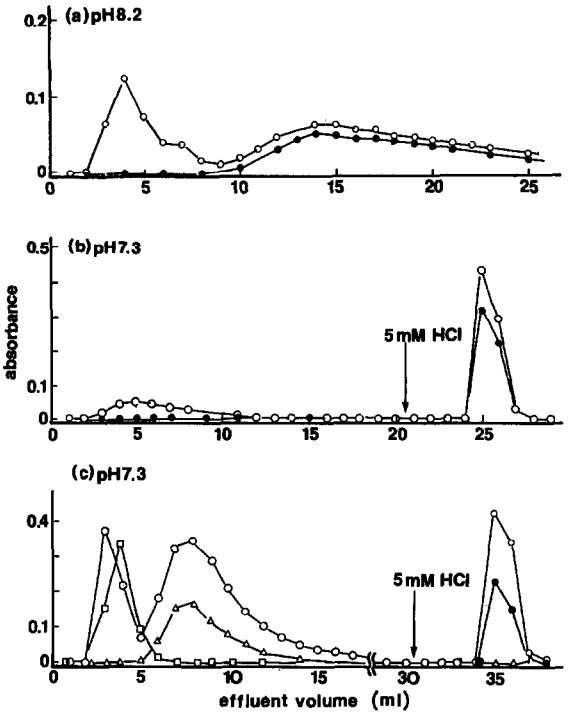

Fig. 3. Chromatography of trypsin and other enzymes on AP-agarose. The column (7 × 0.6 cm I.D.) was equilibrated and run with (a) 0.05 *M* Tris–HCl buffer (pH 8.2) containing 0.02 *M* $CaCl_2$ or (b and c) 0.02 *M* phosphate buffer (pH 7.3). Arrows indicate replacement of the buffers with 5 m*M* HCl. Flow-rate, *ca.* 10 ml/h. All experiments were carried out at 4°C. ○ = protein (A_{280}); ● = trypsin activity; △ = chymotrypsin activity; □ = ribonuclease activity. (a, b) Bovine trypsin (1 mg); (c) mixture of 0.7 mg each of bovine trypsin, chymotrypsin and ribonuclease (From ref. 9).

Material having no enzymatic activity passed through the column, but the enzyme activity coincided with the second protein peak. Thus, although the interaction was not strong enough to cause adsorption of active trypsin, this adsorbent seemed to be effective at least for the removal of enzymatically inactive material. In order to obtain stronger adsorbents, it seemed necessary to block the free carboxyl group of arginine.

However, an unexpected observation was made when the effect of pH was investigated. The chromatogram shown in Fig. 3a was obtained at the optimum pH of trypsin, *i.e.*, 8.2. When chromatography was carried out at lower pH (7.2–4.0), adsorption, instead of retardation, of active trypsin occurred (Fig. 3b). Even after washing with 20 column volumes of buffer, active trypsin did not appear. Only a more acidic eluent such as 5 m*M* HCl could elute the active trypsin. The purity of the eluted fraction determined by active-site titration was found to have increased by more than 95%. Hence AP-agarose proved to be usable as an efficient adsorbent for trypsin if suitable conditions were selected. Blocking of the free carboxyl groups turned out to be unnecessary.

Lines of evidence were obtained that indicated that AP-agarose functioned as a true affinity adsorbent, as described below [9]:

(1) Trypsinogen, an inactive precursor of trypsin, was not adsorbed.

(2) Inactivated trypsin derivatives in which either Ser183 or His46 was modified with active site-directed reagents such as diisopropyl phosphofluoridate (DFP), phenylmethanesulphonyl fluoride (PMSF) and tosyllysine chloromethyl ketone (TLCK) were not adsorbed.

(3) Adsorbed trypsin could be eluted with a competitive inhibitor such as benzamidine.

(4) Chymotrypsin was not adsorbed although it was slightly retarded in comparison with ribonuclease (Fig. 3c).

(5) Urea inhibited the adsorption of active trypsin. This suggested the importance of the native conformation, especially the integrity of the active site of trypsin.

The importance of residues other than the C-terminal arginine of AP was also demonstrated. An agarose derivative having a similar amount of arginine (Arg-agarose) did not adsorb trypsin at any

pH tested. This result suggested that residues located at the N-terminal side of the C-terminal arginine were essential as spacers in order to assist the access of the arginine residue to the active site of trypsin.

AP-agarose, which was obtainable very easily and economically, proved to be very efficient as a purification medium for not only bovine trypsin but also other trypsin-related enzymes such as *Streptomyces griseus* trypsin and plasmin [10].

The basic mechanism of such a pH dependence of adsorption, that is, a low affinity at the optimum pH of enzyme action and high affinity at pH lower than the optimum, should be explained. This is undoubtedly related to the fact that the immobilized ligands were product-type peptides. It is reasonable to assume that there is some mechanism to make the affinity of products lower than that of substrates in order to ensure the turnover of the active site.

One of the most significant differences between the substrate and the product is that the latter has a free carboxyl group which should be negatively charged at the optimum pH of trypsin (*ca.* 8). Probably a negative charge will be located in the active site very close to the newly formed free carboxyl group of the substrate. Hence once the substrate has been converted into the product, the latter should be subjected to repulsion. This putative negative charge does not seem to be a carboxyl group, because it seemed to have disappeared when the pH was lowered from 8 to 7; the pK_a of an ordinary carboxyl group is usually around 4. It is more probable that a group which had a pK_a of about 7.5 became positively charged and neutralized the negative charge responsible for the repulsion of the product (Fig. 4). In other words, it is not because the affinity for the product increased but because the factor that had made the affinity for the product

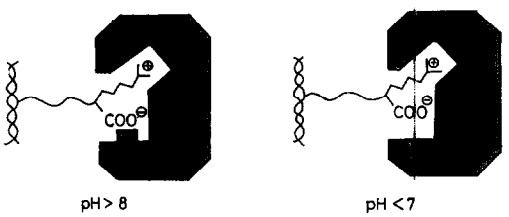

Fig. 4. Possible explanation of the increase in affinity for product-type compounds at lower pH.

low was suppressed. The active site maintains such a state between pH 7 and 4, whereas below pH 3 the conformation of the active site would be destroyed. This would result in the release of trypsin from AP-agarose. Such a pH dependence for product-type compounds was also proved by kinetic experiments in which K_i values of soluble product-type compounds were determined [9].

