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# pH EFFECT ON THE SEPARATION OF α-CHYMOTRYPSIN BY AFFINITY CHROMATOGRAPHY

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**Abstract** — For the separation of enzyme (α-chymotripsin) the characteristics of affinity chromatography were studied. The system employed Sepharose CL-4B as a gel matrix (a packing meterial), 4-phenylbutylamine as a ligand and α-chymotrypsin as a solute.

Moment analysis was used to determine the retention time in a column, in which the longitudinal dispersion in the mobile phase, the radial diffusion inside the porous spherical gel beads and the sorption on the ligands attached to the gel bead were simultaneously taking place.

To analyze the effect of pH on the retention time an ionization model was incorporated into the chromatrgraphic model. Dependence of adsorption equilibrium constant and the retention time on pH were investigated. From the experimental data the approximate pK values could be obtained.

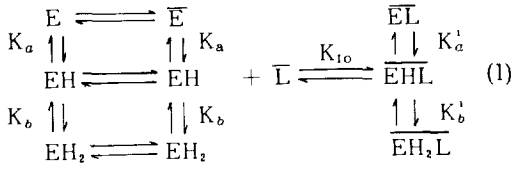
## INTRODUCTION

The effect of pH on the binding of ligand and protein in affinity chromatography actually means the effect of pH on the ionization of functional groups which function as active sites of a solute material. Those ionizable groups are generally located on the side chains of the acidic and basic amino acid residues and may be involved in maintaining the proper conformation of the active site, in binding of the protein with a ligand such as substrate or inhibitor and in releasing the protein from the ligand for elution.

Thus, this phenomenon may be explained and quantified by adopting the type of mechanism equivalent to those describing the pH dependence of enzyme reaction [1].

## IONIZATION MODEL

In this paper the following ionization model is used for the investigation of the pH dependence of adsorption equilibrium constant [2].




(1)

The overbar represents species within the gel matrix. In this scheme the form $EH_2$ obviously bears one more positive charge (or one less negative charge) than EH, and EH has one more positive charge than E(an enzyme). For simplicity the charges will be omitted. It is assumed that the intermediate form EH is the only one that forms a complex with the ligand L(an active site of the gel matrix) and the ionization between the hydrogen ion and the enzyme in the mobile and stationary phases are so fast that equilibrium can be established instantaneously. And a reasonable simplification to this system is the assumption that the ionization is not affected by the presence of the gel. $K_{IO}$ is a pH-independent dissociation constant and $K_a$, $K_a'$, $K_b$, $K_b'$ are ionization constants given by:

$$K_a/[H^+] = [\bar{E}]/[\bar{EH}] \quad (2)$$

$$[H^+]/K_b = [\bar{EH_2}]/[\bar{EH}] \quad (3)$$

$$K_a'/[H^+] = [\bar{EL}]/[\bar{EHL}] \quad (4)$$

$$[H^+]/K_b' = [\bar{EH_2L}]/[\bar{EHL}] \quad (5)$$

$$\text{and } [\bar{L}]/K_{IO} = [\bar{EHL}]/[\bar{EH}] \quad (6)$$

The equilibrium constant, $K_e$, between stationary phase and adsorbed phase is represented by the total enzyme concentrations of both phases.

$$K_e = \frac{[\bar{EH_2L}] + [\bar{EHL}] + [\bar{EL}]}{[\bar{EH_2}] + [EH] + [\bar{E}]} \quad (7)$$

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Dividing by $[\overline{EH}]$,

$$K_e = \frac{[\overline{EH_2L}]/[\overline{EH}] + [\overline{EHL}]/[\overline{EH}] + [\overline{EL}]/[\overline{EH}]}{[\overline{EH_2}]/[\overline{EH}] + 1 + [\overline{E}]/[\overline{EH}]} \quad (8)$$

Since $[\overline{EH_2L}]/[\overline{EH}] = [H^+]/K_b' \cdot [\overline{L}]/K_{IO}$
and $[\overline{EL}]/[\overline{EH}] = K_a'/[H^+] \cdot [\overline{L}]/K_{IO}$,
then substitution yields

$$K_e = \frac{[\overline{L}]}{K_{IO}} \frac{1 + [H^+]/K_b' + K_a'/[H^+]}{1 + [H^+]/K_b + K_a/[H^+]} \quad (9)$$

