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Diaminoalkane as Spacer Arm between Polystyrene and β-Cylodextrin in Affinity Chromatography for α-Amylase Separation

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ABSTRACT
Stationary phase design in affinity chromatography has to fulfill at least two requirements: non-specific interaction between solid support and or spacer arm and protein target or others is minimal and specific interaction between ligand and protein target is maximal. In this study, stationary phases that consist of polystyrene (PS) as solid support, diaminoalkane as spacer arm and with and without βCD as a ligand are performed in α-amylase separation. Therefore, the research was carried out in two steps; stationary phase preparation and adsorption parameter determination towards α-amylase. Stationary phases that consist of PS, 1,6-diaminohexane and βCD (PS–DAH–βCD) and without βCD (PS–DAH) were synthesized. Stationary phase structure was confirmed by FTIR, ^{13}C NMR solid, ^{13}C dan ^{1}H NMR liquid spectroscopy. Other stationary phases, PS–DAP–βCD and PS–DAP that consist of 1,2-diaminopropane (DAP) as spacer arm was synthesized in the previous research. Dynamic α-amylase adsorption isotherm on PS–DAP and PS–DAH shows that both of them did not fix any adsorption isotherm model. The average binding capacity are 0.071 and 0.31 mg/0.1 g resin, respectively. In another hand, PS–DAP–βCD and PS–DAH–βCD fix isotherm adsorption Langmuir model and the adsorption parameters; the maximum binding capacity (q_m) are 5.5 and 2.1 mg/0.1 g resin, adsorption equilibrium constant (K_A) are 0.01965 and 0.01925 μL^{-1} and desorption rate constant (k_2) are 0.201 and 0.774 mg s^{-1}, respectively. Due to adsorption parameters, PS–DAP–βCD offer the best adsorption capacity and selectivity as stationary phase in affinity chromatography for α-amylase separation.
Keywords: α-Amylase, β-Cyclodextrin, Diaminoalkane, Polystyrene

I. Introduction

α-Amylase is one of the most important industrial enzyme such as its use in sugar, brewing, textile, detergent and bioethanol industries [1]. In its separation, some methods have been reported [2—4]. One of the reported methods is affinity chromatography [5—7]. The advantage of this method is it could shorten the separation and purification route that reduces separation and purification cost and improves recovery/yield [8]. Hence, affinity stationary phase design for α-amylase separation is undertaken.

Affinity chromatography is based on specific and reversible interaction between stationary phase and product target in column. Stationary phase or adsorbent in affinity chromatography consists of solid support, spacer arm that gives space between ligand and solid support, and ligand that interacts specifically and reversibly with the product target. Some ligands that have been used in affinity chromatography to separate α-amylase are cibacron blue, starch and β-cyclodextrin. β-cyclodextrin (βCD) is known as one of α-amylase inhibitors [9], so it is used as ligand in affinity chromatography for α-amylase separation and is developed in this research.

To reach a good separation efficiency in affinity chromatography, stationary phase should be high specificity, high mass transfer, high binding capacity, low non-specific adsorption, incompressibility, resistance to alkaline condition for sanitization, chemically stability, low ligand leakage and cost effectiveness [10]. The successful stationary phase design depends not only on ligand but also solid support and spacer arm selection.

Spacer arm that has slight size causes ligand to interact with solid support and solid support hinder the ligand to be accessed. These can deduct the ability of protein target to access the ligand thus reducing binding capacity. Extensive spacer arm can interact nonspecifically with protein target and others so it could reduce specificity. Spacer arm also can be folded that shorten the spacer arm and reduce binding capacity [11]. Spacer arms that used to bind βCD to solid support are isocyanate carbamate, diaminoalkane, epoxyether [12]. These compounds have potential non-specific interaction with protein through hydrogen bond. The compound that has the

weakest non-specific interaction between spacer arm and protein target is diaminoalkane.

Diaminoalkane as spacer arm was used to bind βCD to silica, polyacrylamide, sepharose, and polyvinylamine, but it has not been published yet as spacer arm to bind βCD to Polystyrene (PS). This polymer is resistance at pH more than 7 for sanitization, hydrophobic and suitable for polar mobile phase, incompressibility and has better mechanical rigidity for using at high pressure. Besides, this polymer is quite durable, chemically and immunologically inert, efficient, simple, low-cost and has a long shelf life [13]. These characteristics affect PS as potential solid support in affinity chromatography for α-amylase separation.

