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Resurveying the Tris buffer solution: The specific interaction between tris(hydroxymethyl)aminomethane and lysozyme

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ABSTRACT

An unusual phenomenon, the specific interaction between tris(hydroxymethyl)aminomethane (Tris) and lysozyme (LZM), was demonstrated for the first time by rapid screen analysis of interactions using a quartz crystal microbalance (QCM) biosensor. This phenomenon was also observed in a surface plasmon resonance (SPR) system. Further study using high-performance affinity chromatography (HPAC) confirmed this specific interaction between LZM and immobilized Tris with an apparent dissociation constant (K_D) of 6.7×10^{-5} M. Molecular docking was carried out to identify possible modes of binding between LZM and Tris linked to a binding arm. The estimated binding free energy was −6.34 kcal mol^{-1}, corresponding to a K_D of 2.3×10^{-5} M, which correlated well with the experimental value. Based on the docking model, the three hydroxyl groups of Tris form intermolecular H bonds with Asp52, Glu35, and Ala107 in LZM. This study reinforces the importance of buffer selection in quantitative biochemical investigations. For a lysozyme ligand binding study, it is better to avoid using Tris when the ligands under study are weak binders.

Buffers are important and widely used to maintain stable pH value in many fields, such as chemistry, biology, pharmacology, medical science, and our daily life as well. In life science research, buffers not only maintain pH, but also simulate the in vivo environment to retain the native structures and activities of biomolecules. In addition to its strong buffering ability, a good buffer should also be inert and not have any specific interactions with the biomolecules under investigation.

Because of its vivo-like properties and structure-stabilizing ability on biomolecules, tris(hydroxymethyl)aminomethane (Tris)[2] solution is one of the most widely used buffers [1]. Specific interactions between Tris and proteins have commonly been ignored when it is used as a buffer. However, in our recent experiment, we obtained a false-positive result when we used Tris buffer. In a study of biomolecular interactions using high-performance affinity chromatography (HPAC), a Tris immobilized affinity column was used as a negative control column and the reservation time of lysozyme (LZM) on it was obviously longer than for other proteins [2]. These abnormal phenomena indicate the possibility of some kind of interaction between Tris and LZM that is not yet understood clearly.

Quartz crystal microbalance (QCM) biosensors have been used in the rapid screening of possible interactions between small molecules and proteins. QCM allows real-time monitoring of interactions between biomolecules without labeling requirements and noninvasive measurements [2–5], compared with other extensively employed techniques such as chromatography [6], electrophoresis [7], NMR [8], mass spectrometry [9], ultracentrifugation [10], scintillation proximity assay [11], and biosensors [12]. Because of these advantages, the applications of QCM biosensors for monitoring interactions between small molecules and proteins are expanding rapidly [13,14]. In our previous study, we proposed a novel screening strategy based on the QCM biosensor to select ligands for affinity chromatography [13]. However, the application of the QCM biosensor in the rapid screening of specific interactions between Tris and proteins has not been exploited.

To quantify specific interaction between small molecules and protein, frontal analysis by analytical high-performance affinity chromatography (HPAC) has been employed [15–17]. Through continuous application of various concentrations of protein to the ligand immobilized affinity column, the interaction between protein and immobilized ligand can be quantified. The apparent dissociation constant of the interaction between Tris and protein can be obtained without considering the binding ratio by frontal analysis using HPAC.

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[2] *Abbreviations used:* LZM, lysozyme; Tris, tris(hydroxymethyl)aminomethane; QCM-FIA, quartz crystal microbalance flow injection analysis; QCM, quartz crystal microbalance; SPR, surface plasmon resonance; HPAC, high-performance affinity chromatography; CEW, chicken egg white; OVA, ovum albumin; HSA, human serum albumin; C IgG, chicken IgG; Hemo, hemoglobin; Trans, transferring; Pep, pepsin; Tryp, trypsin; DTT, DL-dithiothreitol; GPS, 3-glycidoxypropyl trimethoxysilane; PBS, 50 mM potassium phosphate buffer containing 100 mM NaCl, pH 7.0.

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In the present study, we investigated possible specific interactions between a commonly used buffer, Tris, and LZM. A quartz crystal microbalance flow injection analysis (QCM-FIA) system was used for rapid screen analysis of specific interactions between immobilized Tris and various proteins in solution. To confirm the results of QCM-FIA, rapid screen analysis was carried out using surface plasmon resonance (SPR) technique, primarily to determine the apparent dissociation constant. The apparent dissociation constant for LZM binding with Tris was also obtained by frontal analysis using HPAC and compared with the constant obtained from SPR analysis. Molecular docking was performed to search for possible binding sites of immobilized Tris on LZM. The calculated binding constant correlated well with experimental data.

