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Modeling and Kinetic Determination in Affinity Precipitation of Trypsin

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ABSTRACT

A mathematical model was proposed to allow the analysis of kinetic enzyme in experimental of affinity precipitation system. The methodology was tested using a system composed of enzyme, ethylene glycol and conjugated PABA-poly (NIPAM). N-isopropylacrylamide (NIPAM) is one of the monomers that have appealed to a great deal of investigation in the recent years. Its homopolymer NIPAM possesses temperature sensitivity and can act as a functional polymer with great potential. A water soluble ligand bound polymer has been synthesized by Electron Beam Irradiation for the purpose of affinity precipitation of trypsin. The affinity polymer was formed by ligand-PABA. The binding efficiency of trypsin to this polymer was dependent upon the ratio of (NIPAM), mercaptopropionic acid (MPA) as a chain transfer reagent and p-aminobenzamidine (PABA) as ligand that used in the polymer synthesis. The amount of precipitated of poly (NIPAM) present in the polymer solution also greatly affected the trypsin binding efficiency. The total binding capacity of trypsin molecules to ligand molecules approached the theoretical value which was considerably higher than that of insoluble gel matrices. Bound trypsin could be easily eluted by the ethylene glycol solution. At low molecular weight of poly (NIPAM), the conjugate polymer solution was very stable and retained its high capacity for trypsin recovery over a long period of time. The proposed analysis and simulation of kinetic parameters may be helpful in affinity precipitation technique for advanced application.

Keywords: modeling, mathematics, enzyme kinetics, PABA-ligand, trypsin, affinity precipitation

INTRODUCTION

In recent years, affinity precipitation has emerged as a useful approach for reducing the number of steps in the purification protocols for proteins (Pecs et al., 1991; Gupta and Mattiasson, 1992; Kamihara et al., 1992). Choosing ligand-trypsin as a model affinity pair like many other previous workers (Luong et al., 1988; Senstad and Mattiasson, 1989; Pecs et al., 1991), we report here affinity precipitation of trypsin from a commercial

crude preparation with the conjugate of STI with Eudragit S-100 which is an enteric coating methacrylate polymer (Kamihara et al., 1992).

The recovery and purification of trypsin from beef pancreas is of industrial interest since this enzyme has been widely used for medical purposes, beer haze removal and meat tenderization. Purification of trypsin by conventional techniques is difficult due to the presence of the enzyme chymotrypsin which is very similar to trypsin. Development of affinity chromatography using specific trypsin inhibitors over the last decade has led to more purified trypsin fractions. However, these systems are based on immobile and insoluble matrices which are laborious, costly and more vulnerable to degradation. Affinity chromatography also employs a packed column which is more susceptible to fouling and clogging, i.e. reducing the system's productivity. As a result, conventional affinity chromatography can only be used for processing fairly clear solutions. There is a recent breakthrough in the isolation and purification of biomolecules such as proteins, enzymes and hormones.

N-isopropylacrylamide (NIPAM) polymer has long been known of its temperature responsiveness and actually it has been employed for the technique in question. (Mattiasson *et al.,* 1998, and Vaidya *et al.,* 1999) have randomly copolymerized NIPAM with a second monomer that provided coupling sites for the ligand. Their preparations have produced fairly successful results. However, their preparations seemed to be heterogeneous in size and to have more than one ligand in each polymer chain. With such polymers, it will be hard to acquire precise and generalized knowledge on fundamental properties the polymer should have to be widely accepted in the technique.

The purpose of this study was to develop a macromolecular water soluble polymer bearing p-aminobenzamidine, a strong trypsin inhibitor, and to determine optimum conditions for recovery trypsin to the conjugate polymer. The procedures for elution of bound trypsin from the polymer were also investigated. Also of interest in this study was experiment performed to examine the stability, reusability of the affinity polymer and develop model kinetic and simulation by using Microsoft EXCEl.

