Mobile phase effects in the high-performance affinity purification of thermolysin

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(First received August 7th, 1990; revised manuscript received February 13th, 1991)

ABSTRACT

The dependence on solvent composition of the affinity of thermolysin (E.C. 3.2.24.4) for an immobilized inhibitor was characterized using zonal, competitive and frontal analytical high-performance liquid affinity chromatography (HPLAC). Affinity, measured as the extent of thermolysin retardation on inhibitor (Gly-D-Phe) columns, was strongly affected by variations in the pH, ionic strength, nature of buffering salts and the presence of organic solvents such as 2-propanol in the eluting buffer. While zonal elution experiments allowed the determination of the influence of the mobile phase composition on the overall process, frontal elutions identified specific effects on the individual affinity properties of the enzyme and the immobilized inhibitor. In agreement with data determined previously completely in solution, the interaction on the solid phase was driven mainly by hydrophobic interactions and was affected negatively by organic salts. Based on these results, more efficient elution conditions for the purification of thermolysin by affinity chromatography were established.

INTRODUCTION

Binding between biological macromolecules usually occurs between structurally well defined entities with a number of specific side-chain groups involved in providing various interactions to stabilize the forming complex. The medium plays an essential role in favouring or unfavouring such interactions. Ionic/hydrophilic contributions to the interaction can be distinguished from hydrophobic contributions by determining the effect of increasing concentrations of salts and organic solvents on the system binding affinity, under conditions that do not alter the three-dimensional structure of the folded macromolecule.

The study of solvent effects on binding processes is also of extreme importance when a careful design of affinity purification methods has to be made. Very often, milder conditions than chaotropic elution can be devised simply by selecting an elut-

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ing buffer composition that reduces the system binding affinity constant by only one or two orders of magnitude.

In this study, the affinity properties of the protease thermolysin with respect to its inhibitor Gly-D-Phe were characterized under different solvent conditions by analytical high-performance liquid affinity chromatography (HPLAC) using zonal, competitive and frontal elutions [1,2].

Thermolysin is a thermostable metallo endopeptidase which catalyses the hydrolysis of proteins and synthetic peptide substrates [3,4]. The interaction with its inhibitor Gly-D-Phe has been previously characterized in solution [5] from enzymatic inhibition data, providing an equilibrium binding constant of 16 mM. The three-dimensional structure of the enzyme is also known [6], and it has been shown that the interaction with the inhibitor Gly-D-Phe is mainly hydrophobic in nature [7]. Affinity chromatography of thermolysin has been carried out previously using various ligands immobilized on soft gels [8–10], eluting bound thermolysin by raising the buffer pH to 9.0.

The effect of different buffer compositions on the binding affinity of the protease/inhibitor interaction was evaluated to determine whether useful information about the interaction process could be obtained by analytical HPLAC and used to devise new procedures for more efficient affinity purifications of thermolysin.

EXPERIMENTAL

Materials

Thermolysin from *Bacillus thermoprotheolyticus* (Rokko), N$^\alpha$-(furylacryloyl) glycyl-L-leucinamide (FAGLA) and Gly-D-Phe were obtained from Sigma (St. Louis, MO, USA) High-performance liquid chromatographic (HPLC)-grade water, acetonitrile and 2-propanol from Fisher Scientific (Fair Lawn, NJ, USA) and trifluoroacetic acid (TFA) from Pierce (Rockford, IL, USA). The epoxy-preactivated affinity support Eupergit C30 N was obtained from Rohm & Haas (Weiterstadt, Germany). Glass columns for packing the derivatized affinity support were purchased from Omni (Cambridge, UK). HPLC and HPLAC separations were performed on a LKB HPLC system. Analytical reagent grade solvents and chemicals were used throughout.

Immobilization of Gly-D-Phe on Eupgergit C30 N

Eupergit C30N (1.5 g) was washed twice with 50 ml of deionized water on a sintered-glass funnel. The resin was then washed twice with 50 ml of coupling buffer (pH 8.0) containing 0.1 M sodium hydrogen carbonate and 0.5 M sodium chloride, and sucked dry by vacuum aspiration. The resin was then suspended in 45 ml of coupling buffer containing 600 mg of Gly-D-Phe and incubated at room temperature for 2 h with shaking. Excess liquid was decanted, 940 mg of glycinamide dissolved in 40 ml of the above coupling buffer were added and the suspension was shaken for 1 h at room temperature. The derivatized resin was washed twice with 50 ml of coupling buffer and twice with 50 ml of water and then dried under vacuum. The column used for frontal analysis experiments was prepared with a lower loading, coupling 40 mg of Gly-D-Phe on 1.0 g of Eupergit C30 N. Solutions used in the derivatization procedure were first sterilized by passing them through a 0.22-μm filter.

