Communication

Preparation and Evaluation of a Molecular Recognition Bionic Solid Phase Extraction Column for Separation of Glucosides

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A molecular recognition bionic solid phase extraction (SPE) column for separation of glucosides has been prepared using a positively charged β-glucosylamidine as the ligand in which a glycon moiety is connected via an N-glycoside linkage. β-Glucosylamidine, highly potent and selective inhibitors of β-glycosidase, is immobilized through a one-step synthesize procedure involving the addition of β-glucosylamine and 2-iminothiolane. HCl simultaneously to a matrix modified with maleimido groups via an appropriate spacer to give a molecular recognition absorbent for β-glucosides. N-octyl-β-D-glucopyranoside and β-D-galactopyranoside or α-D-mannopyranoside was directly chromatographed through the bionic chromatographic column, resulting in a much stronger retention of β-D-glucopyranoside than β-D-galactopyranoside and α-D-mannopyranoside. The retained glucopyranoside could only be eluted by glucose solution. This indicates that the binding of the glucoside was of specific nature that corresponds to the glycon substrate specificity of the glucoside. The ease of preparation and the selective nature of the molecular recognition bionic chromatography should promise a large-scale preparation of the molecular recognition adsorbent for the purification and removal of glucosides according to their glycon substrate specificity.

Keywords: Molecular recognition; Solid phase extraction; Glucosides; Separation.

INTRODUCTION

Chemical derivatives of O-linked glucose at C-1 can produce glucosides. Glucoside is one of a large series of amorphous crystalline substrates, occurring very distributed in plants, rarely in animals, and regarded as influential agents in the formation and disposition of sugars. By the action of ferments, or dilute acids, they break down into some characteristic substrates (such as acid, aldehyde, alcohol, phenole, or alkaloid) and glucose.[1]

Glucosides are tightly related to the chemical and physical properties of the organisms. For example, the glucosides in tea can be hydrolyzed to release volatile aroma compounds that are important sources of tea scent.[2] A review by Hechman pointed out that glucosides were among the most important flavor precursors for tobacco and smoke.[3] But until now there is no effective way to separate glucosides from other carbohydrates.

Biochromatography was developed in 1980s. It is combined with biology science and separation technology.[4] The field of biochromatography employs separation techniques to characterize biomolecules, such as proteins, peptides, nucleic acids and carbohydrates. As an accurate, sensitive, and reproducible alternative to electrophoretic methods, biochromatography has the ability to observe biomolecules while retaining their biological activity (non-denaturation). But as it uses biomolecules as its ligand, the retaining of its bioactivity is a big problem during separation process.

Molecular recognition is based on selectivity and differential integrating between biomolecules. It means researching chemical and biologic combinations on molecular level that widely exist in chemical and biologic systems.[5] Lai et al deeply studied the molecular recognition theory and developed a series of analysis methods of specificity and highly selectivity that can be applied to chemical and biological analysis of complex biological samples.[6]

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Be enlightened by this, we wonder if we can integrate biochromatography and molecular recognition to get molecular recognition bionic chromatography. That means we can biomimetic the molecular structure of the biomolecules and apply them to the ligands of biochromatography. As they are not real biomolecules, this will help us solve the problem of bioactivity loss during separation.

In this paper, we report the preparation and characterization of a molecular recognition bionic chromatography adsorbent for β-D-glucoside. In preparing the adsorbent, a one-pot procedure for in-situ formation and immobilization of β-glucosylamidine has been developed so as to tailor the ligand conveniently to the glycon substrate specificity of glucoside. This new method was successfully used for the separation of glucopyranoside and the selective nature of the ligand-glucoside interaction was proved.

EXPERIMENTAL SECTION

Reagents and apparatus

Dimethyl sulfoxide (DMSO) and pyridine were distilled from CaH_2 to remove water and stored under nitrogen. Other solvents were used as received with no further treat. 2-Iminothiolane.HCl and N-(γ-maleimidobutyryloxy) succinimide ester (GMBS) was purchased from Pierce Chemical Co. TSKgel AF-Amino Toyopearl 650 M was purchased from Tosoh Co., Japan. Other chemicals were obtained from commercial sources and were of the highest purity available. ^{1}H NMR spectra were recorded on a Varian VXR-300 (300 MHz) spectrometer using tetramethylsilane (for D_2O) as an internal standard. Chemical shifts were recorded in ppm with the internal standard at δ_H = 0.00. Elemental analyses were performed on an Elementar Vario EL-III. Mass spectra were obtained on a JEOL JMS700 spectrometer. A Shimadzu UV-1600 UV-Vis spectrophotometer was used for UV-Vis analysis.