The experiment described above emphasized that the most suitable conditions for adsorption and desorption should be sought carefully for each affinity adsorbent. The best conditions often depend on which ligand one has chosen and they have seldom studied in detail before. Flexibility in thinking is also important because some compound which has not been recognized as a suitable ligand might become an excellent ligand if good chromatographic conditions are established. The observations described here were also instructive because they afforded some valuable information on the nature of the active site of trypsin. During the search for suitable conditions for adsorption, pH dependence of the interaction between trypsin and its product which reflects the state of ionization of the active site was revealed unintentionally. Hence affinity chromatography proved to be useful not only for preparative purposes but also for fundamental studies of biomolecules.

3. ROLES OF THE CATALYTIC RESIDUES OF TRYPSIN IN SPECIFIC BINDING

One of strong supports for AP-agarose being a true affinity adsorbent was the observation that only active trypsin had been adsorbed whereas trypsin inactivated with DFP or TLCK had not. However, is it always the case that inactivated trypsin loses affinity for AP-agarose? In other words, is catalytic activity essential for specific binding? Modification of the catalytic residues, serine (Ser183) with DFP and PMSF, or histidine (His46) with TLCK, not only resulted in destruction of the catalytic activity but also may have caused steric hindrance around the substrate binding pocket. Hence other modification procedures that destroy only the catalytic activity should be examined. The use of modifying reagents that are small enough not to cause blocking of the binding pocket was necessary.

His46 has been found to be specifically modified

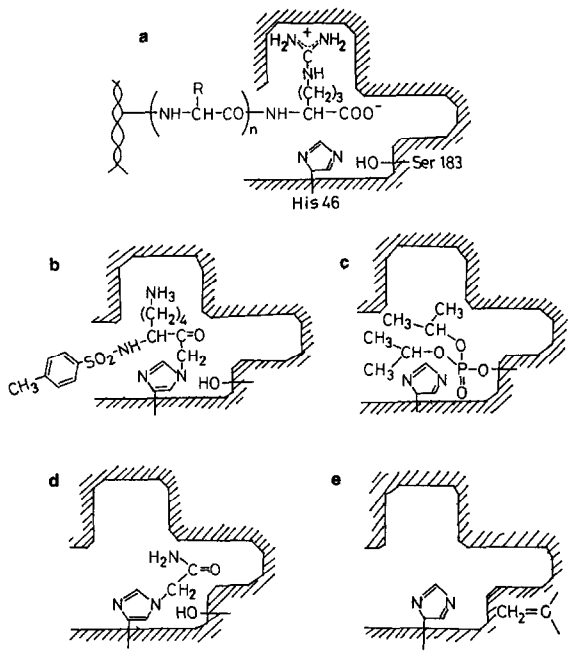

Fig. 5. State of the specific binding site of modified trypsins. (a) Intact trypsin which interacts with AP-agarose; (b) TLCK-trypsin; (c) DFP-trypsin; (d) carboxyamidomethyl-trypsin; (e) anhydrotrypsin.

with monoiodoacetamide in the presence of methylamine, and the resulting carboxyamidomethyltrypsin (CAM-trypsin) lost catalytic activity [11]. Ser183 can be converted into dehydroalanine [12]. To prepare this derivative (called anhydrotrypsin because one water molecule has been removed), trypsin was first modified with PMSF, and the resulting PMS-trypsin was treated with alkali to allow β-elimination of the PMS group. Finally, catalytically inactive anhydrotrypsin was produced. Both of these inactive trypsin derivatives were found to be adsorbed to AP-agarose [13].

These experiments clearly showed that the two sites constituting the active site of trypsin, namely the catalytic site and the binding site, function independently. It was evident that DFP, TLCK and PMSF had sterically hindered the access of the C-terminal arginine to the binding pocket (Fig. 5). Both His46 and Ser183, essential residues for catalysis, were not essential for the specific binding. This also suggests that one must be careful that purification by affinity chromatography does not necessarily guarantee that the enzyme preparation is 100% active.

However, the following points should be noted. These results do not mean that the active site of every enzyme can be divided into catalytic site and binding site, or that these two sites are always completely independent. There will also be many enzymes in which two sites are closely related. Consequently, when one of them is destroyed, the other will also lose its function. Even with trypsin, it was found that the two sites are not completely independent (see later).

4. STRUCTURE OF ARGININE PEPTIDES FAVOURABLE FOR BINDING WITH TRYPSIN

The use of product-type immobilized ligands made AP-agarose an efficient tool not only for purification but also for investigations of the mechanism of specific recognition. However, it had a drawback for basic studies because the immobilized ligands were heterogeneous in terms of both length and sequence. Affinity adsorbents having homogeneous peptide would be much more suitable for detailed studies.

From a practical point of view, the binding site of proteolytic enzymes is often considered to be composed of hypothetical subsites corresponding to amino acid residues of the substrate, as shown in Fig. 6. The S_1 site is the substrate-binding pocket where the specificity-determining residue of the substrate (P_1) interacts. With AP-agarose, the C-terminal arginine binds to this site. Residues located at the N-terminal side of the arginine (P_2, P_3, etc.) should interact with S_2, S_3, etc., respectively. The strength of binding should be influenced by the nature of these residues. Hence these residues play the role not only of a spacer but also of part of the specific ligand.

In order to elucidate the mechanism of interaction more deeply, a number of affinity adsorbents having well characterized arginine peptides were prepared [14,15]. Peptides with different lengths having arginine at C-termini were synthesized and immobilized to agarose. It was also necessary to establish a new methodology to compare quantitatively the binding strengths of these adsorbents. Hence the theory of frontal affinity chromatography was originated and applied to this system. Li-

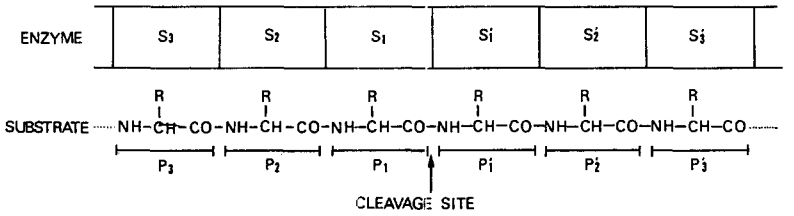

Fig. 6. Subsites of proteolytic enzyme.

gand contents of adsorbents were kept low because weak adsorbents were required for this procedure.

According to the theory of frontal affinity chromatography [16–19], if very dilute enzyme solution is applied, the dissociation constant (K_d) of the complex between the affinity adsorbent and the enzyme can be expressed by an extremely simple equation:

$$K_d = B_t/(V - V_0) \quad (1)$$

where B_t is the amount of the immobilized ligands, V is the elution volume of the front of the target enzyme and V_0 is the elution volume of a substance which does not interact with the immobilized ligand. In practice, it is important to use weak affinity adsorbents which do not cause adsorption because we have to measure the extent of retardation of the target enzyme. The affinity of bovine trypsin for these adsorbents was analysed quantitatively by frontal affinity chromatography (Table 1).