Model Development of Kinetics Enzyme

Monod Kinetics as describing a chain of enzymatically mediated reactions with a limiting step described by Michaelis-Menten kinetics. The basic assumption behind Michaelis-Menten Enzyme kinetics is that enzymes catalyze reactions by first forming an enzyme-substrate complex. This substrate complex will either decay back to enzyme and substrate or irreversibly decay to enzyme and product. These chemical reactions for complex formation and product formation respectively are:

$$S + E \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \qquad (1)$$

$$ES \xrightarrow{k_3} E + P \qquad (2)$$

Where: S = substrate

E = enzyme

ES = enzyme-substrate complex

P = product

k_1 = rate constant for complex formation

k_2 = rate constant for reverse complex formation

k_3 = rate constant for product formation

The rates for the above reactions would be as follows:

$$\left.\frac{d\{ES\}}{dt}\right|_{k_1} = k_1\{S\}\{E\} \qquad (3)$$

$$-\left.\frac{d\{ES\}}{dt}\right|_{k_2} = k_2\{ES\} \qquad (4)$$

$$\frac{d\{P\}}{dt} = -\left.\frac{d\{ES\}}{dt}\right|_{k_3} = k_3\{ES\} \qquad (5)$$

Furthermore, it is assumed the above set of equations are in equilibrium such that:

$$\frac{d\{ES\}}{dt} = 0 \qquad (6)$$

By solving material balance and than:

$$\frac{d\{P\}}{dt} = \frac{k_3 E_T \{S\}}{k_m + \{S\}} \qquad (7)$$

Which is analogous to Monod kinetics, k_3 is analogous to the maximum specific substrate utilization rate, E_T is analogous to biomass concentration, and k_m is analogous to the half saturation constant. Monod kinetics and its variations, along with other bio-kinetic equations will be presented in the following discussion. In competitive inhibition an inhibitory complex can combine with the controlling enzyme in addition to the reaction equation (1). This additional complex prohibits the enzyme from forming the complex with the substrate of interest.

$$I + E \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EI \qquad (8)$$

Where: I = inhibitor

E = enzyme

EI = enzyme-substrate complex

P = product

k_4 = rate constant for complex formation

k_5 = rate constant for reverse complex formation

Equation now looks like:

$$E_T = \{ES\} + \{EI\} + \{E\} \qquad (9)$$

Where: E_T = total complexed and un-complexed enzyme

$\{E\}$ = concentration of free enzyme

$\{ES\}$ = concentration of substrate-enzyme complex

$\{EI\}$ = concentration of inhibitor-enzyme complex

After substitution of equation (8) (with the assumption of equilibrium) equation (9) becomes:

$$E_T = \{ES\} + \left(\frac{k_4}{k_5}\{I\} + 1\right)\{E\} \quad (10)$$

The same derivation for Michaelis-Menten Kinetics as presented above applies:

$$E_T\{S\} = \left(\left(\frac{k_4}{k_5}\{I\} + 1\right)\frac{k_2 + k_3}{k_1} + \{S\}\right)\{ES\} \quad (11)$$

so let

$$k_I = \frac{k_4}{k_5} \quad (12)$$

therefore

$$\frac{d\{P\}}{dt} = \frac{k_3 E_T\{S\}}{k_m\left(1 + \{I\}/k_I\right) + \{S\}} \quad (13)$$

Where: $\{P\}$ = concentration of product

$\{ES\}$ = concentration of enzyme-substrate complex

$\{S\}$ = concentration of substrate

$\{I\}$ = concentration of inhibitor

E_T = total complexed and un-complexed enzyme

k_1 = rate constant for complex formation

k_2 = rate constant for reverse complex formation

k_3 = rate constant for product formation

k_m = "half-saturation" concentration

k_I = "saturation" constant for inhibitor

MATERIALS AND METHODS

Synthesis of Poly (NIPAM) Polymer

A 10 g of NIPAM was dissolved in 20 ml of ethanol. To the solution 0.1 g of 2,2`-Azobis- (isobutyronitrile) (AIBN) was added together with 0.4 ml of MPA. The mixture was incubated at 60°C for 20 hr under atmosphere of nitrogen. The resulting precipitate was isolated by precipitation into diethyl ether. The molecular weight was determined by gel permeation chromatography (GPC). The polymer produced was characterized by FTIR. Precipitation temperature was determined by measuring OD at 470 nm after heating polymer solution in water (0.2% w/v) at various temperatures for a couple of minutes. Dry weight recovery after precipitation was determined by measuring dry weight of the precipitate obtained after heating polymer solution in water (5% w/v) at 38°C for 15 min (Syaubari et al., 2004).