High-performance liquid affinity chromatography
    Derivatized support was dry-packed into a 150×6.6, 100×6.6 or 50×3 mm I.D. glass column. For chromatography, the columns were connected to an LKB liquid chromatograph equipped with a Model 2151 variable-wavelength UV detector. All buffers used for chromatographic elutions were prepared with HPLC-grade water, filter sterilized and degassed under vacuum prior to use.

Enzyme activity
    The activity of thermolysin was measured by monitoring the decrease in absorbance of FAGLA at 345 nm at 25°C. The assay solution (3 ml) contained FAGLA (1 mM) in 10 mM) in 10 mM Tris (pH 7.0), 10 mM calcium chloride and 10^{-5} M zinc acetate, and the catalytic reaction was initiated by adding an enzyme solution (5–50 μl). The decrease in absorbance at 345 nm was followed as a function of time.

Theoretical
    Dissociation constants for the interaction of thermolysim with immobilized Gly-D-Phe were obtained by two methods: zonal elution and continuous (broad zone) elution. In the former instance, the column was equilibrated with an appropriate buffer (see Results), monitoring the effluent by measuring the UV absorbance at 280 nm, until a stable baseline was observed. Zones containing different amounts of thermolysin were then injected and the elution profile was recorded. The elution volume of the zone, V, can be related to the concentration of thermolysin in the zone by

$$\frac{1}{V - V_0} = \frac{K_{M/P}}{M_T} + \frac{[P]}{M_T} \tag{1}$$

where V_0 is the unretarded elution volume (determined with injections of blue dextran), $K_{M/P}$ is the dissociation constant of the immobilized inhibitor–thermolysin complex and M_T is the total amount of immobilized inhibitor. During zonal elution [P] is not easily defined and is not determined. However, is possible to plot the initial concentration of P in the zone, namely $[P]_0$, against $1/(V - V_0)$ and calculate the $K_{M/P}$ value from the intercept at $[P] = 0$. Competitive elutions were also carried out by injecting zoned of thermolysin on the inhibitor column equilibrated with buffers containing different Gly-D-Phe concentrations, $[L]_T$. The variation of V with $[L]_T$ was evaluated by the equation

$$\frac{1}{V - V_0} = \frac{K_{M/P} + [P]}{M_T} + \frac{K_{M/P}[L]_T}{K_{P/L}M_T} \tag{2}$$

where $K_{P/L}$ represents the dissociation constant between the mobile Gly-D-Phe and thermolysin. Total amount of active, immobilized Gly-D-Phe was determined from frontal elution experiments. To the equilibrated column, solutions containing different concentrations of affinity-purified thermolysin were applied continuously and the effluent was monitored by measuring the UV absorbance until a plateau was observed. The variation of the elution volume of the front, V, was plotted according to the equation

$$\frac{1}{[P]_T(V - V_0)} = \frac{K_{M/P}}{M_T} \cdot \frac{1}{[P]_T} + \frac{1}{M_T} \tag{3}$$

From a plot of the first term of eqn. 3 against $1/[P]_T$ it is possible to calculate $1/M_T$ from the intercept at $1/[P]_T = 0$.

RESULTS

Characterization of thermolysin–Gly-D-Phe interaction by analytical HPLAC

The zonal elution affinity chromatography approach was used to evaluate quantitatively the binding process between immobilized Gly-D-Phe and soluble thermolysin. Zones containing different amounts of thermolysin were injected onto the [Gly-D-Phe]Eupergit column equilibrated with 50 mM Tris (pH 7.0), and the elution profile was recorded (Fig. 1). The extent of retardation was found to be linearly dependent on the amount applied (Fig. 1, inset), in agreement with eqn. 1.