TABLE 1

K_d VALUES OF BOVINE TRYPSIN FOR VARIOUS AFFINITY ADSORBENTS HAVING ARGININE-TERMINATED PEPTIDES (pH 6.0, 4°C) (FROM REF. 15)

Immobilized ligand	K_d (mM)
P_5 P_4 P_3 P_2 P_1	
Val–Arg	0.35
Gly–Gly–Arg	0.13
Gly–Ala–Arg	0.048
Gly–Val–Arg	0.14
Gly–Gly–Gly–Arg	0.15
Gly–Gly–Gly–Gly–Arg	0.11
Gly–Gly–Gly–Val–Arg	0.10
Gly–Gly–D–Arg	1.2

The effect of the length of peptides can be assessed by comparison of the K_d values for Val–Arg, Gly–Val–Arg, Gly–Gly–Val–Arg and Gly–Gly–Gly–Val–Arg [15]. A marked difference in affinity between di- and tripeptides was observed; the K_d for Val–Arg was 0.35 mM whereas that for Gly–Val–Arg was 0.14 mM. The addition of one glycine residue resulted in about a twofold increase in affinity for trypsin. This glycine residue at P_3 may either have a role as a spacer or interact with the subsite S_3 of trypsin. Addition of two or three glycine residues did not result in a substantial increase in affinity. These results showed that a tripeptide is long enough to make the C-terminal arginine accessible to the substrate-binding pocket of trypsin (S_1 site). In other words, the minimum length of spacer that can prevent steric hindrance is that of dipeptide. It was also concluded that, in the case of trypsin, only subsites S_1, S_2 and S_3 are worth considering.

The K_d values of three tripeptides having different P_2 residues (Table 1) also showed the impor-

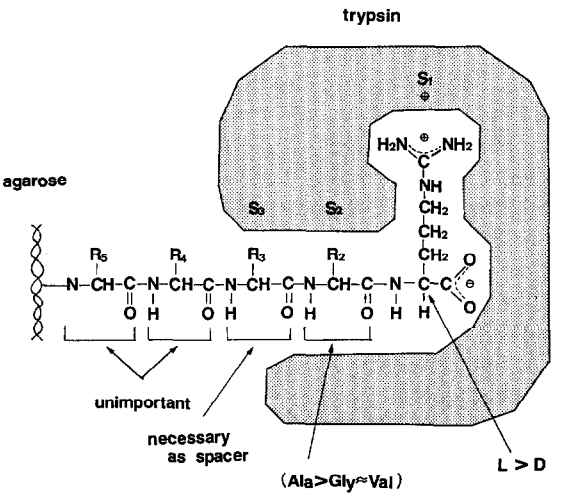

Fig. 7. Interaction of trypsin with AP-agarose.

tance of interactions including the S_2 site. If the P_2 residue was either glycine or valine, almost the same K_d value was observed, whereas alanine at a P_2 site resulted in a significant decrease in K_d. This observation was supported by a kinetic experiment in which inhibition constants (K_i) of soluble tripeptides having the same structure except that their α-amino group was acetylated were determined. A ligand having a C-terminal D-arginine was also examined. The K_d of Gly–Gly–D-Arg was about ten times of that of Gly–Gly–L-Arg. The binding site of bovine trypsin discriminates optical isomers of even product-type compounds and has a stronger affinity for L-amino acids.

Fig. 7 shows schematically a summary of the results obtained here. These results strongly indicate that quantitative affinity chromatography is a powerful tool for direct investigations of specific interactions between biomolecules.

5. ANALYSIS OF EFFECTS OF ENVIRONMENTS ON THE INTERACTION BETWEEN TRYPSIN AND THE AFFINITY ADSORBENT

In the previous section, eqn. 1 was used to compare the binding abilities of affinity adsorbents having different arginine peptides. The same equation can also be applied to study the binding ability of an affinity adsorbent under various conditions. Thus, K_d values under various conditions were determined by using bovine trypsin and Gly–Gly–Arg–agarose. This gave almost equivalent information to the study by using trypsin and soluble product-type compounds [17].

5.1. Effect of pH

K_d values were determined at various pH values (Fig. 8a). The K_d values decreased markedly from pH 8 to 7 and more gradually below pH 7. The minimum value was obtained at pH 4. This result is almost consistent with the qualitative observation previously obtained with AP-agarose that trypsin was adsorbed between pH 7 and 4. The pH dependence of the interaction between trypsin and a soluble product-type compound was also studied by kinetic experiments. Inhibition constants (K_i) of benzyloxycarbonylarginine at various pH values were determined. A very similar pH dependence was observed. Thus, the mode of adsorption of trypsin to

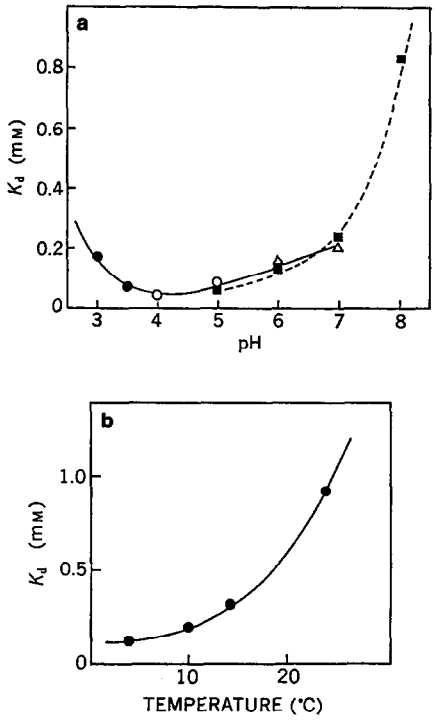

Fig. 8. (a) Effect of pH on the affinity of bovine trypsin for Gly–Gly–Arg–agarose. K_d values were determined by frontal affinity chromatography at various pH values. Buffers used: ■ = 0.1 *M* Tris–maleate; △ = morpholinoethanesulphonate; ○ = acetate; ● = formate. Temperature, 4°C. (b) Effect of temperature on the affinity of bovine trypsin for Gly–Gly–Arg–agarose. 0.1 *M* Tris–maleate buffer containing 0.02 *M* $CaCl_2$ (pH 6.2) was used. (From ref. 17).

Gly–Gly–Arg–agarose proved to reflect exactly the interaction between trypsin and soluble product-type compounds.

5.2. Effect of temperature

Fig. 8b shows the effect of temperature on the K_d values of the binding of trypsin to Gly–Gly–Arg–agarose. The binding strength was found to be very sensitive to changes in temperature; the lower the temperature, the stronger was the interaction. The K_d value at 15°C was about ten times that at 4°C. From this result, it seems desirable to carry out chromatography in a cold room from the point of view of both the affinity and stability of the enzyme.