β-D-Glucosylamine (1)

β-D-glucosylamine was synthesized according to the literature.[8] To 50 mL of anhydrous methanol were added 40 g of D-glucose and 0.5 g of ammonium chloride. And the mixture was treated with ammonia gas at 0 °C until the sugar had all dissolved. The solution was stored in refrigerator until satisfactory crystallization had occurred. The first crystallization was slow, and required storage of the solution for about a month. Subsequent crystallizations with the aid of seed crystals were more rapid, but maximum yield required storage for a week or two longer. The crystals were separated, washed with methanol, and air-dried. The crude product was recrystallized from an equal weight of a 1:10 mixture of concentrated ammonium hydroxide and water, by the successive addition of 2 volumes of methanol and 2 volumes of ethanol. After 48 h, the crystals were separated, washed with methanol, air-dried and stored in refrigerator until use.

^{1}H NMR (300 MHz, D_2O): δ 4.05-4.12 (d, 1H, H-1); δ 3.80-3.90 (d, 2H, H-6, H-7); δ 3.60-3.72 (dd, 1H, H-5); δ 3.45-3.50 (m, 1H, H-4); δ 3.32-3.40 (m, 1H, H-3); δ 3.10-3.18 (m, 1H, H-2). ^{13}C NMR (50 MHz, D_2O): δ 85.21 (C-1). Chemical shifts were uncertain to ± 0.05 ppm. All the data was consistent with the literature data of β-glucosylamine[8,9,13] and α-anomer couldn't be detected.

The calculation data for $C_6H_{13}NO_5$ is: C, 40.2%; H, 7.31%; N, 7.82%. In our experiment, the data we found was: C, 39.93%; H, 7.35%; N, 7.30%. Considering the experiment error and a little glucose impurity that can't be eliminated, we can assure that the found data is consistent with the theoretical data.

As for the mass spectrum experiment, we found the molecular ion peak at 179.0794 m/e, which is consistent with the molecular weight.

The melting point was found as 127-128 °C which is also consistent with the literature reported data.[16]

The new, crystalline β-D-glucosylamine could be kept for short period in refrigerator. But after about a month, some of it had been hydrolyzed. So the glucosylamine was used freshly prepared.

Soluble form of β-D-glucosylamidine

β-D-glucosylamine (1) (179 mg, 1.0 mmol) was suspended in dry pyridine (5 mL) at 0 °C. 2-iminothiolane.HCl (138 mg, 1.0 mmol) was added to the suspension and the mixture was stirred for 4.5 h at 0 °C under a nitrogen atmosphere. The mixture gave a clear solution as the reaction proceeded. The Ellman' reagent (DTNB solution) was prepared by dissolving 39.6 mg of 5,5′-dithiobis (2-nitrobensoic acid) (DTNB) in 10 mL phosphate buffer (pH = 7.0). After the reaction had been completed, the reaction mixture was immediately dilute with 10 mL phosphate buffer (pH = 8.0) and 1 mL of DTNB solution was added. The color developed rapidly (about 2 min). Then the absorbance at 412 nm was determined by UV-Vis spectroscopy.

Solid support modified by GMBS (2)

TSKgel Amino TOYOPEARL 650 M (dry weight, 40 mg; wet volume, 0.2 mL; total titrable NH_2 group, 20 μmol)

was suspended in dry DMSO (0.5 mL). GMBS (44.8 mg, 0.16 mmol, 8 equiv of total NH_2 group) was added. The mixture was shaken at room temperature for 10 h under nitrogen atmosphere. The resulting suspension was filtered, and the gel was washed successively with water, EtOH and hexane, and air-dried.

The moles of the ligand were calculated from the moles of the amino groups of the gel before and after the modification with GMBS. The gel (dry weight, 40 mg; wet volume, 0.2 mL) was suspended in DMSO (0.5 mL), and p-nitropheyl acetate (0.120 g, 0.67 mmol) was added. The mixture was shaken at room temperature for 2.5 h. Then the reaction mixture was diluted with 50 mM Tris-HCl buffer (pH = 8.0). The concentration of pNP was determined by measuring the absorbance at 400 nm. As reference, the reactant of gel and GMBS was dealt with the same procedure.

