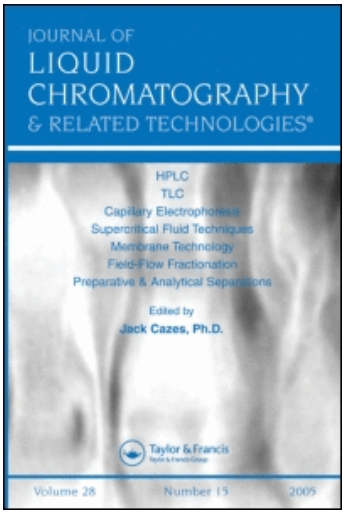

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PREPARATION OF HIGH PERFORMANCE AFFINITY CHROMATOGRAPHY PACKINGS FOR SPECIFIC ADSORPTION TO α-AMYLASE AND PURIFICATION OF CRUDE α-AMYLASE

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SUMMARY

A new packing material used to separate α–amylase in high performance affinity chromatography has been synthesized and its chromatographic characteristics have been investigated. In addition, a procedure for purification of α–amylase has also been established.

This packing material consists of starch and epoxy silicane bonded to silica surface. When a phosphate buffer solution is used as eluent and stepwise elution is operated, a crude α–amylase can be rapidly purified and to obtain a sharp peak which is entirely separated from other proteins. After the purification, the recovery of α–amylase is higher than 88%. Compared with unpurified crude enzyme, its purity is increased upto 20–fold. The loading capacity of the stationary phase for α–amylase is more than 4.6mg/g. The purity of α–amylase purified by this kind of affinity column may be comparable to the standard one made by Sigma Company.

INTRODUCTION

α–Amylase is a kind of hydrolytic enzyme for the catalysis of starch. It mainly exists in pancreas, saliva, blood, urine in animal body, bacteria, mold and various kinds of high

plant. α–Amylase is usually used as food additive agent. α–Amylase with high purity may also be used as an enzyme agent to do research about the characteristics of biological function and the actional mechanism of enzyme as well as the determination of a thermodynamic or a kinetic equilibrium constant of some chemical reactions. The isolation of α–amylase in a plant is usually carried out with organic solvent or ammonium sulfate precipitation technique and α–amylase obtained in this way is a crude one with low purity. Kruger et al. [1], Macgregor et al., [2] and Silvanovich et al. [3] used CM–cellulose and cyclohepta–amylose–epoxy–sepharose 6B column, respectively, to purify α–amylase and obtain it with high purity. Weber et al. [4] bonded starch to Sephadex G–10 gel to obtain affinity packings with specific adsorption to α–amylase. They purified a crude enzyme solution and obtained a homogeneous product with those adsorbents. However, the chromatographic operation must carry out under lower pressure. Therefore, its purification process is not only spending a long time, but also losing a lot of its bioactivity. Though, Szepesy et al. [5] investigated some α–amylases by High–Performance Hydrophobic Interaction Chromatography, the selectivity of the method was low. To overcome these disadvantages, starch with specific affinity to α–amylase was bonded to silica surface to prepare a kind of high performance affinity chromatography (HP–AFC) paking material. Using it, α–amylase with high purity was rapid isolated and purified from crude one. The purified product has high bioactivity and the purification procedure has high recovery as well as the column has long lifetime.

EXPERIMENTAL

Equipment

An LC–6A liquid chromatograph (Shimadzu, Kyoto, Japan) was used which consists of two pumps (LC–6A), a detector (SPD–6AV Variable–Wavelength, UV–Visible), column oven (CTO–6A), controller system (SCL–6A), data system (CRT–3A) and recorder (R–112). Stainless–steel column (4.0 × 100mm) was used and packed with a slurry packing apparatus (Chemico, Japan). PHS–25type pH–meter was obtained from The Second Analytical Instrument Co. (Shanghai, China), CS501 type ultra–thermostat bath (± 0.5℃) was bought from Chongqing Experiment–Equipment Plant.

Chemicals

Standard α–amylase (α–AMY, Bacillus anthracis, typ Ⅱ A) was obtained from Sigma Co. (St. Louis, MO,USA); Crude α–amylase was purchased from Biological and Chemical Reagent Plant of Hangchu University (Hangchu, Zhejiang Province, China); Silica of 7μm particle diameter with pore 50nm was bought from Beijing Chemicals Institute (Beijing, China); γ–glycidoxypropyltrimethoxysilane (GLDP) was obtained from Caixian Chemical Engineering Institute (Gaixian, Liaoning Province). All of other chemicals used in this study are of analytical grade. Pure water was bought and re–distilated.