5.3. Other factors affecting affinity

Frontal affinity chromatography of trypsin on Gly–Gly–Arg–agarose was carried out in the pres-

TABLE 2

EFFECTS OF ADDED COMPOUND ON THE K_d VALUE OF BOVINE TRYPSIN FOR GLY–GLY–ARG–AGAROSE (FROM REF. 17)

Addition	Concentration	K_d (mM)	Eluting buffer
None		0.11	0.1 M Tris–maleate buffer containing
Sodium chloride	0.1 M	0.11	0.02 M $CaCl_2$, pH 6.2, 4°C
	0.3 M	0.16	
	1.0 M	0.16	
Thallium formate	0.1 M	2.2	
None		0.16	Morpholinoethanesulphonate buffer containing
Glycine	0.03 M	0.18	0.02 M $CaCl_2$, I = 0.24, pH 6.0, 4°C
	0.1 M	0.21	
	0.5 M	0.38	
Dioxane	1%	0.21	
	5%	0.43	
n-Propanol	5%	0.14	
Ethylene glycol	10%	0.16	

ence of various compounds which cause changes in the solution environment (Table 2). No detectable change in K_d was observed on the addition of 0.1 M NaCl. However, higher concentrations of NaCl caused an increase in K_d. This suggests that the ionic interaction between Asp177 located in the bottom of the binding pocket of trypsin and the guanidino group of Gly–Gly–Arg–agarose was weakened. Addition of glycine also caused an increase in K_d. This is also attributed to the decreased interaction brought about by the increase in dielectric constant.

Among the organic solvents tested, dioxane was the most effective in increasing K_d. Because of its effect in decreasing the dielectric constant of water, the ordered structure of trypsin may be partially destroyed. *n*-Propanol at the same concentration increased K_d slightly. Ethylene glycol (10%), often used to dissociate proteins from hydrophobic adsorbents, had no effect. This suggested that hydrophobic interactions contribute little to the binding ability of trypsin.

Stroud *et al.* [20] reported an X-ray diffraction study indicating that two thallium ions bind to the substrate-binding pocket and inhibit trypsin competitively. In the presence of 0.1 M thallium formate, an extremely large K_d value (2.2 mM) in comparison with K_d obtained in the presence of the same concentration of NaCl (0.11 mM) was obtained. This strongly suggests the specific binding of thallium ions and supports the results obtained by X-ray analysis.

6. ANALYSIS OF THE EFFECT OF COMPETITIVE LIGANDS

Competitive elution of adsorbed substances is a popular and effective method in affinity chromatography. If we perform frontal affinity chromatography in the presence of a soluble competitive inhibitor, its effect can be quantitatively analysed by measuring the extent of the reduction of the elution volume of the target molecule (trypsin in the present instance). In this instance, an equilibrium constant equivalent to the inhibition constant (K_i) obtainable by enzyme kinetics is obtained by a simple equation:

$$V_i = V_0 + K_i \cdot \frac{V_m - V_i}{[I]_0} \quad (2)$$

where V_i is the elution volume of trypsin in the presence of a soluble competitive inhibitor, V_m is that in the absence of the inhibitor and $[I]_0$ is the concentration of the inhibitor.

If we perform frontal affinity chromatography of trypsin with various concentrations of a competitive inhibitor, measure the elution volumes (V_i) and plot these values against $(V_m - V_i)/[I]_0$, a straight line will be obtained and K_i and V_0 can be determined from the slope and the intercept on the ordi-

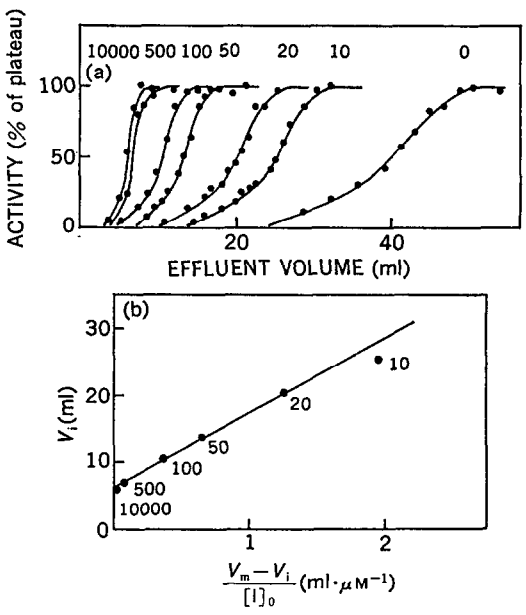

Fig. 9. (a) Effect of benzamidine on the pattern of frontal chromatography of bovine trypsin. A column of Gly–Gly–Arg–agarose (10 × 0.6 cm I.D.) was equilibrated and run with 0.1 *M* Tris–maleate buffer containing 0.02 *M* $CaCl_2$ (pH 6.0). Temperature, 4°C. The concentrations of benzamidine are indicated in *μM*. Concentration of the enzyme, 0.2 *μM*. (b) Plot of V_i against $(V_m - V_i)/[I]_0$ for the data obtained from (a). (From ref. 17).

nate, respectively. Moreover, once V_0 and V_m have been determined, K_i for any competitive inhibitor can be obtained by only one operation of chromatography in the presence of the inhibitor.

To verify the validity of this theory, the effects of competitive inhibitors of trypsin on frontal affinity chromatography were studied by using Gly–Gly–Arg–agarose [17]. Fig. 9a shows the effect of benzamidine on bovine trypsin. The plot of experimental data according to eqn. 2 fitted a straight line very well (Fig. 9b). A similar experiment was also carried out on *S. griseus* trypsin. K_i values of benzamidine for these two trypsins at pH 6.0 and 4°C were determined as 14 and 1.5 *μM*, respectively. These values were also determined by enzyme kinetics although the conditions were unfavourable because the enzyme reaction proceeded slowly owing to the low pH and low temperature. Values of 15 and 1.0 *μM* were obtained. Thus, K_i values obtained by these two completely different methods were very close.

These results show that affinity chromatography can provide almost the same information as enzyme kinetics on specific interactions. Moreover, it has advantages because it is applicable to systems or conditions where enzyme kinetics are no longer applicable, *e.g.*, far from the optimum pH or at very low temperature. Binding constants for even inactivated enzymes can be determined. This means that it is applicable not only to enzyme systems but also to every interacting biomolecule system, *e.g.*, specific interactions between lectins and complex carbohydrates. Frontal chromatography has been applied to a variety of interacting systems and has contributed to the elucidation of their mechanism [21–25].