Matrix with β-glucosylamidine as the ligand (3)

TSKgel Amino TOYOPEARL 650 M (dry weight, 400 mg; wet volume, 2 mL; total titrable NH_2 group, 200 μmol) was suspended in dry DMSO (5 mL). GMBS (448 mg, 16 mmol, 8 equiv of total NH group) was added, and the mixture was shaken at room temperature for 10 h under nitrogen atmosphere. The resulting suspension was filtered, and the gel was washed successively with water, EtOH and hexane, and air-dried. Unreacted amino groups were blocked by shaking the gel in a mixture of acetic anhydride (2 mL) and pyridine (2 mL) for 3 h. The resulting gel (2) was filtered and washed successively with water, EtOH and hexane. To a suspension of the resulting gel in dry pyridine (5 mL) were added β-glucosylamine (1) (180 mg, 1 mmol) and 2-iminothiolane.HCl (138 mg, 1 mmol) at room temperature. The mixture was shaken at room temperature for 24 h under nitrogen atmosphere and filtered. The resulting gel was washed successively with water, EtOH and hexane. The unreacted maleimido group was blocked by shaking the gel with 2-mercaptoethanol (0.34 mL, 5 mmol) in dry pyridine (5 mL) at room temperature for 4 h. The resulting gel was filtered and washed successively with water, EtOH and hexane, and air-dried. This final gel was stored in citrate buffer (pH = 6.0) until use.

Separation function of the synthesized molecular recognition bionic chromatography

We packed the white powder synthesized in the above procedure into a stainless glass column (2 mm i.d, 5 cm length) to get a desired molecular recognition bionic chromatography column. Firstly, we balanced the column with citrate buffer (pH = 6.0). Secondly, we passed 2 mL of complex solution of p-nitrophenyl-β-D-galacto-pyranoside (0.1 mol/L) and n-octyl-β-D-gluco-pyranoside (0.1 mol/L) in citrate buffer (pH = 6.0) and collected the outflow (outflow 1). Thirdly, we used 5 mL citrate buffer (pH = 6.0) to wash the column and also collected the outflow (outflow 2). At last, we eluted the column with 5 mL 10% glucose-citrate solution and collected the eluent.

Then the two outflows and the eluent were hydrolyzed in disodium hydrogen phosphate-citric acid buffer (pH = 2.6) at 80 °C for 3 h respectively. Each of the hydrolyzed solution was equally divided into two parts. One part was extracted with dichloromethane and then analyzed by GC-MS. The other part was basified with 10% NaOH solution to pH 8.0 and then analyzed by UV-Vis spectrometer to determine the absorption at 400 nm of pNP.

The same procedure was applied to the separation of n-octyl-β-D-glucopyranoside and metyl-α-D-mannopyranoside except that methanol was detected by GC-MS.

RESULTS AND DISCUSSION

Synthesis of β-glucosylamine

Ammonia can directly react with sugar to yield glucosylamine (Fig. 1).

In a typical synthesis procedure, a sugar is dissolved in an alkanol medium at a temperature of 0 °C-10 °C and ammonia gas is introduced into the medium. The ammoniated solution is maintained under cooling conditions for a period of a month or more. Usually the glucosylamine product crystallizes out of the alkanol medium during the reaction period.[9]

Condensation of the sugar and ammonia can presumably take place either through the neutral, carbonyl modification of the sugar or through the acyclic cation
formed by acid catalysis, but the acyclic cation is far more reactive (Fig. 2). This hypothesis accounts for the effec-

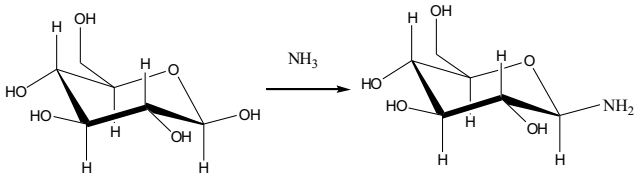

Fig. 1. Reaction of D-glucose and ammonia.

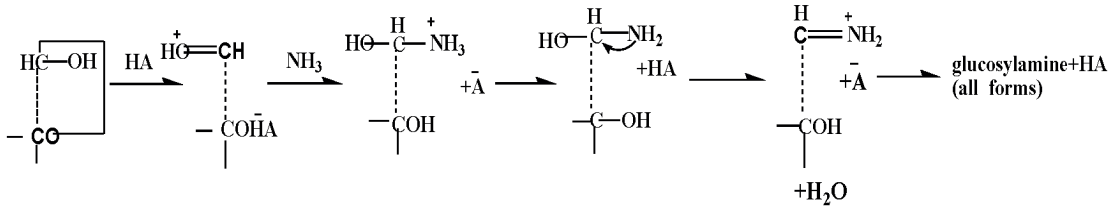

Fig. 2. Mechanism of formation of glucosylamine.

tiveness of general acid catalysts (NH_4Cl) in promoting formation of glucosylamine. Condensation of the acyclic cation with ammonia does not take place in strongly acidic solution, because under these conditions, the nucleophilic character of the ammonia is satisfied by combination with hydrogen ion (ammonium salt formation). The high ammonia concentration can hasten crystallization.