In general, affinity chromatography was represented mostly by Langmuir adsorption model to describe protein adsorption on adsorbent [14]. The adsorption parameters such as q_m or maximum banding capacity gives information about maximum amount of adsorbate that can be adsorbed per amount of adsorbent and *K*d or dissociation constant gives selectivity information of affinity system [15].

The use of βCD as a ligand in α-amylase separation has been reported, however none of the publication reported the use of diaminoalkane as spacer arm between PS and βCD in α-amylase separation. Here, we inform the characteristics of stationary phase consists of PS as solid support, βCD as ligand and 1,2 diaminopropane (DAP) that has been reported in previous work [16] and 1,6 diamine hexane (DAH) as spacer arm in α-amylase separation. Adsorption parameters such as q_m, *K*d and desorption constant rate were determined to describe the interaction between α-amylase and stationary phase.

II. Material and Methods

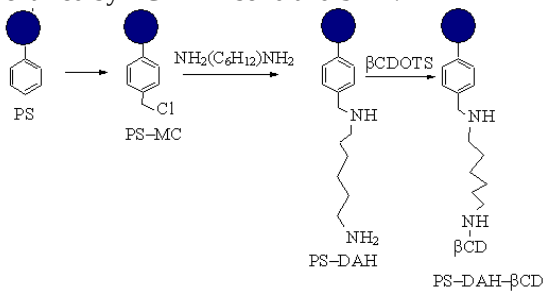
2.1 Material

Linear PS was given by Abestyrindo Indonesia, α-amylase, diaminohexane, and DMSO were purchased from Wako (Japan), βCD, glycin, succinate acid, sodium phosphate monobasic, DMF, pyridine, CTAB, p-toluensulfonyl chloride or tosyl chloride, soluble starch and toluene were purchased from Sigma and Bradford reagent was purchased from Thermo Scientific.

2.2 Methods

2.2.1 PS–DAH and PS–DAH–βCD preparation

PS–DAH and PS–DAH–βCD were prepared (Scheme 1) described as PS–DAP and PS–DAP–βCD preparation in previousl report [16]. First, PS was activated to produce PS–MC. After that, chloride in PS–MC was substituted by DAH. About 1.00 g PS–MC was poured to three neck flask that contained 120 ml DAH. The mixture was refluxed at 80^{o}C for 12 hours. The mixture was added drop wise by sulphate acid 0.9% to remove un-reacted DAH. White precipitated was filtered and washed by NaOH solution till pH of solution was 7, distilled water and alcohol, respectively. White precipitated was characterized by FTIR, ^{13}C dan ^{1}H NMR liquid. To activate βCD, it was reacted with tosyl chloride (ClOTS) that produced βCDOTS. The last step was grafting βCD to PS–DAH as follow: about 1.00g PS–DAH was dissolved in DMF in three necked flask. βCDOTS was added to the solution. The mixture was stirred and refluxed at 80^{o}C. The reaction took 24 hours. Reaction mixture was poured into distilled water while stirred. The white precipitate was filtered, washed with distilled water and alcohol and dried at 50^{o}C for 7 hours. The resin obtained was identified by ^{13}C NMR solid and SEM.



Scheme 1. PS–DAH, and PS–DAH–βCD preparation

2.2.2 Dynamic adsorption test

The dynamic adsorption test of different stationary phase was conducted as follow: 0.10 g of dried PS–DAH, PS–DAP, PS–DAH–βCD or PS–DAP–βCD is packed into 100 mm x 2.5 mm i.d. glass chromatography column. Buffer and α-amylase solution were filtered and all columns were equilibrated by universal buffer pH 7 before using. The dynamic adsorption test experiment was carried out according to the reported procedure [17]. 50, 100, 150, 200, 300, and 400 µL of α-amylase solution (14.8 mg/mL in universal buffer pH 7) were introduced to each column at 0.25 mL/min using peristaltic pump (Masterflex, Cole palmer). The column was dried by pumped air and then adsorbates were eluted using universal buffer pH 7. Protein concentration in eluate was determined by Bradford methods using spektrofotometer UV-Vis (HP 8453 Agilent Technologies). Columns were cleaned by distilled water and kept at 4^{o}C after used. The procedure was repeated two times in different column and the average values of adsorbate amount are reported in this paper.