Conjugation of PABA to Poly(NIPAM) Polymer

A 1 g of carboxylated poly(NIPAM) was dissolved in 10 ml of distilled water. A 450 mg of PABA was added into it, and the pH was brought to 6.5 with NaOH. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (550 mg) was added in 3 portions over 12 hr stirred at room temperature. The PABA-linked polymer was precipitated by increasing the temperature above the LCST. The precipitate was washed twice with cold distilled water and once with 10 mM Tris-HCl buffer (pH 8.1). The polymer was dissolved in distilled water to get 10% w/v of stock solution. The conjugation of PABA was estimated spectrophotometrically (Syaubari et al., 2004).

Recovery of Trypsin

One ml of PABA-polymer solution in water (10%, w/v) was mixed with 1 ml solution was mixed with 1 ml solution containing 48 μg of trypsin and 48 μg of chymotrypsin in 50 mM Tris, 10 mM $CaCl_2$, pH 8.1. The mixture was incubated at 25°C for 15 min and precipitate was recovered by centrifugation. The precipitate was dissolved in 2 ml solution containing 50 mM Tris, 10 mM $CaCl_2$, pH 8.1 plus 50% v/v ethylene glycol. The PABA-polymer was thermoprecipitated and separated by centryfugation. The residual activity of trypsin in the supernatant was assayed according to Erlanger *et al.*,(1961) using BAPNA as a substrate for trypsin and for chymotrypsin using N-benzoyl-L-tyrosine ethyl ester according to published methods. The elution solution for recovery of trypsin is used the ethylene glycol.

RESULTS AND DISCUSSION

PABA was covalently linked to the pendant carboxyl groups of these polymers via the amide link using water soluble carbodiimide. One gram of polymer was dissolved in 10 ml of distilled water at cold room 10±5°C, and EDC and PABA were added to it. The reaction mixture was stirred at 10±5°C for 12 h. The molar ratios of EDC:carboxyl groups and PABA:carboxyl groups were both 10:1. The PABA-linked polymer was precipitated by increasing the temperature above its LCST. The polymer was washed three times with cold double distilled water and dried-up in oven at 80°C overnight. The conjugation of PABA to polymer was estimated by UV-spectrophotometer at optical density 300 nm. The results of PABA conjugation were reported including optimally of conjugation by adjusting effect of molecular weight (Figure 1),

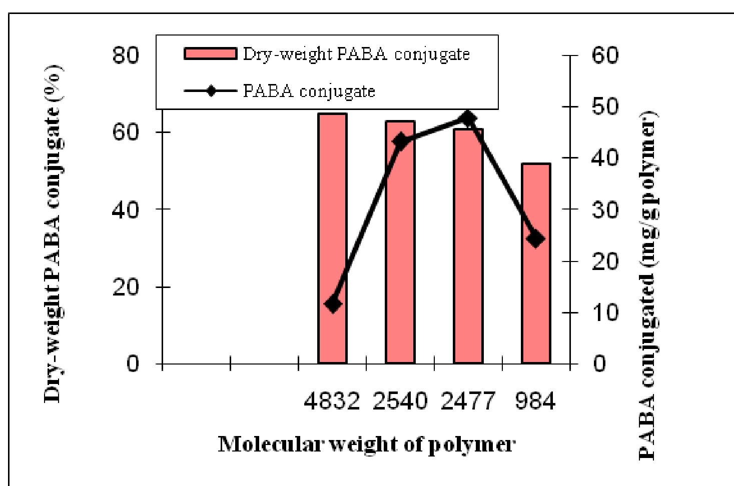

Figure 1: Effect of Molecular Weight for Conjugation PABA to Poly(NIPAM)

As indicated by several researchers, the trypsin inhibition kinetics of p-aminobenzamidine (PABA) with respect to the substrate N'-benzoyl-L-arginine p-nitroanilide is of a competitive manner. In this study, it was further observed that the polymer containing PABA exhibited the same mechanism of trypsin inhibition. The inhibition constant, Ki, was determined to be 0.60 + 0.05 mM (expressed in monomer molarity) for polymers containing a small percentage of PABA. However, Ki increased significantly. When the polymer contained a high percentage of PABA.