Materials and methods

Materials

Analytical reagent-grade Tris was purchase from Amresco (Solon, OH, USA). Chicken egg white (CEW) LZM, ovalbumin (OVA), human serum albumin (HSA), chicken IgG (C IgG), hemoglobin (Hemo), transferrin (Trans), gamma globin, myoglobin, pepsin (Pep), trypsin (Tryp), RNase A, and DL-dithiothreitol (DTT) were all from Sigma (St. Louis, MO, USA). 3-Glycidoxypropyltrimethoxysilane (GPS), 1,4-butanediol diglycidyl ether, and sodium borohydride were supplied by Aldrich (St. Louis, MO, USA). All other chemicals were of analytical reagent grade, and deionized water was used for preparation of aqueous solution. All solutions prepared were filtered (0.45 μm) before use.

Methods

The homemade QCM biosensor setup consisted mainly of two parts, the QCM system and the FIA system, as described previously [13]. Ten-megahertz AT-cut quartz crystal (14-mm diameter) chips with gold electrodes (8-mm diameter, geometric area 50.2 $mm^2 \times 2$) on both sides were purchased from Beijing 707 Factory (Beijing, China). The gold surface of the crystal electrode was smoothed using a mirrorlike finishing process to a roughness of less than 5 μm.

An Agilent 1100 series liquid chromatography system (Palo Alto, CA, USA) consisting of a quaternary solvent delivery system, a manual sampler, and Agilent 1100 VWD detector coupled with an analytical workstation was used.

SPR experiments were performed using the Biacore 3000 system (Biacore International AB, Uppsala, Sweden).

Immobilization of Tris on QCM sensor chips

The gold electrodes were cleaned with a boiling solution consisting of 30% H_2O_2, 30% ammonia, and deionized water in a 1:1:5 volume ratio to remove adsorbed components disturbing the self-assembly of thiols [18]. The solution was dripped onto the gold, kept there for 10 min, and then thoroughly washed with deionized water. The freshly cleaned sensor chip was modified with dithiothreitol self-assembled monolayer and activated to an epoxy group as described before [13]. The prepared crystal was immersed in 5 mL of Tris–HCl (pH 8.5, 1.0 M), and kept at 40 °C for 2 h. Finally, 5 mL of Tris–HCl (pH 8.5, 1.0 M) was used to block the residual reacting sites on the chips. The reaction was kept for 30 min at room temperature.

Screening of the interaction between proteins and immobilized Tris using a QCM biosensor

In all these screening experiment, PBS was used as loading buffer. The freshly coated chip was mounted in the flow-through cell and rinsed with 60 μL min^{-1} loading buffer continuously until the baseline had stabilized. Then, 200-μL volumes of various protein solutions (0.5 mg mL^{-1}) were injected into the flow-through cell, respectively, with the injection valve. The interaction between immobilized Tris and proteins was monitored in real time by recording the frequency shift-versus-time curves. If there was a decrease in frequency, to refresh the sensor chip for the next binding, 600 μL elution solution (50 mM potassium phosphate buffer containing 500 mM NaCl, pH 7.0) was injected into the system to dissociate the bound protein.

Surface plasmon resonance detection

Tris was immobilized on a Biacore sensor chip (bare Au) using the same procedure for QCM sensor chip preparation. LZM was dissolved in PBS at various concentrations (0.2, 0.4, 0.6, 0.8, 1.6, and 3.2 μM) for binding analysis using SPR. Measurements were performed with a blank chip as reference at 25 °C. The flow rate was 30 μL min^{-1}.

Preparation of the affinity column using Tris as a ligand

Two grams of Uetikon macroporous silica sphere (granule diameter 5 μm, pore diameter 300 Å, specific surface area 40 $m^2\,g^{-1}$) was modified by GPS as described before [13]. A 0.2-g amount of Tris was reacted with epoxy-activated silica in 10 mL of potassium phosphate buffer (2.5 M, pH 7.9) at 60 °C for 48 h. Then, 10 mL of Tris–HCl (pH 7.9, 1.0 M) was used to block the residual reacting sites on the surface of the silica sphere for 3 h. The prepared packing was packed into stainless-steel columns (4.0 mm × 70 mm i.d.) under 16 MPa pressure.