2.2.3 Desorption test

Desorption test was performed to determine desorption rate constant as follows: 300 µL of α-amylase solution (14.8 mg/mL in universal buffer pH 7) were introduced to chromatography column that

packed with PS–DAH–βCD and PS–DAP–βCD at 0.25 mL/min. The column was dried by pumped air. Adsorbates were eluted using 3 mL universal buffer pH 7 at 0.25 mL/min. Every 100 µL eluate was taken and protein concentration of eluate was determined.

III. Theory

The adsorption isotherm study was performed to evaluate stationary phase affinity to α-amylase. Isotherm Langmuir is often used to describe adsorption data of protein. The adsorption parameter such as q_m and K_A was determined by adsorption process and desorption rate constant was determined by elution process.

$$A + \alpha\text{-Amylase} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} A\text{—}\alpha\text{-Amylase}$$

The forward and reverse reaction rate constants k_1 and k_2, respectively, will be defined with respect to A (free surface area).

Adsorption rate:

= - dA/dt

∞ (adsorbate concentration) (free surface area)

∞ (adsorbate concentration) (total surface area – used surface area)

A is equal to amount of adsorbate that adsorb to adsorbent

$= dq/dt \propto C (k_1 q_m - k_1 q)$ (1)

q = amount of adsorbate that adsorb to adsorbent/g adsorbent

q_m = maximum amount of adsorbate that can be adsorb per g adsorbent

C = α-amylase concentration that introduced to adsorbent in constant volume

Reverse or desorption reaction rate:

= dA/dt ∞ used surface area or

$= dq/dt \propto k_2 q$ (2)

Total adsorption rate

$= C (k_1 q_m - k_1 q) - k_2 q$ (3)

In equilibrium state, total adsorption rate = 0,

$k_1 C (q_m - q) = k_2 (q)$, (4)

$q = q_m k_1 C/(k_2 + k_1 C)$ (5)

$K_A = k_1 / k_2$, K_A is isotherm equilibrium constant

$q = q_m K_A C/(1 + K_A C)$ (6)

or $C/q = 1/(q_m \times K_A) + C/ q_m$ (7)

For constant concentration and varied volume that introduced to column, the equation (7) will be

$V/q = 1/(qm \times K_A) + V/qm$ (8)

From (8), q_m and K_A could be determined by plot curve V/q to V.

Reverse or desorption reaction rate

$dq/q = k_2 dt$ (2)

$\int dq/q = \int k_2 dt$ (9)

$Ln(q) = k_2 t$ (10)

From (10), desorption rate constant can be determined by plot curve Ln(q) to t. Adsorption rate constant can be determined from K_A value.

IV. Result and Discussion

4.1 PS–DAH and PS–DAH–βCD characteristics To study the interaction between α-amylase and stationary phase, PS–DAP, PS–DAH, PS–DAP–βCD and PS–DAH–βCD, stationary phases were prepared. The presence of amine functional group in PS–DAH was marked by wave number at 3460 cm^{-1} (d) in FTIR spectra. Doublet peak indicates that it is free primary amine functional group.

The presence of DAH carbon atom in PS–DAH was confirmed by ^{13}C NMR spectra (Fig.1). The presence of quaternary aromatic carbon is exhibited by chemical shift at 145-146 ppm. The peak at 128 and 41 ppm relates to tertiary aromatic carbon and aliphatic main chain methylene and methine carbons, respectively. The shoulder at methylene-methine peak at 41 ppm is assigned to chloromethyl carbon. The peak at 27 ppm is attributed to methyl carbon present in the precursor. These peaks almost present in PS ^{13}C NMR spectra [18]. There are 6 peaks at 42—48 ppm which is characteristic of aliphatic hexine carbon in DAH.

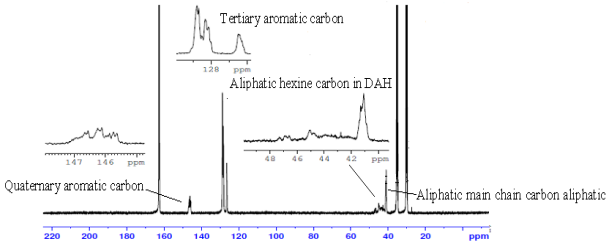


Figure 1. ^{13}C NMR spectra of PS–DAH

The presence of hexine hydrogen in DAH is confirmed by ^{1}H NMR spectra (Fig.2). The main assignments are aromatic hydrogen at 7.2 and 6.7 ppm and aliphatic hydrogen at 1.3—2.2 ppm [19]. The peaks at 3.4, 3.19, 2.8, 2.6, 2.59 and 2.58 ppm indicate aliphatic hexine hydrogen in DAH. FTIR, ^{13}C and ^{1}H NMR spectra indicate that grafting DAH to PS was successfully performed.