Since the concentration of the enzyme in the gel matrix is dilute, one can assume that $[\overline{L}]$ is constant and equal to the total ligand concentration $[\overline{L}_o]$. Therefore, the equilibrium constant is expressed:

$$K_e = \frac{[\overline{L}_O]}{K_{IO}} \frac{1 + [H^+]/K_b' + K_a'/[H^+]}{1 + [H+]/K_b + K_a/[H^+]} \quad (10)$$

## CHROMATOGRAPHIC MODEL AND MOMENT ANALYSIS

A long column is considered to be uniformly packed with spherical gel beads (a packing material). The transient material balances of a solute are

$$\frac{\partial C}{\partial t} + U\frac{\partial C}{\partial Z} - D_z\frac{\partial^2 C}{\partial Z^2} + k_f(KC - C_s|_{r=r_s}) = 0 \quad (11)$$

for the mobile phase, and

$$\frac{\partial C_s}{\partial t} = Dr\left(\frac{\partial^2 C_s}{\partial r^2} + \frac{2}{r}\frac{\partial C_s}{\partial r}\right) - \frac{\partial q}{\partial t} \quad (12)$$

for the stationary phase. Assuming a finite rate of adsorption on the internal porous surface of spherical gel beads with a linear isotherm, one has

$$\frac{\partial q}{\partial t} = k_A C_s - k_D q \quad (13)$$

for adsorption kinetics. The velocity profile of the mobile phase is assumed to be of a plug shape. The dispersion in the mobile phase is assumed to be in a longitudinal direction only and its dispersion coefficient, $D_Z$, is also assumed to be constant. The solute transfers into the stationary phase through the interface layer by a mass transfer process with a constant mass transfer coefficient $k_f$.

If the square pulse is injected at the top of the column as a sample solution, the initial and boundary conditions are

$$C(Z,t) = O \qquad \text{for } t = O \quad (14)$$
$$C_s(r,Z,t) = O \qquad \text{for } t = O \quad (15)$$
$$q(r,Z,t) = O \qquad \text{for } t = O \quad (16)$$
$$C(Z,t) = C_O[u(t) - u(t-t_o)] \qquad \text{for } Z = O \quad (17)$$
$$C(Z,t) = O \qquad \text{for } Z = \infty \quad (18)$$

where u(t) is a unit step function and $t_o$ is injection time of one pulse. At the surface of the adsorbent (gel matrix) the mass transfer rate from mobile phase to the stationary phase is equal to the diffusion rate at the surface of the adsorbent, that is,

$$k_f(KC - C_s|_{r=r_s}) = -\frac{3(1-\varepsilon)\varepsilon_s}{\varepsilon r_s} Dr \frac{\partial C_s}{\partial r}\Big|_{r=r_s} \quad (19)$$

for $r = r_s$. The symmetry of the adsorbent implies that

$$\frac{\partial C_s}{\partial r} = 0 \quad \text{for} \quad r = 0 \quad (20)$$

It is difficult to solve analytically the set of differential equations subjected to the initial and boundary conditions, if at all possible. However, the moments of the solution can be found by using either the Laplace or Fourier transformation. And the first moment was derived by Chung [3] as follows:

$$m_1 = \frac{t_o}{2} + \frac{l_b}{U}(1 + K\phi\,(1 + K_e)) \quad (21)$$

where $\phi = \frac{(1-\varepsilon)\varepsilon_s}{\varepsilon}$ and $K_e = k_A/k_D$

For a nonadsorbable solute Eq. (21) reduces to the below equation since $K_D = \infty$.

$$m_1 = \frac{t_o}{2} + \frac{l_b}{U}(1 + K\phi) \quad (22)$$

## MATERIALS AND METHODS

Sepharose CL-4B (Pharmacia Fine Chemicals, Sweden) was used as gel matrix (a stationary phase). The following reagents were purchased from Sigma Chemicals Company (U.S.A.): α-chymotrypsin (an enzyme), 4-phenylbutyl amine (used as a ligand), blue dextran, bicine, TRIZMA hydrochloride, and TRIZMA base.