Amino acid analysis of the Gly-D-Phe-derivatized affinity support indicated the presence of 500 μmol of immobilized inhibitor. Assuming this to be a correct value for the functional capacity, M_T, a chromatographically calculated dissociation constant of 14 mM is obtained. To verify the absence of aspecific interactions on the column, competitive elutions of thermolysin were carried out on the [Gly-D-Phe]Eupergit column with different amounts of Gly-D-Phe dissolved in the eluting buffer. According to eqn. 2, under these conditions it is possible to obtain not only $K_{M/P}$, but also $K_{P/L}$, the dissociation constant for the interaction between soluble Gly-D-Phe and thermolysin. The results are summarized in Fig. 2. The chromatographically calculated value of $K_{M/P}$ (14 mM) agrees fairly well with the $K_{P/L}$ value (16 mM), both calculated under the same conditions, indicating that aspecific interactions with the support, if they occur, are negligible. These values agrees well also with the inhibition constant of
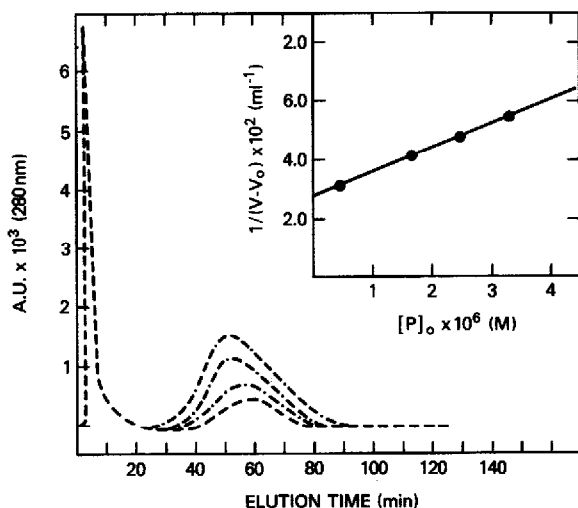

Fig. 1. Zonal elution HPLAC analysis of thermolysin binding to immobilized Gly-D-Phe. Zones (200 μl) containing different amounts of HPLAC-purified thermolysin were eluted on the [Gly-D-Phe]Eupergit column (75 × 6.6 mm, M_T 500 μmol) equilibrated with 20 mM Tris–10 mM CaCl$_2$ (pH 7.1) at a flow-rate of 0.5 ml/min. The inset shows the variation of V with the amount of thermolysin in the applied zone. Data are plotted according to eqn. 1.
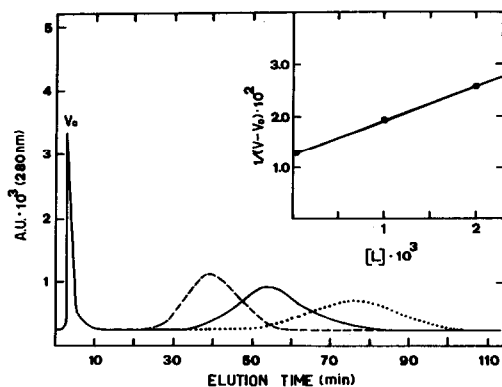
Fig. 2. Competitive zonal elution HPLAC analysis of thermolysin binding to Gly-D-Phe. Zones of 28 μg of HPLAC-purified thermolysin in 200 μl were eluted on the [Gly-D-Phe]Eupergit column (6.6 × 150 mm I.D., M_T 1 mmol) equilibrated with 20 mM Tris–10 mM CaCl$_2$ (pH 7.1) at a flow-rate of 1.0 ml/min containing soluble Gly-D-Phe at concentrations of 0, 1 and 2 mM. Data are plotted according to eqn. 2.

soluble Gly-D-Phe for soluble thermolysin (K_i = 16 mM), determined completely in solution [5]. The lower affinity observed for the interaction in solution may well reflect the previously reported diminished inhibition of thermolysin enzymatic activity by dipeptides with free α-amino groups [5].

Dependence of mobile phase composition on thermolysin–inhibitor interaction

      The extent of thermolysin retardation on the [Gly-D-Phe]Eupergit column was strongly dependent on the composition of the eluting buffer (Fig. 3). An increase in the Tris concentration in the buffer negatively affected the binding, reducing the interaction strongly. Similarly, but to smaller extent, 4-(2-hydroxyethyl)-1-piperazi-neethanasulphonic acid (HEPES) influenced the retardation. Inclusion of 2-propanol also reduced the binding, indicating that hydrophobic interaction phenomena play an essential role in the process, as lowering of the surface tension in an aqueous phase on inclusion of an organic solvent has been shown to reduce greatly hydrophobic interactions between molecules [11].