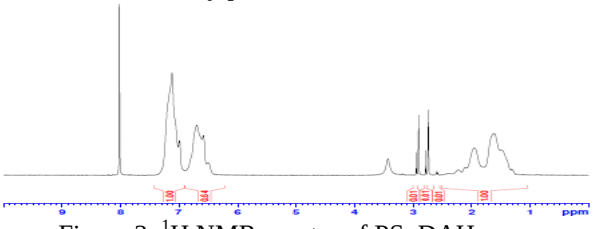


Figure 2. ^{1}H NMR spectra of PS–DAH

The presence of βCD in PS–DAH–βCD is characterized by the use of solid state NMR technique of ^{13}C NMR CP-MAS. In the ^{13}C NMR

CP-MAS spectra of PS–DAH–βCD (Fig.3) the peak chemical shifts are similar to ^{13}C NMR chemical shift at PS–DAH. The peak chemical shift at 145-150, 125-135, 35-45 and 10 ppm attributed to quaternary aromatic carbon, tertiary aromatic carbon, overlapping aliphatic hexine and main chain methylene and methine carbon and methyl carbon present in the precursor, respectively. Broad chemical shift at 182-210 ppm indicates spinning side band (SSB) and peak at 165-175 ppm indicates carbonyl function in polymethylmethacrylate rotor cap. These similar peak chemical shifts are found in PS ^{13}C NMR CP-MAS Spin spectra [20]. The spectra obtained clearly shows glucopyranosyl carbon. Signals at 104-110 and 65-70 ppm are attributed to C1 and C6 carbons in hexapyranoses, respectively. The overlapping signal at about 85-95 ppm is associated with C2, C3, C4, and C5 carbons hexapyranoses in βCD. It is clear that grafting βCD to PS–DAH is successfully carried out.

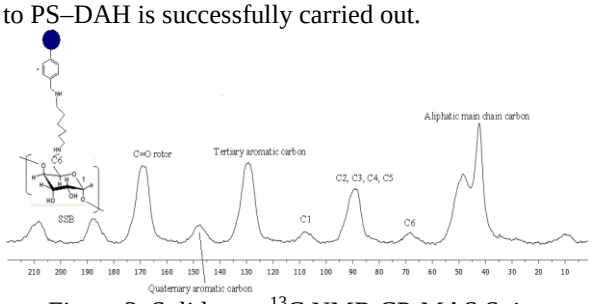


Figure 3. Solid state ^{13}C NMR CP-MAS Spin 5000Hz spectra of PS–DAH–βCD

SEM photograph shows that the particle size is almost the same as PS–DAP–βCD, in between 50—70 μm [16].

4.2 Adsorbent characteristics of stationary phase

The adsorption isotherm study was performed to evaluate the equilibrium capacity of the α-amylase adsorb to PS–DAP, PS–DAH, PS–DAP–βCD and PS–DAH–βCD at specific α-amylase volume using dynamic system. Figure 4 shows the adsorption isotherm of α-amylase on PS–DAP and PS–DAH. None adsorption isotherm model was fixed and the average amount of α-amylase adsorb per g PS–DAP and PS–DAH are 0.071 and 0.31 mg, respectively.

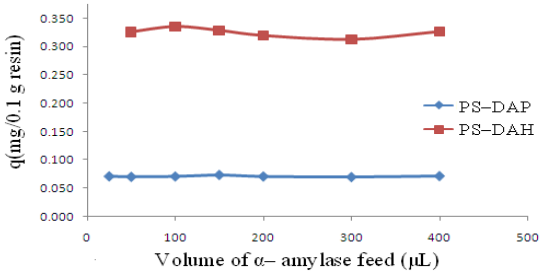


Figure 4. Adsorption isotherm of PS–DAP and PS–DAH

The q average value of PS–DAH is 4.4 times q average value of PS–DAP. It can be explained as follow: α-amylase interacts nonspecifically with free primary amine functional group in PS–DAH and PS–DAP. Primary and secondary amine that is near can do interaction with each other through hydrogen bond in PS–DAP (1,2–diaminopropane), see Figure 5. Thus, it reduces the ability of free primary amine functional group to capture α-amylase from the bulk. On the other hand, primary amine is far from secondary amine in PS–DAH (1,6–diaminohexane), so it is difficult for them to do interaction with each other through hydrogen bond. It might cause the free primary amine functional group to conveniently capture α-amylase from the bulk.