7. COVALENT AFFINITY CHROMATOGRAPHY OF TRYPSIN BY USING ARGININAL DERIVATIVES

Leupeptin, which is produced by several strains of *Streptomyces*, was found to have an extremely strong inhibitory ability for trypsin-family proteases [26]. Leupeptin is basically a mixture of derivatives of leucylleucylargininal. The α-amino group is acylated with either an acetyl of propionyl group (Fig. 10). The strong inhibitory action is explained by the formation of a covalent hemiacetal adduct between the aldehyde group in the inhibitor and the hydroxyl group of the catalytic Ser183 of trypsin [27]. The complex thus formed can be regarded as a model of a hypothetical tetrahedral intermediate of trypsin action and the bound leupeptin is sometimes called a "frozen substrate". It was of great interest to use compounds of this type as immobilized ligands of affinity adsorbents for trypsin-family enzymes, because they are situated between product and substrate. It was also expected to be able to prepare very strong affinity adsorbents because the formation of a reversible covalent bond was expected.

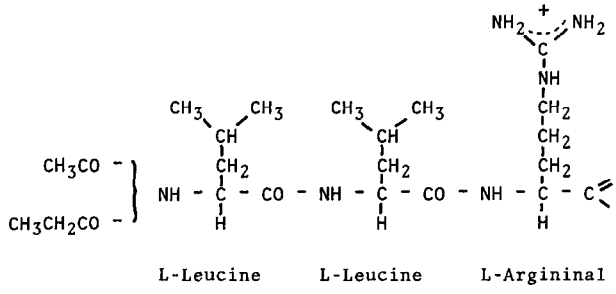

Fig. 10. Structure of leupeptin.

As both the guanidino and aldehyde groups of leupeptin are essential for its function, it was necessary to create a suitable reactive group for an immobilization reaction. It was considered that if a peptide bond in leupeptin could be hydrolysed, a free amino group would appear. Then, thermolysin was found to hydrolyse exclusively the leucyl–leucyl bond [28]. However, the direct thermolysin digestion of leupeptin gave complex products and yield of the expected leucylargininal was very low. This was considered to be due the intramolecular reaction of the exposed α-amino group with the aldehyde group. Therefore, the aldehyde group was protected as a form of dibutylacetal prior to the thermolysin digestion and the product was handled in the protected form throughout a series of reaction steps. After the final immobilization reaction to agarose, the aldehyde group was regenerated.

Leucylargininal dibutylacetal was reacted with agarose gel derivatives which had been coupled with a spacer such as aminohexanoic acid and Gly–Gly by using water-soluble carbodiimide. Affinity adsorbents prepared by such a procedure (LA-agarose) showed a strong affinity for trypsin-family proteases, as had been expected. Enzymes adsorbed by LA-agarose were bovine trypsin, *Streptomyces griseus* trypsin, plasmin, kallikrein, urokinase, tissue plasminogen activator and clostripain, etc.

The importance of the aldehyde group for adsorption was evident. The agarose derivatives adsorbed proteases only after the regeneration of the aldehyde group by acid treatment. The adsorbents completely lost their ability when the aldehyde group was converted into the corresponding alcohol by treatment with $NaBH_4$. The importance of Ser183 for the interaction was also demonstrated. Trypsin derivatives which lost the function of Ser183, namely DIP-trypsin and anhydrotrypsin, were not adsorbed. These results strongly suggest that adsorption of trypsin is based on both the affinity of the binding site and the formation of a covalent bond at the catalytic site.

The formation of the covalent bond was also suggested from the observation that elution of adsorbed trypsin was almost impossible with the procedures usually used, such as elevation of ionic strength, lowering of pH or addition of denaturants. Successful elution was achieved only by the addition of leupeptin to the eluent at low concentration (*e.g.*, 0.2 m*M*). However, the transfer of adsorbed trypsin to this mobile phase proceeded only slowly, so the flow-rate should be kept very low. In practice, a leupeptin-containing buffer was introduced into the column to fill the void, allowed to stand overnight without flow and elution was started again the next day. Another difficulty was that the eluted trypsin formed a tight complex with leupeptin which could not be removed by dialysis or gel filtration. Therefore, leupeptin was inactivated by converting its aldehyde group into alcohol with dilute $NaBH_4$ (*e.g.*, 2 m*M*). To regain the intact enzyme, it is important that the objective enzyme tolerates such reductive conditions. Trypsin was stable under these conditions, and about 90% of the activity originally applied to the column was recovered.

It was difficult to determine quantitatively the affinities of the ordinary adsorbents of this type by frontal analysis because enzymes were adsorbed too tightly. Therefore, an adsorbent of low ligand content was prepared and the dissociation constants were determined. A preparation of LA-agarose whose ligand content was 3.2 nmol/ml ($3.2 \cdot 10^{-6}$ *M*) was used to determine K_d values for various trypsin-family enzymes. The K_d values for bovine α- and β-trypsin and *S. griseus* trypsin were 1.1, 0.33 and 0.025 μ*M*, respectively, at 25°C and pH 8.2. The K_d for bovine trypsin increased slightly when the pH was lowered from 8.2. At pH 6 the K_d value was 1 μ*M* and at pH 4 it was 3.2 μ*M*, that is, about ten times larger than at pH 8.2 [29]. Such a pH dependence also suggested that LA-agarose adsorbed trypsin by a different mechanism from adsorbents having product-type ligands.

In place of a leupeptin derivative, a synthetic argininal derivative, Gly–Gly–argininal, was also prepared and used as an immobilized ligand [30]. Gly–Gly–argininal-agarose showed very similar properties to LA-agarose. Although the importance of Ser183 of trypsin was again demonstrated, His46 proved to be non-essential for the interaction with the adsorbent. CAM-trypsin, a catalytically inactive derivative in which His46 was specifically modified, was found to be adsorbed.

The usefulness of leucylargininal dibutylacetal was not limited to the field of affinity chromatography. It was an excellent starting material for a variety of reagents for investigation. For example, dan-

sylated derivatives were found to be effective fluorescent affinity labelling reagents for trypsin-family proteases [31]. A number of affinity adsorbents and soluble inhibitors having different P_3 amino acid residues were also synthesized. They were used effectively to assess the function of proteolytic enzymes in biological regulation such as fertilization and development [32,33].

8. IMMOBILIZED TRYPSIN AS AN AFFINITY ADSORBENT

As demonstrated in the previous sections, quantitative affinity chromatography became an excellent investigative tool for specific interactions of biomolecules, because the high resolution ability of chromatography and the specific recognition of the affinity technique were effectively combined. In order to explore the potential of frontal affinity chromatography, application to immobilized enzymes was undertaken. Interaction of protein molecules in the mobile phase with immobilized small ligands had been studied, but the reverse case should also be possible. Immobilized enzymes have been used for a variety of purposes. However, in the present instance, only the analysis of specific interactions with small ligand molecules was considered. Thus, trypsin was immobilized to agarose gel and the behaviour of small specific ligands was studied.