Determination of column capacity

      Although with the zonal approach a convenient determination of equilibrium binding constants is provided, no information about the accessibility of the immobilized inhibitor can be obtained. This information is critical when evaluation of the mobile phase composition has to be made. Frontal elutions allow the determination of the amount of functionally active inhibitor immobilized on the column and at the same time also equilibrium binding constants. The dependence of chromatographically calculated M_T values on the concentration of (A) Tris and (B) HEPES is illustrated in Fig. 4. From the graphical analysis, the intercept according to eqn. 2 corresponds to the reciprocal of the column capacity, M_T, and the slope is function both of $K_{M/P}$ and M_T. In the presence of Tris, the capacity of the column, M_T, decreases with increasing Tris concentration, whereas in the presence of HEPES it remains unaffected, even if the reduction in the binding constant is evident from the change in
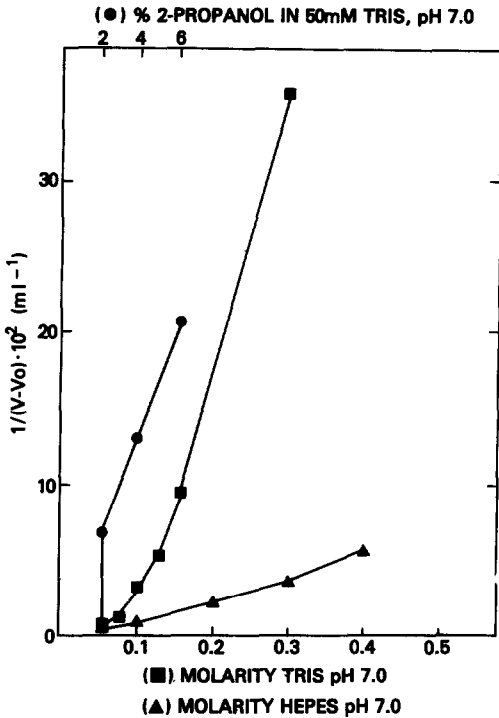
Fig. 3. Affinity dependence on the composition of the mobile phase. Extent of binding is expressed as $1/(V - V_0)$ obtained by extrapolation to [P] = 0. The [Gly-D-Phe]Eupergit column was equilibrated at a flow-rate of 1.0 ml/min with ($\bullet$) 50 mM Tris–10 mM CaCl$_2$ (pH 7.0) and various concentrations of 2-propanol, ($\blacksquare$) 10 mM CaCl$_2$ (pH 7.0) and various concentrations of Tris and ($\blacktriangle$) 10 mM CaCl$_2$ (pH 7.0) and various concentrations of HEPES.
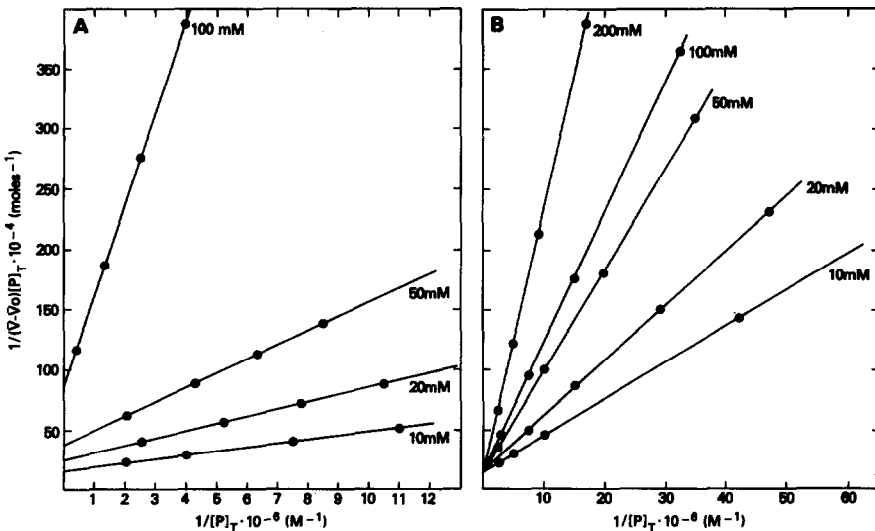