Preparation of Reagents

1. Eluent: solution A: water; solution B: 0.12 mol / L phosphate buffer solution with pH 7.00

2. Preparation of standard solution of α–amylase: Weigh 6.35mg standard α–amylase and put into a tube. Dissolve it with 1.00ml pure water and then make centrifugation at 13,000r / min. for five minutes and then to transfer the clear solution into another tube.

3. Pre–treatment of crude α–amylase: The procedure for preparation of crude α–amylase solution is the same as that for standard one described above.

Synthesis of affinity packings

The procedure of synthesizing the packings was divided into two steps: The first step is to bond one kind of silicane containing epoxy group, γ–glycidoxypropyltrimethoxysilane (GLDP), to silica to obtain it with epoxy group on its terminal. The second step is to bond the specific substrate, starch, of α–amylase to the silica–GLDP to obtain the HPAFC packings.

1. Synthesis of silica–GLDP

A. Pre–treatment of silica: Put 10 gram silica into a beaker containg 50ml of 1.0 mol / L HCl and then make it vibration with ultrasonic for 15–20 minutes to remove micro air bubble in capillary or in pore of silica particles thoroughly. Having be filtered waste hydrochlorid solution, we transfered the wet silica into a 250ml flask containing 100ml of 1.0M HNO_3 , and then, refluxed it about 30 minutes to remove the soluble impurities in acid solution and to break the epoxy–bond on the surface of silica. After the suspension solution had been cooled, filtered on a sintered glass fileter crucible (No.4) with suction and washed with pure water until the filtrate had appeared neutrality, we made it dry in a constant–temperature at 90 to 110℃ for four hours.

B. Bonding GLDP to silica:

The procedure used to couple GLDP with the silica was a modification of a earlier published method [6] . All instruments should be previously dried. The porous silica (3g) was suspended in 50ml of sodium–dried toluene and 5.0ml of GLDP solution was added. In order to catalyse the reaction, 0.1ml of triethylamine (potassium hydroxide–dried) was added. After the suspension solution was shaked thoroughly, the slurry was refluxed for six hours. Then, the substituted silical was filtered off with suction by using a sintered glass filter crucible. The synthesized silica–GLDP was washed with a few solvents in the order: toluene, acetone and ether, 100ml for each, respectively, and then, it was dried in vacuum drier overnight.

2. Bonding of starch to silica–GLDP

The solid starch (1g) was added to a suspension of GLDP–silica (2g) in 40ml of sodium bicarbonate solution with pH 8.0. After removing air bubble with ultrasonic for 15 minutes, we made the starch and silica–GLDP react for six hours at 50℃ with continuous stirring. The suspension solution was filtered with suction by using a sintered glass filter crucible (No. 4) and then it was washed with methanol and water thoroughly. The specific

HPAFC packings for α–amylase synthesized according to the foregoing procedure then was dried in vacuum drier overnight.

Packing column and chromatographic conditions

The specific HPAFC packings (1.3 to 1.7g) was packed into 4.0 × 100mm stainless steel column by using slurry method with isopropanol at 5500 to 6000 P.S.I. And then, the packed column was equilibrated with eluent A and B for one hour, respectively, at flow rate 1.0ml / min. After sample solution was injected into the column, the impurity proteins in sample were eluted with the mixture solution of 99% eluent A and 1% eluent B for eight minutes. α–Amylase was eluted by stepwise elution with 100% B eluent only for one minute. Fig. 1 shows the chromatogram of α–amylase purified from crude and standard enzyme with the foregoing chromatographic conditions. The peak 1 in it is of impurity and the peak 2 is of the α–amylase peak.From the fig. I –(b), we know that the standard enzyme from Sigma Co. can be further purified.