Affinity chromatography conditions

Protein solutions were prepared with loading buffer (PBS) at a concentration of 0.5 mg mL^{-1}. These solutions were filtered through a 0.45-mm membrane filter before use. The injection volume was 50 μL with a standard 100-μL loop and 50-μL syringe. All separations were carried out through the column by passing the loading solution for 8 min, the elution solution (50 mM potassium phosphate buffer containing 600 mM NaCl, pH 7.0) for 12 min, and then the loading solution within 10 min at a flow rate of 0.5 mL min^{-1}, after injection of the protein solution, if not otherwise explained. The chromatographic profiles were monitored at 280 nm. All chromatographic experiments were performed at room temperature.

Frontal analysis on the affinity column

The concentrations of the LZM solutions were 0.25, 0.50, 0.75, and 1.00 mg mL^{-1}, respectively. Frontal analysis was performed by injecting various LZM solutions continuously with the quaternary pump as loading buffer. Profiles were monitored until the platform of maximum absorbance was observed.

Molecular docking

To construct the binding models of LZM with Tris linked with a binding arm, and to predict their binding affinities, molecular docking was performed using the AutoDock 3.05 program [19].

The structure of CEW lysozyme in complex with MPD (taken from the protein data bank, PDB code 1DPW) was used in all docking studies, and was prepared with the molecular modeling program SYBYL 6.91 (Tripos, Inc.). All water moleucles were removed, with polar hydrogen atoms added geometrically and Kollman united-atom partial charges assigned. The initial structures of the Tris and part of the binding arm were generated using SYBYL 6.91 in the all-atom representation, and were used in docking. The structure was fully optimized using a conjugate gradient procedure based on the TRIPOS force field and Gasteiger and Hückel charges.

Because of the uncertainty of the binding site between the LZM and Tris with a binding arm, first, blind docking was performed to predict the possible ligand binding pocket on the whole protein surface. Affinity (grid) maps of 126 × 126 × 126 grid points, centered on the protein center, and 0.375-Å spacing generated by the Autogrid program, were used to cover the whole protein structure, and the Lamarckian genetic algorithm was used for all molecular docking simulations. Each simulation was performed for 200 times, yielding 200 docked conformations. The genetic algorithm parameters for docking were as follows: population size = 300, mutation rate = 0.02, and crossover rate = 0.8. Simulations were performed using up to 2.5 million energy evaluations with a maximum of 270,000 generations. The root mean squared deviation (RMSD) was calculated for the resulting 200 structures using the ligand structure of the lowest binding energy as reference; a 1.0-Å tolerance was used to form clusters of the closest structures. The possible binding site was identified by the binding conformation with the lowest binding energy.

Further refined molecular docking was carried out to explore the binding model more precisely, based on the identified binding pocket. Smaller grid maps, 60 grid points in each Cartesian direction, were used, and maps were centered around the lowest binding energy conformation. The other parameters used were the same as described above.

Results and discussion

QCM biosensor rapid screening of the interactions between Tris and proteins

Immobilized Tris was chemically linked to the gold surface. The structure of the modified electrode is illustrated in Fig. 1. The gold surface of the crystal electrode was coated with a DTT self-assembled monolayer. The sulfhydryl groups were activated to epoxide groups with ethanediol diglycidyl ether under weak base conditions. The amino groups of Tris bound directly to the epoxide groups. Thus, Tris was immobilized on the QCM sensor chips. Because of the self-assembled monolayer of thio compounds, the gold surface of the crystal electrode was coated with hydroxy groups to prevent nonspecific adsorption of the proteins, and the hydrophilic modified surface was suitable for specific interactions between biomolecules.

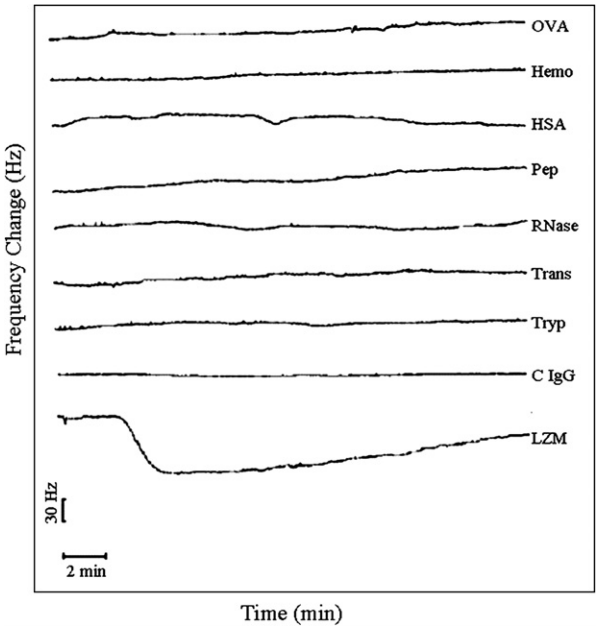

Fig. 2. Rapid screening of the interaction between immobilized Tris on a QCM sensor chip and various proteins in solution (0.5 mg mL^{-1}).