Trypsin-agarose was prepared by the ordinary cyanogen bromide method [18]. The amount of trypsin molecules retaining their binding ability was determined by using soybean trypsin inhibitor (STI). In a typical example, 50% of immobilized trypsin retained the binding ability for STI. This adsorbent was packed in a column and frontal chromatography of benzamidine was carried out. As indicated in Fig. 11a, a sharp elution front was observed. When benzamidine solution was replaced with buffer, the tail was not as sharp as the front. These profiles suggested that the adsorption of benzamidine to the trypsin–agarose is of Langmuir type. In the presence of leupeptin (50 μg/ml), the elution volume of the front decreased to almost that of the pass-through volume, and long tailing was no longer observed (Fig. 11b). This suggested that leupeptin had completely inhibited the interaction between the immobilized trypsin and benzamidine.

In the experiments described in the previous section, it had been difficult to analyse the effect of the concentration of solute molecules on the elution volume, because proteins were used as the target molecules. However, if we use a column of immobilized trypsin, the concentration of a counterpart molecule such as benzamidine in the applied solution can be varied over a wide range. Thus, elution volumes of the front of benzamidine solutions of various concentrations were experimentally determined and analysed by using the following equation:

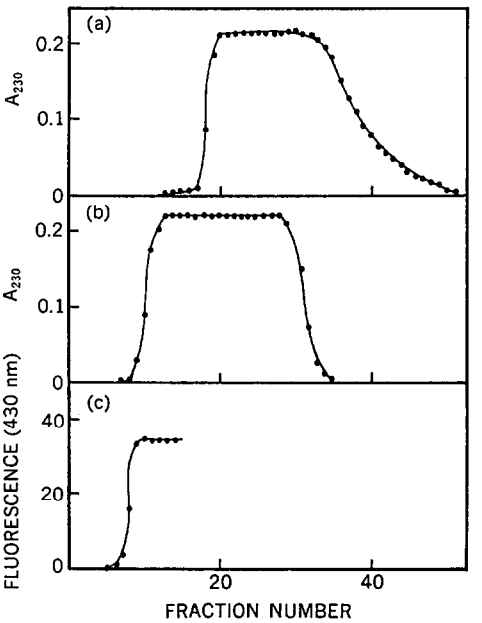

Fig. 11. Frontal chromatography of benzamidine on trypsin-agarose (a and b). The column (10.5 × 0.9 cm I.D.) was equilibrated and run with 0.05 *M* acetate buffer containing 0.01 *M* $CaCl_2$ (pH 6.0). Temperature, 4°C. Fractions of 1 ml were collected. (a) 21.5 ml of 19.9 μM benzamidine were applied; (b) same as (a) except for the addition of leupeptin (50 μg/ml). (c) Control experiment using 10 μM glycine. (From ref. 18).

$$[A]_0(V - V_0) = \frac{B_t\,[A]_0}{[A]_0 + K_d} \tag{3}$$

where $[A]_0$ is the concentration of the interacting molecule (benzamidine in the present instance) in the applied solution. This equation is equivalent to the Michaelis–Menten equation in enzyme kinetics. $[A]_0(V - V_0)$ represents the degree of saturation of the immobilized ligand, which corresponds to the velocity in the case of enzyme kinetics. B_t represents

the maximum binding ability of the column, which corresponds to the maximum velocity (V_{max}). $[A]_0$ and K_d correspond to the substrate concentration and Michaelis constant, respectively. This is not surprising because both enzyme kinetics and affinity chromatography are based on a Langmuir-type adsorption phenomenon. Hence the effect of concentration on frontal affinity chromatography provides almost equivalent information to that from enzyme kinetics.

Elution volumes were determined for various $[A]_0$ values, and a plot of $[A]_0(V - V_0)$ *versus* $[A]_0$ was made (Fig. 12b). B_t and K_d correspond to the coordinates of two asymptotes of the hyperbolic curve. In Fig. 12b, a procedure for the determination of B_t and K_d which was proposed by Eisenthal and Cornish-Bowden [34] (direct linear plot) is also shown. These value can also be determined by a double reciprocal plot (eqn. 4) analogous to a Lineweaver–Burk plot in which data points form a straight line (Fig. 12a):

$$\frac{1}{[A]_0(V - V_0)} = \frac{K_d}{B_t} \cdot \frac{1}{[A]_0} + \frac{1}{B_t} \quad (4)$$

The K_d value of benzamidine for trypsin at pH 6.0 and 4°C was 13 μM, which is very close to that obtained by competitive elution and enzyme kinetics described in the previous section.

The B_t value obtained corresponded to 73% of the amount of immobilized protein (determined by amino acid analysis). This value was large compared with the binding capacity towards STI, which had corresponded to 50% of the total amount of immobilized trypsin. This result suggests that a portion of the immobilized trypsin molecules could bind small competitive inhibitors only. These trypsin molecules might have been immobilized to agarose in the vicinity of the active site. Hence, although the interaction with benzamidine was normal, that with STI was inhibited by steric hindrance.

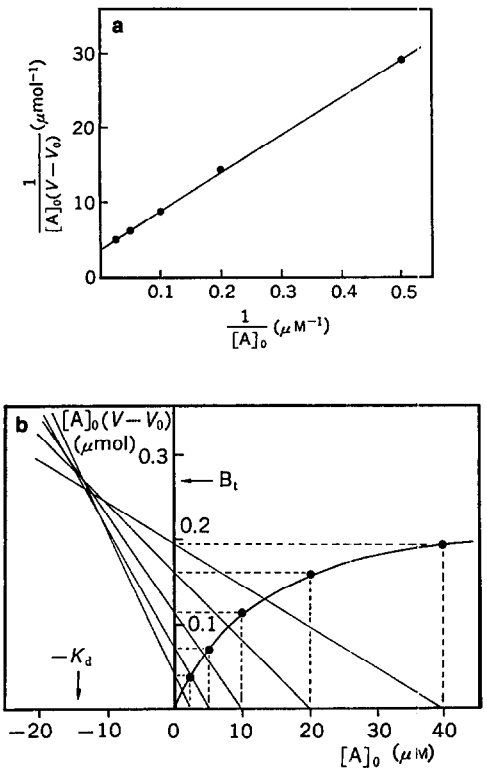

Fig. 12. Plot of the data from Fig. 11. (a) Plot of $1/[A]_0(V - V_0)$ *versus* $1/[A]_0$ for benzamidine; (b) plot of $[A]_0(V - V_0)$ *versus* $[A]_0$ for benzamidine. (From ref. 18).