In the final reaction solution, the D-glucosylamine establishes equilibrium mixtures consisting almost entirely of the β-pyranose anomer (as the optical ratio showed). The stability of the β-pyranose forms of D-glucosylamine presumably arises from its assuming normal chair conformation, in which all of the groups attached to the ring, except the hydrogen atoms, lie in equatorial positions.[11-12]

We studied the IR spectrum of the product (Fig. 3).

As we can see from Fig. 3, when using 0.5 g catalyst and 1.0 g catalyst, the position and the intensity of the peaks are totally the same. The peak at 1620-1625 cm^{-1} is the characteristic peak of N-H bond bending vibration (δ_{N-H}). The peak at 1200-1030 cm^{-1} is the characteristic peak of C-N bond stretching vibration (ν_{C-N}). The stretching vibration peak of N-H (ν_{N-H}) is at about 3400 cm^{-1} which is overlapped by the broad peak of O-H stretching vibration (ν_{O-H}).

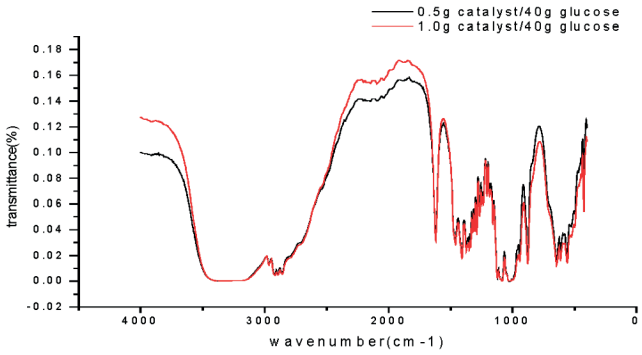

Fig. 3. The IR spectrum of glucosylamine.

Crude glucosylamine (contaminated with little sugar) was used without further purification because glucose didn't interfere with next synthesis steps.[14]

Synthesis of soluble form of β-glucosylamidine

Before immobilizing the β-glucosylamidine as the molecular recognition ligand, a soluble form of the ligand was synthesized to examine the immobilization method. Since β-glucosylamidine was synthesized readily from the corresponding β-glucosylamine and thioimidate without protecting the hydroxyl groups,[7] a one-pot procedure for in situ formation of the β-glucosylamidine was developed by using a cyclic thioimidate (2-iminothiolane.HCl). It is shown in Fig. 4.

2-Iminothiolane.HCl, also called Traut's reagent, is a useful reagent for chemical modification of proteins to yield amidinated derivatives which contain sulfhydryl groups that can be used in the next steps, for example, reacting with maleimido groups. It reacts with primary amines ($-NH_2$) to introduce sulfhydryl (-SH) groups while maintaining charge properties similar to the original amino

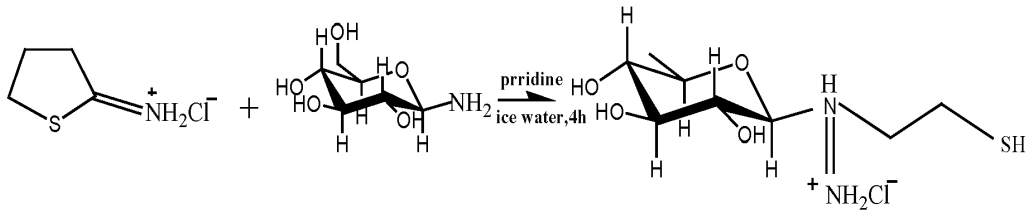

Fig. 4. Formation of soluble form of β-glucosylamidine.

groups.[6]

The reaction was clear after a period of reaction. When the reaction finished, the reactant was immediately treated with Ellman's reagent (DTNB) to determine the existence of free sulfhydryl groups by UV-Vis absorbance at 412 nm (Fig. 5).[14]

From the intensive absorbance at 412 nm after treated with Ellman's reagent, we can see that the product contained sulfhydryl groups. But because the sulfhydryl groups can be oxidated easily in air, the test should be conducted immediately.