CNBr activation of Sepharose and ligand coupling were performed as described earlier [4-5].

Glass tube (15x1.1cm) with two flow adapter at both top and bottom was employed as a column for affinity chromatography. Eluent was passed in down flow through the column by a microtube pump (Cole-parmer, U.S.A.), the suction side of which was connected to the outlet of the column through the detector. The detector consisted of a spectrophotometer (Beckman, Model 35) equipped with a flow cell (Beckman, 0.5 ml) and a recorder. The effluent from the column was collected automatically by fraction collector (Buchler Fractomette Alpha 200). Experiments were carried out in a cold room at 5°C to maintain the enzyme activity.

## RESULTS AND DISCUSSION

Chromatographic peaks for pulse input of an aqueous mixture of NaCl (0.6 mole/l) and blue dextran (2.0 g/l), and for that of chymotrypsin (0.1 mg/ml) were measured in packed bed of unsubstituted Sepharose CL-4B gels. 0.05M bicine buffer with pH 8.0 was used as the carrier. The concentration of blue dextran was detected with a spectrophotometer at 620mm and the concentration of NaCl was measured with a conductivity bridge (conductivity bridge model 31, YSI).

And for ligand coupled gel bed, moment analysis of chymotrypsin peak was used to evaluate $K_e$ according to the variation of pH.

Let the partition of blue dextran into the gel be zero and that of NaCl be one, then the following equations from Eq. (22) should hold for blue dextran pulses, sodium chloride pulses and chymotrypsin pulses [6];

$$m_1 - \frac{t_o}{2} = \varepsilon l_b/U_o \quad \text{for blue dextran} \quad (23)$$

$$m_1 - \frac{t_o}{2} = \varepsilon (1+\phi) l_b/U_o \quad \text{for NaCl} \quad (24)$$

$$m_1 - \frac{t_o}{2} = \varepsilon (1+\phi K) l_b/U_o \quad \text{for chymotrypsin} \quad (25)$$

when unsubstituted gel beads were used as a packing material. And for the gel bed coupled with ligands, the following equation should hold for chymotrypsin:

$$m_1 - \frac{t_o}{2} = \varepsilon [1+\phi K(1+K_e)] l_b/U_o \quad (26)$$

Thus $\varepsilon$ is calculated from blue dextran data and $\varepsilon_s$ is obtained from NaCl data using the value of $\varepsilon$. From chymotrypsin data, the partition coefficient of chymotrypsin for unsubstituted Sepharose CL-4B is calculated. Lastly with these values $K_e$ could be calculated using Eq. (26).