As reacting sites of the residual epoxyl groups had been blocked and the gold surfaces were hydrophilic, the nonspecific adsorption of these hydrophobic proteins on the gold surfaces was almost impossible [13]. Furthermore, the arm formed by the activated monolayer provided an appropriate spacer to facilitate the interactions between the small ligands immobilized on the gold surfaces and large proteins by alleviating steric hindrance [20,21].

Eight proteins coexisting in CEW were analyzed by QCM-FIA to rapidly screen for interactions between these proteins and immobilized Tris. HSA was used as a standard reference. The rapid screening analysis results are illustrated in Fig. 2. Under rinsing with the loading buffer within the tested 20 min, the frequency decreased about 60 Hz when LZM was injected into the flow cell and then returned to the initial level. For all other proteins, frequency remained at the baseline level. This indicated that

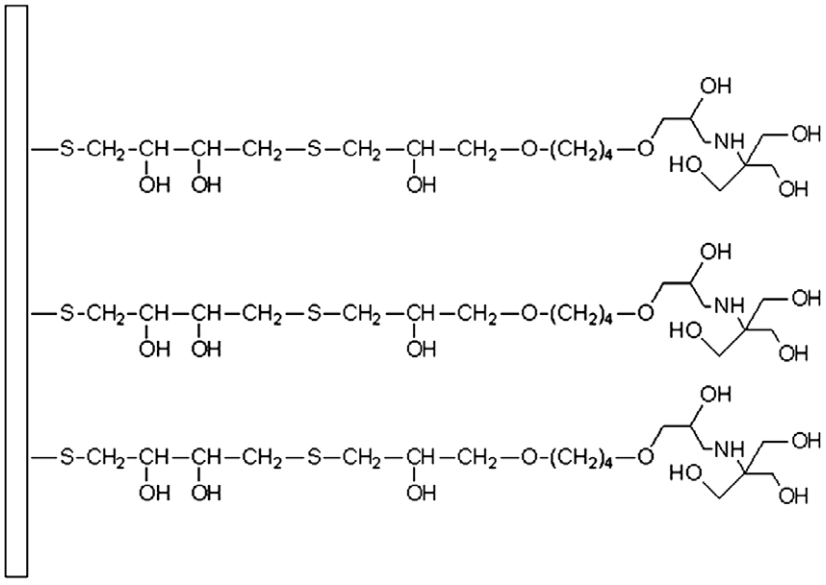

Fig. 1. Structure of biorecognition layers of Tris-modified sensor.

among the proteins tested, only LZM binds specifically to immobilized Tris under physiological ionic conditions.

From these results, it can be inferred that using Tris as a buffer for these proteins, except LZM, does not influence the experimental results. For LZM, whether the interaction influences the experimental result depends on the strength of the interaction between LZM and the ligand under investigation. If the specific interaction between LZM and ligand is much stronger than that between Tris and LZM, the influence on arm detection could be ignored.

In Fig. 2, the frequency returned to the initial baseline after one test cycle, indicating that the chips were quite reproducible. The good reproducibility of the chips was very important for the repetition of the sensor. The stable frequency around the baseline value while HSA was injected clearly indicates there is no nonspecific adsorption of these proteins on the modified sensor chip under physiological ionic conditions.

Surface plasmon resonance characterizing the interaction between Tris and LZM

SPR measurements were performed to prove the interaction between Tris and LZM and further determine the thermodynamic constants. Measurement with the SPR biosensor is a nondestructive analysis technique that has the important advantage of being capable of monitoring the interaction between molecules in real time [22]. OVA was used as a standard reference. As shown in Fig. 3, the response signal of SPR increased with the increase in concentration of the injected LZM solutions. When OVA was injected, the signal stayed on the baseline, which demonstrated that the sensor chip has low nonspecific adsorption. These results clearly indicate that LZM interacts specifically with immobilized Tris.

In addition, the apparent equilibrium dissociation constant K_D was estimated by curve fitting the steady-state binding level as a function of LZM concentration using the equation [23]

$$R_{eq} = C \cdot R_{max}/(K_D + C \cdot n), \quad (1)$$

where R_{eq} is the steady-state binding level, R_{max} is the theoretical binding capacity, C is the concentration of LZM, and n specifies the number of averaged binding sites on one analyte molecule [23] (in this research, we assumed n to be 1, which would be proved subsequently). The program Origin 7.0 (OriginLab Corp., MA, USA) was adopted for nonlinear regression analysis determining the apparent K_D using Eq. (1). Values for the apparent equilibrium dissociation constant K_D calculated by SPR analysis (Fig. 4) averaged 1.0×10^{-5} M.