Modification of Amine Matrix with GMBS

The soluble form should be attached to a solid support when performing separation analysis. A "spacer arm" which can conjugate molecules together by a covalent bound should be used in the attachment process. GMBS is a heterobifunctional cross-linker that contains N-hydroxysuccinimide (NHS) ester and maleimide groups that allow covalent conjugation of amine- and sulfhydryl-containing molecules. NHS esters react with primary amines (in fact on its deprotonated form) at pH 7-9 to form amide bonds, while the maleimide reacts very specifically with sulfhydryl groups (nucleophilic attack of the thiolate anion) at neutral pH 6.5-7.5 to form stable thioether bonds.[15]

As for the solid support, we choose a gel with amino groups (2) (TSKgel AF-Amino Toyopearl 650 M). Toyopearl gel remains dimensionally stable within wide extremes of pH and ionic strength. It can be used to couple ligands using their carboxylate groups (peptide bond formation) or aldehyde groups (reductive amination). The gel can be treated with GMBS to introduce maleimido groups via a spacer of four carbon atoms in length (Fig. 6).

To calculate the modification efficiency of GMBS, we reacted the gel before and after modification with pNPA (p-nitrophenyl acetate) respectively, and measured the absorbance at 400 nm of the result pNP (p-nitrophenol) to determine the moles of amine groups on the amine gel (Fig. 7).

From the result of UV-Vis spectrum, we can calculate that 71.5% of the amines on the amine gel have been modified. The hydrolysis of GMBS that increases with pH and the high dilutions of the molecule can account for the efficiency of the reaction (Fig. 8).

Also, we compare the IR spectrum of GMBS and that of the gel after modification. The wide peak at 3400-3500 cm^{-1} becomes stronger and wider in modified gel, which shows that the amine group has been attached to GMBS.

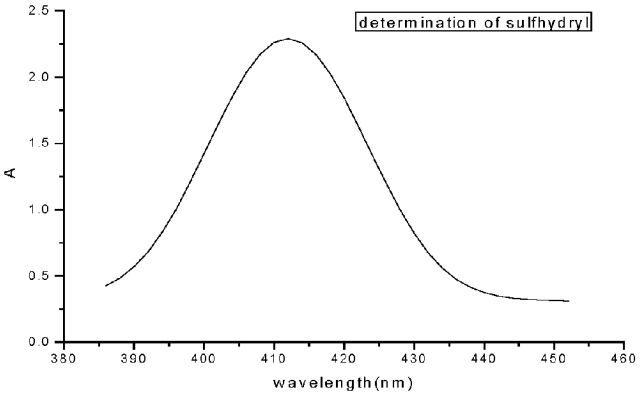

Fig. 5. Determination of sulfhydryl groups.

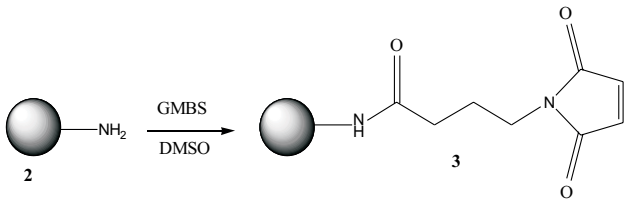

Fig. 6. The reaction of amine gel and GMBS.

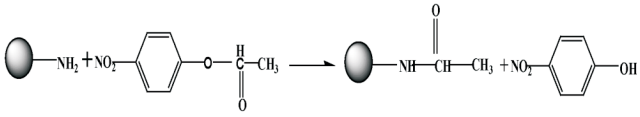

Fig. 7. The reaction of amine and pNPA.

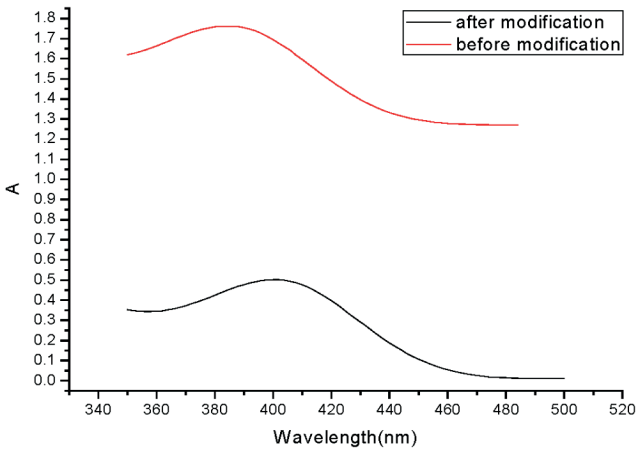

Fig. 8. The UV-Vis spectrum before and after modification.