Fig. 4. Frontal analysis of the dependence of the interaction on the mobile phase composition on the [Gly-D-Phe]Eupergit column (50 × 5 mm I.D., M_T 56 μmol) equilibrated at a flow-rate of 0.5 ml/min with 10 mM CaCl$_2$ (pH 7.0) and (A) various concentrations of Tris and (B) various concentrations of HEPES. Data are plotted according to eqn. 3 to calculate values of M_T by extrapolation to $1/[P] = 0$.
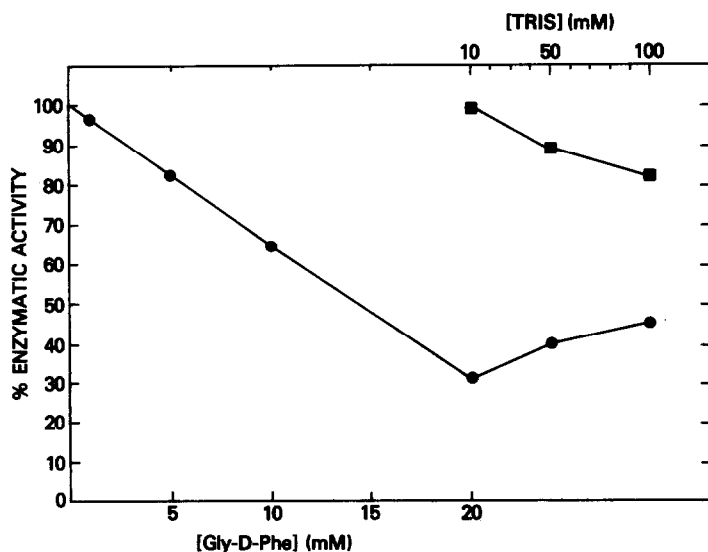
Fig. 5. Effect of Tris on the enzymatic activity of thermolysin. The decrease in enzymatic activity of thermolysin in the presence of Gly-D-Phe was measured in 10 mM Tris–10 mM CaCl$_2$ (pH 7.0). When the activity had decreased to 20% of the original value, the Tris content was raised and the variation in activity monitored (●). The effect of Tris on the enzymatic activity of TLN was also monitored in the absence of inhibitor (■).

slope. From these results it is clear that the large reduction in binding affinity in the presence of Tris is due mainly to a reduction in the amount of functionally active inhibitor (*i.e.*, functional capacity) and minimally to an effect on the protease.

Dependence of thermolysin enzymatic activity on Tris concentration

To verify that the effect of increasing Tris concentration on the thermolysin–Gly-D-Phe interaction is not limited to the solid phase, an evaluation of enzymatic activity of thermolysin towards its substrate FAGLA in the presence of inhibitor and Tris was carried out, as shown in Fig. 5. The thermolysin activity decreased with increasing Tris concentration, but in the presence of Gly-D-Phe as inhibitor the overall effect is an increase in enzymatic activity, at least in the range of Tris concentrations tested.

Preparative HPLAC purification of thermolysin

Purification of thermolysin has been carried out previously by affinity chromatography mainly on soft gels with immobilized inhibitors [8–10]. Elution of bound protease from the support is usually achieved by a buffer pH change to 9.0, disrupting the thermolysin salt bridges His-231/Asp-226 and His-142/Asp-170, which are an integral part of the active site of the enzyme [12].

The possibility of using different elution conditions, based on the previously observed effects of the mobile phase on the binding affinity, was evaluated in order to establish milder and more convenient ways to purify the protease. Commercially obtained thermolysin was first adsorbed on the column equilibrated with 10 mM
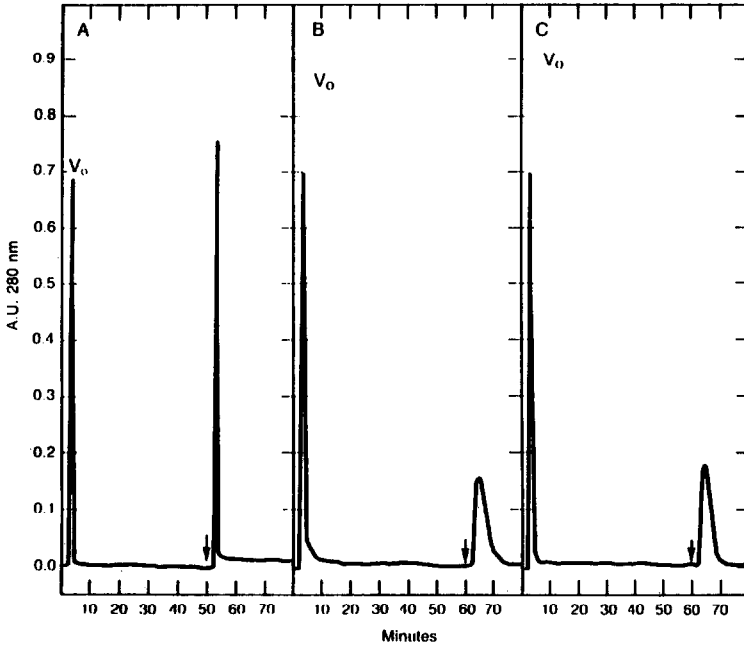
Fig. 6. Preparative HPLAC purification of commercial thermolysin on [Gly-D-Phe]Eupergit. The columm (150 × 10 mm I.D.) was equilibrated at a flow-rate of 1.0 ml/min with 10 mM CaCl$_2$ (pH 7.0) and 4.0 mg of commercial thermolysin dissolved in 200 μl of eluting buffer were injected. At the points indicated by the arrows, the eluent was changed to (A) 0.1 M Tris–10 mM CaCl$_2$ (pH 9.0); (B) 5% 2-propanol–10 mM Tris–10 mM CaCl$_2$ (pH 7.0); (C) 0.4 M Tris–10 mM CaCl$_2$ (pH 7.0).