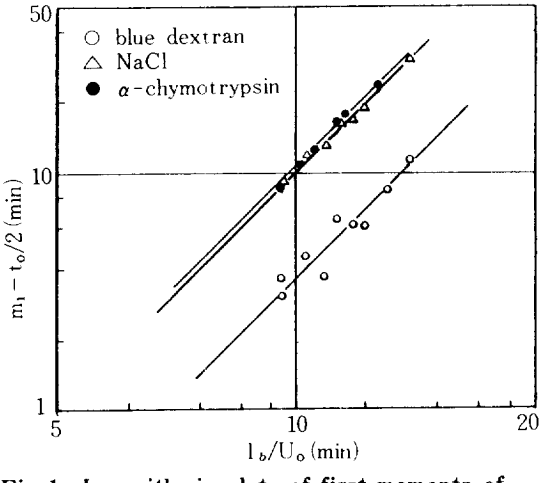


**Fig. 1. Logarithmic plots of first moments of $(m_1 - t_o/2)$ versus $l_b/U_o$.**

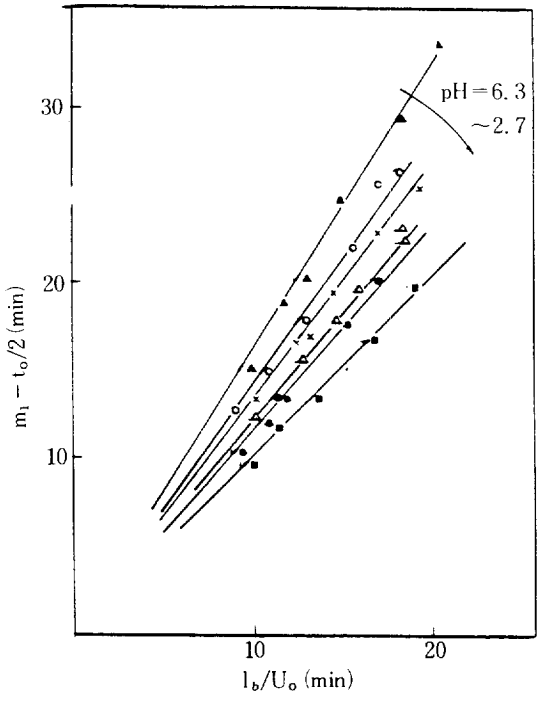


**Fig. 2. First moment plots for various values of pH.**

Fig. 1 indicates the plots of $(m_1-t_o/2)$ versus $l_b/U_o$ for unsubstituted gel bed. Apparently there are linear relations between $(m_1-t_o/2)$ and $l_b/U_o$. Void fraction $\varepsilon$ and porosity $\varepsilon_s$ for Sepharose CL-4B are 0.36 and 0.98, respectively. The partition coefficient of chymotrypsin, K, is 1.127. This implies that chymotrypsin is slightly retarded by Sepharose CL-4B gels even though Sepharose CL-4B is considered to be inert matrix.

Fig. 2 shows first moment plots with the variation of pH of carrier fluid for the ligand coupled gel bed. Apparently there is a linear relation between $(m_1-t_o/2)$ and $l_b/U_o$. The following equation is derived from Eqs. (10) and (21):

$$V = V_m(1+K\phi) + V_m K\phi \frac{[L_o]}{K_{lo}} \frac{1+[H^+]/K_b+K_a'/[H^+]}{1+[H^+]/K_b+k_a/[H^+]} \quad (27)$$

where $V = (m_1 - \frac{t_o}{2})U_o$ and $V_m = l_b\varepsilon$.

The elution volumes are calculated by using the above equation with the data of $K_e$ obtained by moment analysis. When these volumes are compared with ex-