Evaluation of the bioactivity of the purified α–amylase

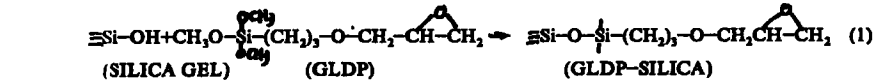
Take a suitable volume of the fraction 1 and 2 in Fig.1 and eluent A into three color tubes, respectively, each contains 5.0ml of 0.020 mol / L phosphate buffer solution and 2.0ml of 0.1% starch solution. Then the mixture solution was warmed exact 15 minutes at 37℃ . After that, the three color tubes were cooled immediately in a ice –bath and then 0.40ml of 0.0050mol / L iodine solution was added in it, respectively. At last, pure water was added in until to each mark. The color tube 1 appeared dark blue and had the same intensity with that for blank. The color tube 2 only appeared light yellow. These facts explain that the peak 1 is of impurity and peak 2 is α–amylase.

The foregoing procedure is only qualitative justification for that which peak is α–amylase. The quantitative determination for bioactivity recovery of α–amylase may be measured by spectrophotometric method [7] . This method studied by one of the authors is better than that with color reagent, 3.5–dinitrosalicylate.

RESULTS AND DISCUSSION

Reaction of silica and GLDP

In order to diminish the nonspecific adsorption of the silica surface to α–amylase and the influences of steric and exclussion effects, a spacer should exist between silica and starch. One terminal group of the spacer should have siloxane, and another one should have an active group which can react with the ligand. The γ–glycidoxypropyltrimethoxysilane is usually used as spacer in affinity chromatography. We also used it in this study. The reaction equation is as follows



This reaction may carry out in both aqueous [8] and non–aqueous [9] phases. The former is simple and rapid but the bonding density of GLDP on silica surface is low, and

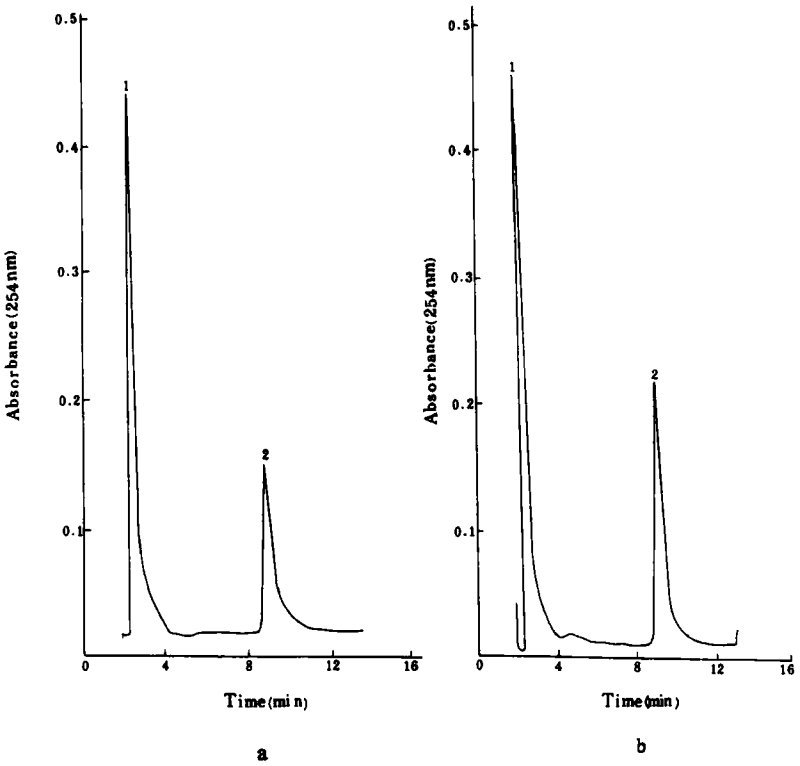


Fig. 1 Chromatogram of α—amylase with the affinity column

(a) crude enzyme (b) standard enzyme (sigma)

Conditions:

Column: HPAFC—silica (100 × 4.0mmI.D.)

mobile phase: A(H_2O) B(0.12M phosphate buffer pH7.0)

flow rate: 1.0ml / min

wave length (UV Detector): $\lambda = 254$nm

elution condition with stepwise: 0—8min A = 99%, B = 1%

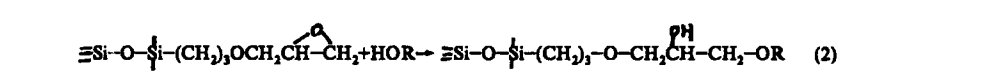
8—9min B = 100%

> 9min A = 99%, B = 1%

sometimes, when reaction conditions are unsuitable, such as the concentration of GLDP and reaction time etc., agglomerate will form during reaction process. The latter is tedious and the environment of the reaction must be absolutely dried. Because of traces of water will result in polymerization of epoxysilane coupled to the surface of silica, and produce a thick molecular coating on the silica particles. Thus, the efficiency of the chromatographic column will evidently decrease due to slow diffusion of solute molecular, especially for biopolymers, such as proteins and polypiptides. However, the bonding density of GLDP on the silica is 1.4 fold higher than that in the former one. In order to obtain higher bonding density, we used the latter case in this paper. This surface of GLDP–silica appears hydrophobic characteristics. However, this nonspecific adsorption to protein can be diminished with bonding starch.