Tris–10 mM calcium chloride (pH 7.0). Elution of bound protein was achieved by a buffer change to disfavour, or strongly reduce, the binding process. Fig. 6 shows typical chromatograms obtained for the purification of 4 mg of crude thermolysin. Bound protein was eluted by (A) of pH change to 9.0, (B) the inclusion of 5% 2-propanol and (C) 0.4 M Tris (pH 7.0). At pH 9.0 the affinity between thermolysin and Gly-D-Phe is completely abolished and bound protease is eluted as a sharp, narrow peak. Elution with 5% 2-propanol or 0.4 M Tris allows the recovery of thermohysin in a much wider peak, reflecting the residual binding affinity under these conditions. The presence of contaminants or autolytic products in the purified thermolysin preparations was then checked by reversed-phase HPLC [13] (Fig. 7). While the purified thermolysin preparation obtained by elution with 5% 2-propanol and 0.4 M Tris (pH 7.0) are essentially contaminant free, the small amount of degradation products in the pH 9.0-eluted thermolysin preparation were derived from the slow autolysis of the protease occurring at pH values above neutrality [14]. Consequently, the specific activity of the protease purified using neutral buffers was also higher than that from pH 9.0 purification (Table I). In any case, the recovery of activity was high under all the conditions employed. The same column has been used for more than 50 runs with no apparent loss of capacity.
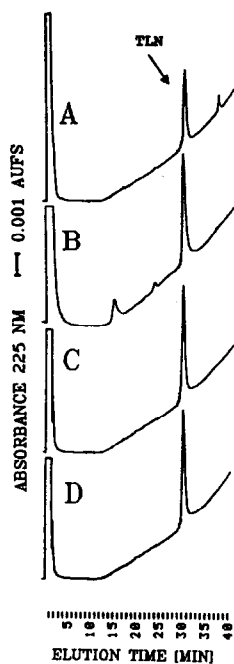
Fig. 7. Reversed-phase HPLC profiles of commercial thermolysin (A), and affinity-purified thermolysin eluted by (B) 0.1 M Tris–10 mM CaCl$_2$ (pH 9.0), (C) 10 mM Tris–10 mM CaCl$_2$–5% 2-propanol (pH 7.0) and (D) 0.4 M Tris–10 mM CaCl$_2$ (pH 7.0).

TABLE I

RECOVERY OF ACTIVITY OF HPLAC-PURIFIED THERMOLYSIN

Elution conditions[a]	Activity recovery[b] (%)	Specific activity[c] [(mg/ml)$^{-1}$s^{-1}]
(A) 0.1 M Tris (pH 9.0)	78	0.43
(B) 0.4 M Tris (pH 7.0)	83	0.48
(C) 10 mM Tris (pH 7.0)–5% 2-propanol	90	0.55

[a] Containing 10 mM CaCl$_2$.
[a] Determined using FAGLA as substrate.
[c] Specific activity of the commercial thermolysin sample was 0.4 (mg/ml)$^{-1}$s^{-1}.

CONCLUSIONS

Examination of solvent effects is of great importance in studying binding processes, as preliminary information can be obtained on the mechanisms involved in the interaction.