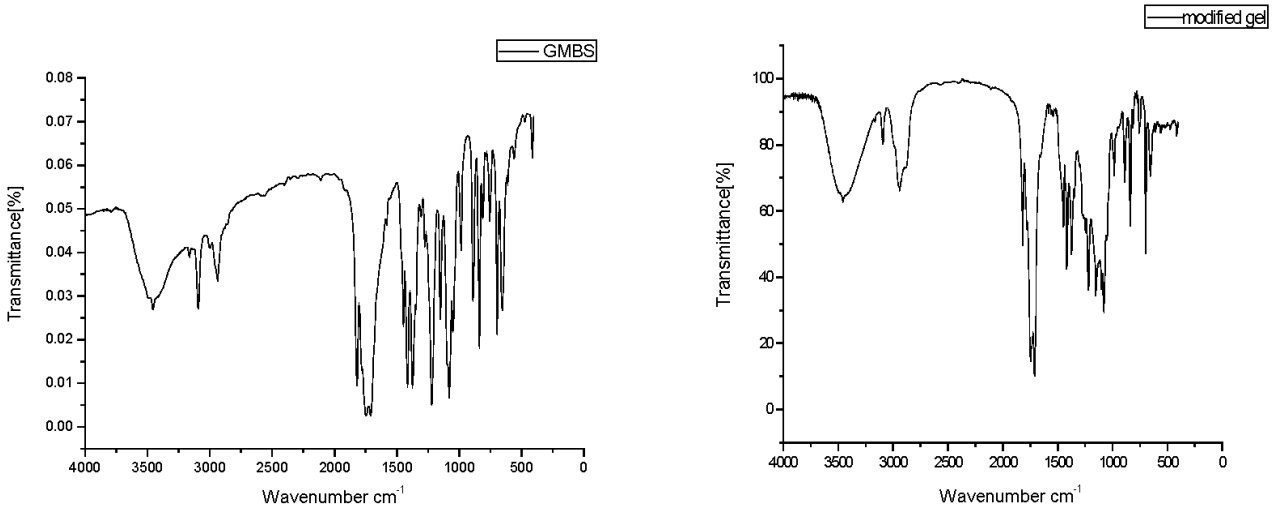

Fig. 9. The IR spectrum of GMBS and the modified gel.

And the peak at 1818 cm^{-1} becomes much weaker in modified gel, which shows that the NHS group of GMBS has been eliminated by the reaction. Larger alkyl peaks of the modified gel are due to the polymeric alkyl of the amine gel (Fig. 9).

In conclusion, we can affirm that GMBS has been attached to the amine gel through peptide bond formation reaction.

Synthesis of molecular recognition bionic chromatography with β-glucosylamidine as the ligand

If the modified gel, 2-iminothiolane.HCl and β-glucosylamine were all mixed together at once, the separation functional groups could be easily attached to the solid support in one pot.

The block of the unreacted amino groups of the TSKgel was very important for the one-pot procedure. Because 2-iminothiolane.HCl could also react with the amino groups on the gel. So before add of 2-iminothiolane.HCl, the unreacted amino groups of the matrix were blocked with excessive acetic anhydride.

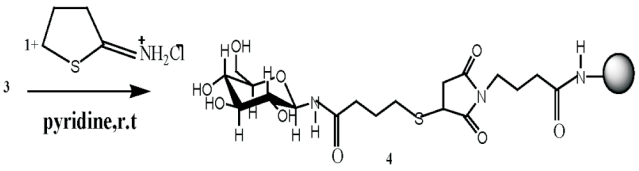

Fig. 10. One-pot synthesis procedure of molecular recognition biochromatography.

The resulting gel 3 was suspended in dry pyridine and was treated with an excess amount of β-glucosylamine (1) and 2-iminothiolane.HCl simultaneously to immobilize the β-glucosylamidine as a ligand to yield the molecular recognition adsorbent 4 (Fig. 10).

A spacer arm between the ligand and the solid support is highly important in reducing the unfavorable steric interaction between the glucoside and the matrix.