A balance, hence, must be made between the number of available binding sites (high PABA content) and the strength of the binding force (corresponding to low Ki). The polymer having an NIPAM/AIBN/MPA of 100 : 1 : 6 apparently offered a good compromise, it was therefore chosen for further studies.

Trypsin Recovery of the PABA-Poly (NIPAM) Polymer

Following the addition of conjugate PABA-poly (NIPAM) (10%, w/v) 1 to a mixture of trypsin and chymotrypsin (48 μg of each enzyme, in 50 ml Tris buffer containing 10 mM CaCl2). After 15 minutes of the experiment, the activity of chymotrypsin detected in the precipitate and the supernatant was 5 and 95%, respectively. Meanwhile, the precipitate contained ~86% of trypsin activity, the remaining 10% was in the supernatant. It is, therefore, evident that the polymer possesses specific affinity toward trypsin. The p-Aminobenzamidine has been experimentally determined to be a competitive inhibitor (Ki = 0.930 mM, data not shown) with respect to the substrate N~-benzoyl-L-arginine p-nitroanilide for trypsin.

TABLE 1: Enzyme Activity

Enzyme Activity (%)						
Binding					**Recovery**	
	Trypsin		**Chymotrypsin**		**Trypsin**	
No.	Precipitate	Supernatant	Precipitate	Supernatant	Precipitate	Supernatant
1	92	8	5	95	6	86

Simulation Profiles of Kinetic Parameters in Affinity Precipitation Enzyme

The System of differential equations developed here forms the basis of a time profile for the same derivation for Michaelis-Menten Kinetics. A simulation profile was generated using kinetic parameters from experimental data for recovery of trypsin by affinity precipitation technique. By using kinetic parameters as shown in Table 2 and applied to equation 13, it shows simulation profile in Figure 2.

TABLE 2: Kinetic Parameters are used in the Construction of Simulation

Kinetic Parameters	Unit	Values
K_3	mM/h	1
E_t	mM	1.2
S	mM	2
K_m	mM	2.835
I	mM	2.4
K_i	mM	0.93

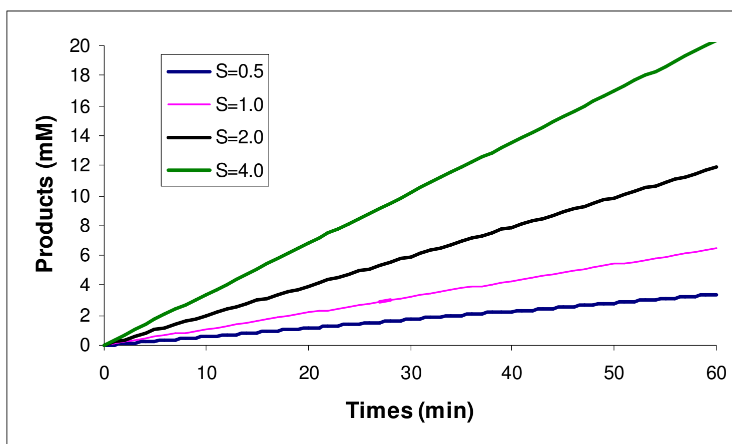

Figure 2 Simulation Profiles of Kinetic Parameters, Effect of Substrate concentration

CONCLUSIONS

In this study, a conjugate polymer was synthesized and used together with ethylene glycol as dissociation solution to recover of trypsin from a mixture of trypsin and chymotrypsin. The synthesis of the water soluble conjugate poly (NIPAM) is simple, utilizes no spacers as commonly required to produce insoluble matrices for conventional affinity chromatography. Furthermore, the enzyme-ligand binding occurs in a homogenous phase, so is probably more effective than in the heterogeneous systems of conventional affinity chromatography. At low molecular weight of poly (NIPAM), the conjugate polymer solution was very stable and retained its high capacity for trypsin recovery over a long period of time. This study also developed model kinetic of enzyme in affinity precipitation system and simulated of kinetic parameters.

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