Analytical HPLAC leads to detailed information that does not require the interaction to be studied to produce secondary effects, such as enzymatic activity or

chromophoric transitions. In this particular instance, equilibrium binding constants determined on the solid phase by analytical HPLAC for thermolysin–Gly-D-Phe interaction were in good agreement with those determined completely in solution [5]. The effect of organic salts on the enzymatic activity of thermolysin has been previously studied in solution [15] using the neutral substrate FAGLA. While K_{cat} is affected negatively, K_m remains essentially unaltered, thus indicating that the thermolysin–neutral substrate interaction is not influenced by the presence of organic salts. In our study, the affinity properties of thermolysin for its inhibitor Gly-D-Phe, immobilized through its amino group on the solid support and thus with a free carboxyl group, are strongly influenced by the presence of Tris in the buffer. This effect is mainly due to the reduction of accessible inhibitor, and minimally to a reduction in the binding properties of the protease. Frontal elution data indicated that in the presence of 50 mM Tris only half to the total amount of immobilized Gly-D-Phe is accessible to the protease. Hence the Tris molecule is competing with thermolysin for the immobilized inhibitor, with an equilibrium binding dissociation constant of $ca.$ 50 mM. The mechanism of this interaction can be postulated as an ionic interaction between the free carboxyl group of the inhibitor and the charged amino group of the Tris moiety. The same effect was observed in solution by evaluating the influence of Tris on the enzymatic activity of thermolysin towards FAGLA in the prensence and absence of Gly-D-Phe as inhibitor. The Tris molecule has a limited effect on the enzymatic activity, whereas the inhibition by Gly-D-Phe is effectively decreased by the presence of increasing concentrations of this organic salt. In agreement with data obtained on the solid phase by zonal and frontal analytical HPLAC, the interaction between thermolysin and Gly-D-Phe is also inhibited in solution by the Tris molecule. Finally, the evaluation of the effect of solvent composition on the thermolysin–Gly-D-Phe interaction indicated that an efficient reduction in binding affinity could be obtained by including 5% 2-propanol or 0.4 M Tris in the eluting buffer. These conditions could be used for affinity purification of thermolysin, appearing to be more convenient than elution by pH alteration, as the protease at pH values above neutrality slowly undergoes to autolytic processes leading to fragmentation [14].

ACKNOWLEDGEMENTS

REFERENCES

1 I. M. Chaiken (Editor), *Analytical Affinity Chromatography*, CRC Press, Boca Raton, FL, 1987.
2 G. Fassina and I. M. Chaiken, *Adv. Chromatogr.*, 27 (1987) 247.
3 K. Morihara, H. Tsuzuki and T. Oka, *Arch. Biochem. Biophys.*, 123 (1968) 572.
4 J. Feder and J. M. Schuck, *Biochemistry*, 9 (1974) 2784.
5 J. Feder, L. R. Brougham and B. S. Wildi, *Biochemistry*, 13 (1974) 1186.
6 B. W. Matthews, L. H. Weaver and W. R. Kester, *J. Biol. Chem.*, 249 (1974) 8030.
7 B. Holmquist and B. L. Vallee *Biochemistry*, 15 (1976) 101.
8 N. Nishino and J. C. Powers, *Biochemistry*, 18 (1979) 4340.
9 T. Komiyama, T. Aoyagi, T. Takeuchi and H. Umezawa, *Biochem. Biophys. Res. Commun.*, 65 (1975) 352.

10 N. K. Pangburn, Y. Burstein, P. H. Morgan, K. A. Walsh and H. Neurath *Biochem. Biophys. Res. Commun*, 54 (1973) 371.

11 C. J. Van Oss, D. R. Absolm and A. W. Neumann, in T. L. Gribnau, J. Visser and R. J. R. Nivard (Editor), *Affinity Chromatography and Related Techniques*, Elsevier, Amsterdam 1982, p. 29.

12 P. M. Colman, J. N. Jansonius and B. W. Matthews, *J. Mol. Biol.*, 70 (1972) 701.

13 G. Fassina, C. Vita, D. Dalzoppo, M. Zamai, M. Zambonon and A. Fontana, *Eur. J. Biochem.*, 156 (1986) 221.

14 A. Fontana, G. Fassina, C. Vita, D. Dalzoppo, M. Zamai and M. Zambonin *Biochemistry*, 25 (1986) 1847.

15 M. Fukuda and S. Kundugi, *Biocatalysis*, 2 (1989) 225.