The synthesized glucosylamidine is stable in acidic media.[7] The stability of the glucosylamidine probably comes from the fact that the highly basic amidine is completely protonated under acidic conditions, thereby pre-

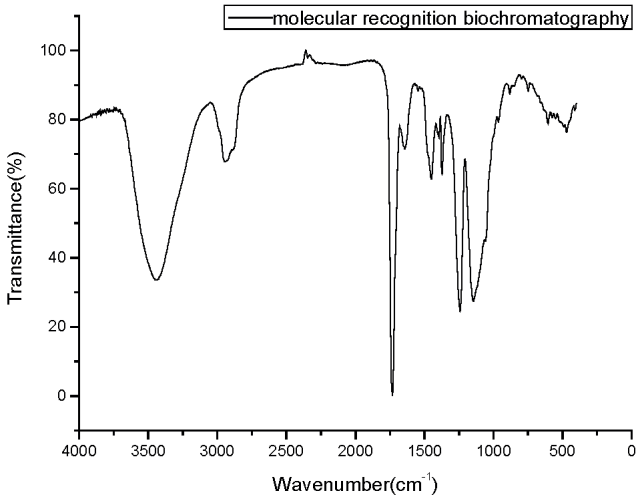

Fig. 11. The FTIR spectrum of the molecular recognition adsorbent.

venting the acid-catalyzed opening of the pyranose ring as would easily occur with the parent glucosylamine. The molecular recognition adsorbent was washed with 0.1 M citrate buffer (pH = 6.0) and suspended and stored in the same buffer.

We analyzed the white powder of molecular recognition adsorbent with FTIR spectroscopy (Fig. 11).

As compared to the IR spectrometry of gel 3 (modified gel), we can clearly see that the three characteristic peaks of maleimido ring have disappeared. It means that the maleimido ring has disappeared by reacting with sulfhydryl group.

Separation function of the molecular recognition bionic chromatography

To determine the separation function of the molecular recognition adsorbent, first of all, we passed a mixture solution of n-octanol and n-octyl-β-glucopyranoside in citrate buffer (pH = 6.0) through the biochromatography column. As the GC-MS result showed, all the n-octanol passed through the column without retention and most of the n-octyl-β-glucopyranoside was retained by the adsorbent (data not show).

To determine the substrate recognition of the adsorbent, then, we applied it to the separation of n-octyl-β-glucopyranoside and p-nitrophenyl-β-D-galactopyranoside (Fig. 12).

After hydrolyzing outflow 1 and outflow 2 and comparing the absorption at 400 nm, we could see that 83% of the p-nitrophenyl-β-D-galactopyranoside had passed through the column without retention (Fig. 13). By GC-MS analysis of n-octanol, we can see that only 12% of the n-octyl-β-D-glucopyranoside passed through the column. By experimenting GC-MS analysis of the hydrolyte of outflow 2, we can see that no n-octyl-β-glucopyranoside is washed down by citrate buffer (pH = 6.0).

At last, we eluted the column with 10% glucose-citrate solution. The surplus 88% n-octyl-β-glucopyranoside was eluted (GC-MS data not show).

Through separation analysis, we could see that most of the p-nitrophenyl-β-D-galactopyranoside passed through the column without retention. But most of the n-octyl-β-D-glucopyranoside was retained by the column and could-n't be washed down by the citrate buffer. The retained β-D-glucopyranoside could just be eluted by stronger conditions such as glucose solution.

We also applied the molecular recognition bionic chromatography column to the separation of n-octyl-β-D-glucopyranoside and metyl-α-D-mannopyranoside. With the same procedure, we could get almost the same result. 95% of the metyl-α-D-mannopyranoside passed through the column without retention and just 8% of the n-octyl-β-D-glucopyranoside passed through the column without retention.

These results suggest that the β-glucosylamidine served as a substrate analog, where the glycon moiety was primarily recognized as substrates by the glucoside. These

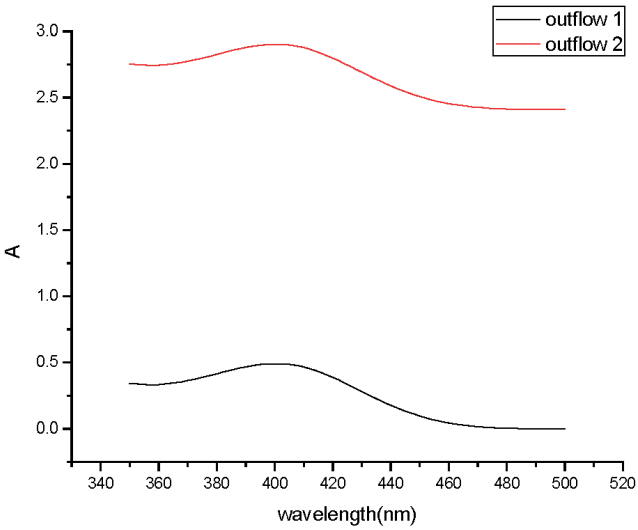

Fig. 13. UV-Vis spectra of outflow 1 and outflow 2.