9. ANHYDROTRYPSIN-AGAROSE

As described before, anhydrotrypsin which had lost the function of Ser183 still retained a specific binding ability to AP-agarose. However, anhydrotrypsin turned out to have gained a more interesting property. Detailed studies revealed that its binding ability to AP-agarose had greatly increased [35–37]. Careful examination by frontal analysis and other methods showed that its binding strength for only product-type compounds increased about 50 times compared with that of native trypsin. No increase in binding ability was observed for both substrate-type compounds and small competitive inhibitors such as benzamidine. This means that anhydrotrypsin acquired an unusual binding ability only for product-type compounds. Hence, although it had once been concluded that the hydroxyl group of Ser183 does not make any important contribution to specific binding, its removal still resulted in modification of the binding ability.

An explanation of this phenomenon has not yet been provided on a molecular level. Disappearance of the hydroxyl group of Ser183 may have caused a subtle conformational change in the active site and the binding site may have gained an ability to attract the negative charge of the free carboxyl group

of the C-terminal arginine of the products, in contrast to the native enzyme which repels the negative charge. Anhydrotrypsin did not show an increased affinity for arginine residues if they were located in internal positions of polypeptides. Thus, anhydrotrypsin proved to be a unique tool which is able to discriminate arginine residues according to their position in a polypeptide chain and binds only the C-terminal arginine. Peptides having lysine residues were recently also found to be recognized by anhydrotrypsin by the same mode, although the binding strength was slightly inferior. To take advantage of such unique specificity, anhydrotrypsin was immobilized to agarose [38,39].

9.1. Application to isolation of C-terminal peptides [40,41]

The usefulness of anhydrotrypsin-agarose as a tool for the isolation and detection of peptides having arginine or lysine at the C-terminus such as some peptide hormones has been shown. As it could distinguish the peptide derived from the C-terminal part of a protein after protease digestion, its usefulness for protein sequence determination and cloning of cDNA has been demonstrated. If a target protein, the C-terminus of which is neither arginine nor lysine, is digested with trypsin and the digest is applied to an anhydrotrypsin-agarose column, all peptides except the C-terminal one will be adsorbed or retarded. Hence the pass-through fraction should contain almost exclusively the peptide derived from the C-terminus. This should greatly facilitate the isolation and sequencing of the C-terminal peptide, and consequently cloning of the cDNA because cDNA libraries are expected to contain more abundantly cDNA fragments corresponding to the downstream part of the coding region. Alternatively, if the C-terminus of a target protein is arginine or lysine, only the peptide derived from the C-terminal part will be adsorbed to anhydrotrypsin-agarose if the protein is digested with a protease other than trypsin. Successful application has already been reported for several proteins [42–44].

9.2. Application to isolation of recombinant proteins; arginine-tail method [45]

The usefulness of anhydrotrypsin-agarose for the specific isolation of recombinant proteins has also been demonstrated. This is one variation of methods often called affinity tag, affinity flag or affinity tail methods. This is based on the introduction of a particular amino acid sequence into a recombinant protein aiming at its efficient purification by affinity chromatography. Such methods will be of great value particularly when a target protein loses characteristic properties such as enzyme activity, specific binding ability or antigenicity as a consequence of site-directed mutation. This principle was successfully combined with the unique property of anhydrotrypsin as follows: (1) insertion of an arginine (or lysine) codon immediately upstream of the termination codon of the gene for the target protein; (2) production of the mutant recombinant protein having arginine (or lysine) at the C-terminus; (3) specific adsorption of the mutant protein with anhydrotrypsin-agarose; (4) specific elution of the protein; and (5) removal of the added C-terminus by using carboxypeptidase B.

The utility of this procedure was shown in the case of a recombinant animal lectin. An expression vector for human β-galactoside-binding lectin (14 000 dalton lectin) [46–49] which codes 134 amino acid residues was used. A single arginine codon (CGC) was inserted before the termination codon by site-directed mutagenesis, and the mutant lectin protein (designated as R135) was expressed. The mutant protein was purified and used for experiments aimed at establishing this new methodology (R135 retained saccharide-binding ability, hence it could be purified by a conventional affinity adsorbent). However, R135 was not adsorbed to anhydrotrypsin-agarose. It might have been difficult for the C-terminal arginine of R135 to access the binding site of anhydrotrypsin because the C-terminus may be only partially exposed or buried between the interface of the dimer structure. Therefore, R135 was dissolved in a buffer containing 4 *M* urea in order to unfold the molecule partially and applied to the column. This resulted in adsorption of R135, and it was eluted by the addition of a specific inhibitor, Bz–Gly–Arg (10 m*M*). It could also be eluted with 5 m*M* HCl. Neither wild-type lectin nor R135 treated with carboxypeptidase B was adsorbed even in the presence of urea.

When a crude *Escherichia coli* lysate containing R135 was applied to anhydrotrypsin-agarose in the presence of urea, R135 was also adsorbed and eluted with the inhibitor. However, in addition to R135, minute amount of several proteins of the host cell were also adsorbed. These proteins seemed to have

arginine or lysine at their C-termini. By this procedure, R135 was enriched more than tenfold. For efficient removal of the co-purified host proteins, use of an adsorbent coupled with antibody for *E. coli* proteins is now under examination.

One of advantages of this procedure is that both the structure and function of the target protein will be only slightly affected because a single arginine residue is added. Moreover, a hydrophilic amino acid such as arginine or lysine at the C-terminus is likely to be exposed on the surface of the protein molecule (unfortunately not the case in R135). Although removal of the added parts from the fusion proteins generally requires specific, but unfamiliar, proteases such as enterokinase and faxtor X, in the present procedure only a widely available exopeptidase, carboxypeptidase B, is needed.

Anhydrotrypsin thus provided a new concept of immobilized ligands for affinity adsorbent. Although it is derived from an enzyme, not the catalytic function but only the specific binding ability was utilized. Other inactivated enzymes, not limited in proteases, may serve as specific recognition molecules. The preparation and characterization of anhydro derivatives of other serine proteases have also been pursued, and it has been shown that both anhydrochymotrypsin and anhydroelastase also gained a stronger affinity for C-terminal residues. They also proved to become efficient tools for specific peptide separations [50–52].

10. HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY OF TRYPSIN AND RELATED ENZYMES

Combination of the principle of specific recognition with the techniques and equipment developed for high-performance liquid chromatography should extend considerably the range of applicability of affinity chromatography. A rigid and microparticulate matrix instead of agarose gel became available as a supporting material for affinity ligands in the mid-1980s. As a variety of hydrophilic packings for high-performance gel permeation chromatography based on synthetic polymer were produced by Japanese manufacturers and had advantages in comparison with silica-based materials, we attempted to develop them as supports for affinity adsorbents.