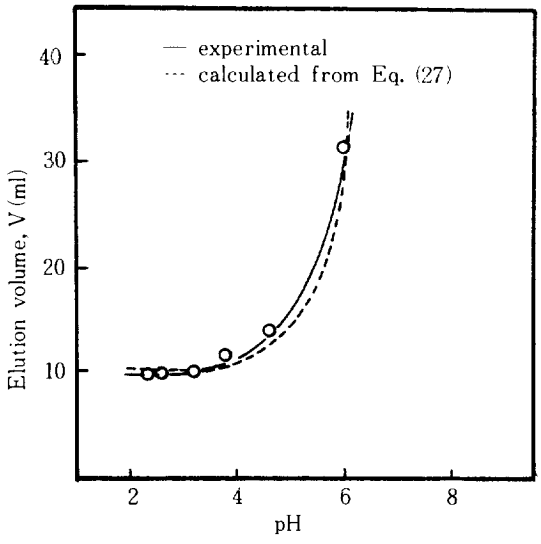


**Fig. 3. Influence of pH on elution volume (column length=11cm and flow rate=48ml/hr).**

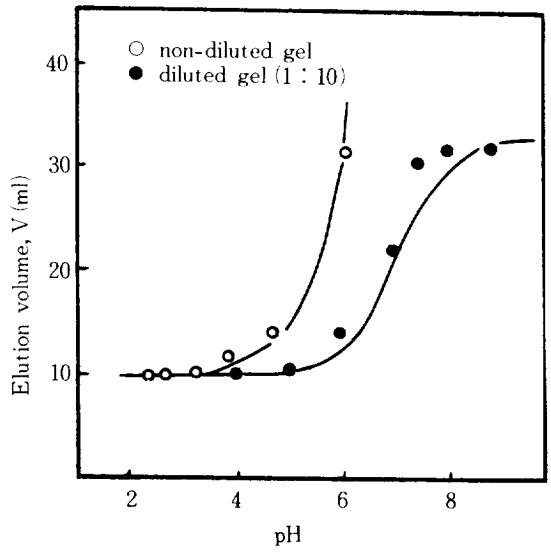


**Fig. 4. Plots of elution volume versus pH for the gel bed with two different ligand concentrations.**

perimental results, they agree very well as shown in Fig. 3. The decrease of elution volume with pH decrement may indicate the adsorption capacity decrement by lowering the values of $K_e$. The approximate pK values derived from the experimental data using the regression method are $pK_a = 7$, $pK_b = 5$, $pK_a' = 8$, and $pK_b' = 4$.

To explain the effect of ligand concentration of gel, two kinds of gels were used: the first had a high ligand concentration of gel which was used to get the previous experimental data and the second had ten times lower ligand concentration than the first one. Fig. 4 shows the experimental data with diluted gels as well as non-diluted gels. As seen in this figure, the ligand concentration influences greatly the performance of affinity chromatography.

Elution by altering the pH of the solvent is the most widely used elution method in many affinity chromatographic systems. In this study, the dependence of the adsorption and desorption of an enzyme onto, or from the immobilized ligand on the amount of hydrogen ion in the solution was assured.

An understanding of the mechanism of action of the enzyme involves a knowledge of what amino acid residues are essential for activity, and of what role each of these groups plays in binding. Some evidence as to the nature of the ionizing groups that are involved in enzyme action is provided by a consideration of the magnitudes of the pK values. The pH behavior of retention time of chymotrypsin is affected by two ionizing groups of pK values 7 and 5. The only group in proteins having a pK of about 7 is histidine and groups of pK of about 5 are aspartate and glutamate. But glutamate does not exist in the neighborhood of the active center of chymotrypsin [7]. Thus, it can be concluded that both histidine and aspartate should participate in chymotrypsin-4-phenylbutylamine binding.

Although many of the enzyme-substrate or enzyme-inhibitor complex were investigated by means of X-ray work, the affinity chromatography technique can also be used as seen in this study.

## CONCLUSIONS

The proposed model of adsorption mechanism explains very well the effect of pH of eluent on the adsorption of α-chymotripsin through an affinity chromatograph.

The approximate pK values were obtained from the experimental data. The knowledge of the difference and the magnitude of each constant may permit to draw conclusions about the nature of enzyme-ligand complex.

The achievement of affinity chromatography is greatly affected by the ligand concentration of gels.

## NOMENCLATURE

| | | |
|---|---|---|
| $C$ | : | concentration of a solute in the mobile phase |
| $C_0$ | : | feed concentration of a solute |
| $C_s$ | : | concentration of a solute in the stationary phase |
| $D_r$ | : | effective intraparticle diffusivity of a solute |
| $D_z$ | : | axial dispertion coefficient of a solute |

E : enzyme
EH : enzyme and hydrogen ion complex
EL : enzyme-ligand complex
K : partition coefficient of a solute between mobile and stationary phases
$K_a, K_a'$ : acid ionization constant
$K_b, K_b'$ : base ionization constant
$K_e$ : equilibrium constant of a solute between stationary and adsorbed phases
$k_A$ : adsorption rate constant
$k_D$ : desorption rate constant
$k_f$ : mass transfer coefficient
$l_b$ : bed length
$\bar{L}$ : immobilized ligand
$m_1$ : first noncentral moment (min)
q : concentration of the adsorbed solute to gel matrix
r : radial coordinate in the gel
$r_s$ : radius of the gel
t : time
$t_o$ : time required for one sample square pulse (min)
U : interstitial velocity
$U_o$ : cross-sectional velocity
u(t) : unit step function
V : elution volume of eluent
$V_m$ : mobile phase volume in a column of affinity chromatography
[·] : concentration of
$\varepsilon$ : void fraction of the bed
$\varepsilon_s$ : porosity of the gel
$\phi$ : $(1-\varepsilon)\varepsilon_s/\varepsilon$

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