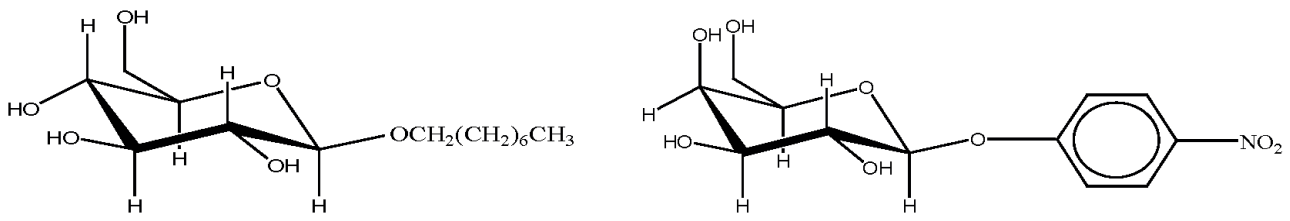

Fig. 12. n-Octyl-β-glucopyranoside and p-nitrophenyl-β-D-galactopyranoside.

characteristics are suitable for a molecular recognition ligand for separating the glucosides according to their glycon substrate specificities.

A question may arise here whether the glucoside was separated by ion-exchange effects of the adsorbent, because the ligand is permanently and positively charged at the pH used for the chromatography. But most of the galactopyranoside and mannopyranoside were not retained by the chromatography. This adsorptive characteristic clearly indicates that the glucopyranoside is bond to the ligand with specific interaction between the glycon moiety of the ligand and the glucopyranoside. A little amount of galactopyranoside and mannopyranoside were also retained by the adsorbent. It may be because of the weak interaction resulting from the similarity of the glucose pyranose and galactose pyranose or mannose pyranose. This can also explain the fact that the α-mannopyranoside was retained less than β-galactopyranoside by the chromatography.

The adsorptive characteristics of the molecular recognition bionic chromatography are affected by several factors such as the steric interaction between the glucoside and the matrix, the accessible surface area, the particle size and the ability of the solute to diffuse in and out of the microporous environment.[16-17] We suppose that the bond between β-glucosylamidine and β-glucoside may be hydrogen bond, static electronic bond, hydrophobic reaction, van der Waals force or some other kind of weak bond. The mechanism is still under research.

But in our experiment, the column could just be used for one time of separation experiment if it was eluted by glucose solution. After it was eluted by glucose solution, the separation function of the molecular recognition bionic chromatography column decreased dramatically. We suppose the reason is that after the column was washed with glucose solution, the function spots of the molecular recognition bionic chromatography were blocked by glucoses. And until now, we haven't found an efficient way to wash these glucoses down and reproduce the separation function of the column. As the expense for the column is large, this is a big disadvantage of this method.

CONCLUSION

In conclusion, the β-glycosylamidine was found effective as a molecular recognition ligand for separation β-glucoside according to the glycon substrate specificity of the glucoside. This adsorptive characteristic reflected the fact that the β-glucosylamidine serves as an substrate analog, and the analog strongly interacting with the glucoside by the presence of a positive charge near the anomeric carbon to mimic the oxocarbenium ion intermediate of enzyme reaction.[7] Hence the β-glucosylamidine ligand successfully recruited the binding energy of the glucoside towards the ligand to achieve selective binding to the corresponding glucosides. This property, along with the ease of synthesis, should promise useful applications not only for purifying a target glucoside, but also for remove an undesirable contaminating glucoside from the sample, based on the glycon substrate specificities.

Many other molecular recognition bionic chromatography adsorbents can be readily synthesized by choosing appropriate carbohydrates added at the last stage of the synthesis. Since the β-glycosylamidine was formed directly from the corresponding β-glucosylamine, this method is applicable, in principle, to any glycosylamidine ligand including those derived from oligosaccharides, provided the precursor glycosylamines are available.[16] Neither the isolation of the intermediate β-glycosylamidine nor the protection of the hydroxyl and the amido groups was necessary for the immobilization.

ACKNOWLEDGEMENT

This work was completed in National Synchrotron Radiation Laboratory of China and China Tobacco Jiangxi Industrial Co. Ltd. The authors thank to National Natural Science Foundation of China (No.20405013) for the financial support.

Received July 28, 2009.

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