The ligand used in affinity chromatography has two broad branchs. The first one is a universal ligand. It can simultaneously react with some groups of protein, such as dye, may selectively adsorb biopolymers, such as kinase, dehydrogenase, ribonuclease and glycoprotein. The second one is a specific ligand which can only react with a given enzyme or protein. This kind of ligand is usually a substrate, an inhibitor and a coenzyme of protein purified. Because the specific ligand bonded to silica only adsorbs its corresponding enzyme , the second one has very high selectivity and high enrichmental and purificational ability. Of course, to select the substrate of α–amylase as the ligand of the HPAFC packings in this study is also necessary and starch should be the best choice.

The reaction equation of GLDP–silica and starch is shown as:



As shown in eq.2, the epoxy bond is broken during reaction process, and reacts with starch and forms the final stationary phase.

Bonding density of GLDP

The bonding density of GLDP on the surface of silica has a great influence on the amount of starch linking to it, so does the loading capacity of the HPAFC. The greater the density of GLDP bonding to silica, usually, the higher the loading capacity is. However, too much GLDP linked to silica would cause worse separation, such as changing the peak shape.

As usually, the determination of a ligand may be carried out with chemical analysis, spectrophotometry, element analysis and radiochemical analysis. The amount of the bonded GLDP on silica was determined by element analysis in this paper. From the results obtained, we know that the ratio value of hydrogen to carbon determined very closes to the theoretical one. The theoretical value is 2.33, and the determined value is 2.37. According to the calculation, the bonding density of GLDP on silica is 6.4 μmol / m^2 which coincides with the optimization range, 5.0 to 6.5 μmol GLDP / m^2 in literature [10]. This fact shows that GLDP tightly linked to the surface of silica and its bonding condition may be suitable.

Table I comparison of bioactivity of α–amylase before and after purification

items	total amount of protein (500μg)	total bioactivity (500μg)	specific bioactivity (U / mg)	recovery of protein (%)	recovery of bioactivity (%)	purified fold
before purification	70	17	34			
after purification	67	15	670	96	88	20

The bonded amount of starch was determined by iodine–spectrophotometry. The bonding density of starch to silica–GLDP was 4.6μmol / g dry silica.

Determination of loading capacity of α–amylase

The determination of loading capacity of a column is usually accomplished with either static equilibrium, [11] or dynamic frontal chromatography [12] . Both have their advantages and disadvantages themselves. To save α–amylase with high purity, we used a continuous injection method. The procedure is as follows. The standard α–amylase solution was continuously injected to a micro–type column (2.0× 50mm) packed with HPAFC packings synthesized in this study and made elution with the mixture solution of 1% eluent B and 99% eluent A at flow rate of 0.30ml / min until approaching adsorption equilibrium. This can be monitored by iodine assay. Appearing α–amylase in eluate explains that its adsorption by the column has approached to saturation. Stopping the injection of the standard solution of α–amylase and to elute it with 100% B eluent at flow rate of 1.0ml / min. until all of α–amylase was washed out. Collect the eluate and determine its amount in it with iodine–spectrophotometry. According to the amount of adsorbed α–amylase by the synthesized packing material, we can calculate the loading capacity of the packings to be 4.6mg α–amylase / g dry silica.

Recoveries of total protein and bioactivity of the purified α–amylase

The magnitude of recoveries of total protein and the bioactivity of the purified protein is a vital parameter for evaluating an affinity column to purify biopolymers. The determination of the total protein recovery was accomplished with the ratio of the peak area of α–amylase purified with the column to that only with empty column. Its bioactivity recovery was determined with the ratio of its bioactivity before and after purification with the column. Table I shows the comparison of their bioactivity in this way on the HPAFC column synthesized in this paper.

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