One of the merits of high-performance affinity chromatography (HPAC) will undoubtedly be its potential usefulness especially for clinical analysis because of its rapidly, sensitivity and reproducibility. The determination of plasmin and plasminogen in human blood was chosen as the first target. Plasmin, one of the trypsin-family enzymes playing an important role in fibrinolysis, consists of a heavy chain (M_r 56 000–58 000) and a light chain (25 000) held together by two disulphide bonds. It is formed from an inactive precursor plasminogen by selective cleavage of peptide bonds by urokinase or plasminogen activator. Monitoring of the state of this enzyme system is very important from the viewpoints of diagnosis, prevention and therapy of various disorders occurring in blood vessels, such as thrombosis, myocardial infarction and disseminated intravascular coagulation syndrome.

Plasminogen has a lysine-binding site which has a key role in the interaction with fibrin. As the lysine-binding site is not removed after activation, plasmin has two types of specific binding sites, the catalytic site in the light chain and the lysine-binding site in the heavy chain. Thus, a benzamidine derivative (*p*-aminobenzamidine) was chosen as an immobilized ligand because it was expected to have affinity for both plasmin and plasminogen.

First, Toyopearl, a microparticulate hydrophilic vinyl polymer gel (particle diameter 40–60 μm) (Toso Industry), was used as a support. Toyopearl contains a considerable amount of hydroxyl groups, which were found to be easily activated by methods already applied to agarose gel. *p*-Aminobenzamidine was immobilized to Toyopearl via different spacers such as 6-aminohexanoic acid (AHA) and chloroacetylglycylglycine. These preparations proved to be effective affinity adsorbents for trypsin [53]. The time required for one cycle of chromatography was considerably reduced and trypsin was eluted from the column as a very sharp peak. Hence their application to the analysis of the fibrinolytic system seemed promising.

In HPAC, the importance of rapid and sensitive detection is high, in addition to effective separation. Moreover, if specific recognition is added to the principle of detection, the total system will become much more powerful because we can take advantage of the synergism of specific isolation and specific detection. Therefore, an on-line assay system for plasmin activity was devised. The effluent from

the affinity column was first passed through a detector for protein (usually a fluorescence monitor measuring fluorescence due to tryptophan). Then the effluent was mixed with a solution of a specific and sensitive substrate (Boc–Glu–Lys–Lys–AMC) for plasmin, which produces fluorescence when hydrolysed by plasmin.

To a column of Toyopearl coupled with *p*-aminobenzamidine via chloroacetylglycylglycine, purified plasmin and plasminogen were applied. Both proteins were adsorbed, and plasminogen was eluted with AHA (a competitive soluble ligand). However, plasmin was bound much more tightly. It could be eluted only after addition of 20 m*M* AHA solution containing 3 *M* urea. Enzyme activity was found only in this fraction. This monitoring system made it possible to detect less than 1 μg of plasmin. These observations suggested that plasmin interacted with the adsorbent through both the lysine-binding site and the catalytic site whereas plasminogen interacted only through the lysine-binding site.

Adsorbents for plasmin and plasminogen were also prepared by using Asahipak GS gel (9-μm particles) (Asahi Chemical Industry) [54–56]. Careful examination of the conditions for separation revealed that plasminogen could be resolved into two fractions. It was known that the plasminogen preparation isolated from human blood often contained two molecular species, one having N-terminal glutamic acid (Glu-plasminogen), which is the intact molecule, the other lacking a portion of N-terminal peptides and with N-terminal lysine (Lys-plasminogen). The latter has been suggested to be an autodigestion product of the former during the purification process. Although its presence in blood had not been confirmed, its detection and determination seemed interesting from the viewpoints of diagnosis and therapy because it is known that it has a stronger affinity for fibrin and can be converted into active plasmin much more rapidly than Glu-plasminogen. It might have a crucial role in triggering the fibrinolytic process.

The high-performance affinity adsorbent based on Asahipak showed an ability to separate these two subspecies according to the difference in affinity between their lysine-binding sites. Glu-plasminogen was bound to the adsorbent more loosely than Lys-plasminogen. The former could be eluted from the column with more dilute AHA solution.

Although two plasminogen species were clearly separated as far as purified preparations were used, the actual detection of Lys-plasminogen turned out to be extremely difficult when human plasma was directly applied to the system, because its content was too low to be detectable even by the fluorescence monitor. Hence a specific and sensitive detection procedure should be devised.

This problem was finally solved by constructing an on-line system which carried out successively the activation of plasminogen and the specific assay of plasminogen [57,58]. Effluent from the affinity column was mixed with urokinase solution and plasminogen was activated during passage through the activation coil. The activity of the plasmin thus formed was monitored by mixing with a fluorescent substrate as described previously. The new total system, combining specific separation by affinity chromatography and specific detection of proenzyme, made it possible to detect and determine even the minor component of plasminogen. A human plasma sample of only 50 μl was sufficient and the time required was less than 30 min. Plasma could be injected directly into the affinity column without any pretreatment. This system first enabled the minor component of plasminogen to be detected and determined. The content of Lys-plasminogen in normal human plasma was determined to be about 3–4 μg/ml whereas that of Glu-plasminogen was about 0.13–0.15 mg/ml. This system was further improved by incorporating an immobilized urokinase column instead of addition of urokinase solution for activation of plasminogen [59]. This resulted in a great increase in reproducibility and at the same time a great reduction in the consumption of urokinase. The principle of this HPAC system should be applicable to other regulation systems where activation of proenzymes plays a critical role, such as blood clotting, renin–angiotensin and complements.

11. CONCLUSIONS

The experiments on trypsin which began from the use of product-type adsorbents led us to consider a variety of aspects of affinity chromatography which had never been expected. The new findings have repeatedly forced us to change our way of thinking and also to invent new methodology. The necessity

to analyse the interaction between the binding site of trypsin and the product-type ligands gave rise to the establishment of the theory of frontal affinity chromatography. The use of leupeptin as a starting material for adsorbents having peptide aldehydes was based on the extension of the product-type adsorbents. Even the target enzyme trypsin proved to be transformed into an extremely unique ligand anhydrotrypsin, and this provided a new concept of ligands for affinity adsorbent. If we had not begun these studies with product-type ligands, the peculiar properties of anhydrotrypsin might have never been found. The series of studies described in this review have undoubtedly contributed to the development of affinity chromatography in general. However, it must also be emphasized that it has become increasingly evident that affinity chromatography is one of the most suitable research tools for molecular recognition.

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