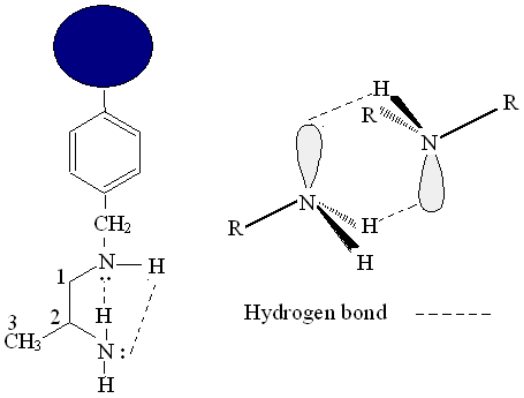


Figure 5. Hydrogen bond between primary and secondary amine in PS–DAP

Figure 6 shows that α-amylase adsorption on PS–DAP–βCD and PS–DAH–βCD fix Langmuir model. The parameter of adsorption was determined by equation (8) through plotted V/q to q. The parameter of desorption was determined by (10) through plotted ln(q) to time. Table 1 shows the parameter of adsorption and desorption isotherm.

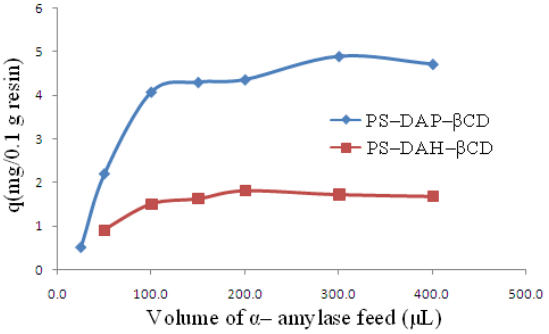


Figure 6. Adsorption isotherm of PS–DAP–βCD and PS–DAH–βCD

The binding capacity of PS–DAP–βCD and PS–DAH–βCD is increased drastically than average binding capacity of PS–DAP and PS–DAH. It might be caused by specific interaction between α-amylase

and βCD in PS–DAP–βCD and PS–DAH–βCD retain them in column. The q_m value of PS–DAP–βCD is 2.6 times value of PS–DAH–βCD. This case might occur because of the spacer arm that has six carbons in PS–DAH–βCD was folded and the βCD become near to PS. It prevents βCD to capture α-amylase from the bulk. It is confirmed by K_D (K_A^{-1}) value. Dissociation constant (K_D) value of PS–DAP–βCD is smaller than K_D value of PS–DAH–βCD. It indicates that the interaction between α-amylase and PS–DAP–βCD is more specific than the interaction between α-amylase and PS–DAH–βCD. This argument is confirmed by k_2 value that k_2 value of PS–DAH–βCD is greater than k_2 value of PS–DAP–βCD. It indicates that the interaction between α-amylase and βCD in PS–DAH–βCD is not as stable as in PS–DAP–βCD, thus α-amylase is desorbed easily.

Table 1. Isotherm adsorption parameter of synthesized adsorbent

adsorbent	qm (mg/0.1 g resin)	K_A (μL^{-1})	k_2 (mg det^{-1})	k_1 (mgμL^{-} det^{-1})
PS–DAP	0.071	-	-	-
PS–DAH	0.31	-	-	-
PS–DAP–βCD	5.4945	0.01965	0.201	0.004
PS–DAH–βCD	2.1053	0.01925	0.327	0.006

V. Conclusion

The stationary phases that consist of PS as solid support, diaminohexane as spacer arm and βCD as ligand is successfully synthesized. The presence of βCD in PS–DAH–βCD and diaminohexane in PS–DAH and PS–DAH–βCD are confirmed by spectroscopy methods. Adsorption parameter describes that the interaction between PS–DAP–βCD and PS–DAH–βCD and α-amylase based on specific interaction. Non-specific interaction is occurred through interaction between α-amylase and amine group in spacer arm. Due to q_m, k_2 and K_D value, PS–DAP–βCD is preferable as stationary phase in affinity chromatography for α-amylase separation.

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