The results obtained using the SPR biosensor supported the rapid screening results obtained with the QCM biosensor. Primarily the thermodynamic constant of the specific interaction between LZM and immobilized Tris was characterized. Compared with the QCM biosensor, the total time spent performing the interaction analysis experiment was similar, from immobilization of Tris to acquisition of the final data, although the response time of the

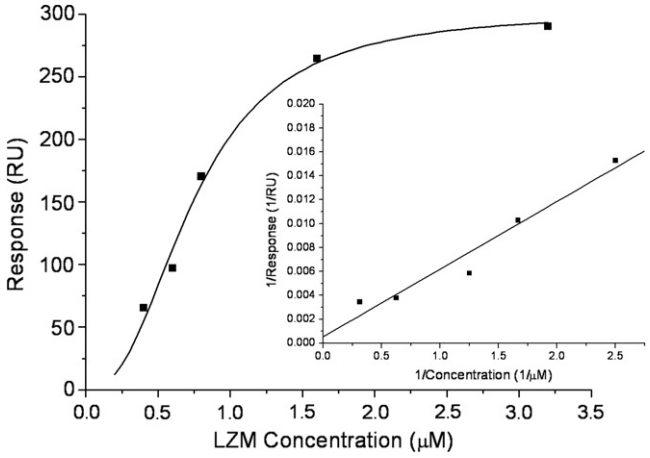

Fig. 4. Curve fitting of the steady-state responses in SPR analysis to estimate the apparent dissociation constant (K_D) of the complex of immobilized Tris and LZM.

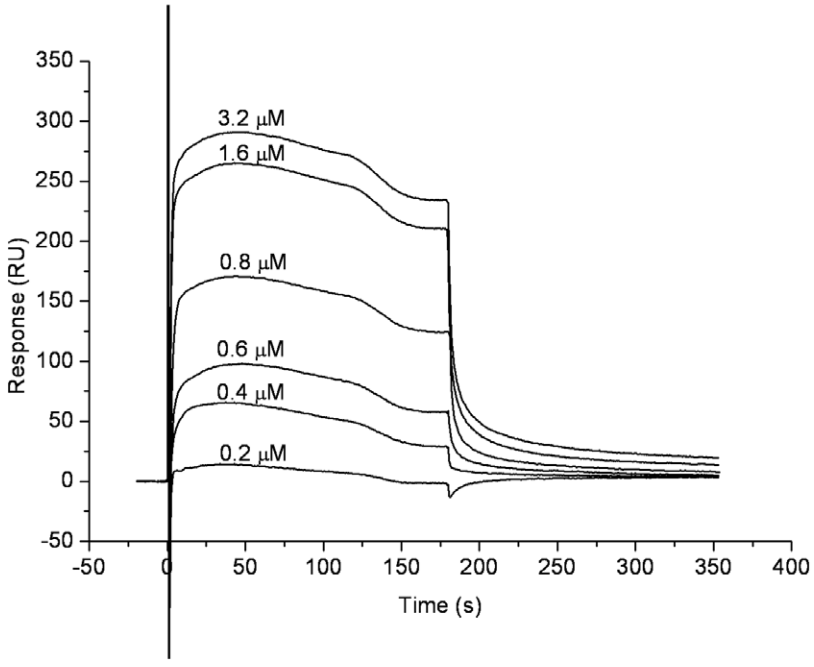

Fig. 3. Interaction between immobilized Tris on a sensor chip and various proteins in solution detected by SPR. The concentrations of LZM were 0.4, 0.6, 0.8, 1.6, and 3.2 μM, respectively. The standard reference was 3.2 μM OVA solution.

SPR biosensor (less than 30 s) is shorter than that of the QCM biosensor (about 60 s). For chip functionalization, the amount of reagent was equal. But in the SPR biosensor study, the injection volume was smaller because of the use of microfluidic technology. In addition, because of the higher signal-to-noise ratio of the SPR biosensor compared with the QCM biosensor, a much lower concentration of the protein was required for SPR analysis. But after all, the QCM biosensor has a price advantage.

Apparent dissociation constant determination for the interaction between Tris and LZM by HPAC

In an effort to provide a more definite quantitative analysis, we used HPAC to detect the apparent dissociation constant of this interaction. The immobilization of Tris onto an epoxy-activated stationary phase under base conditions has been proven to be an effective method [13]. In our experiment, the Tris molecule was immobilized on epoxy-activated silica in a high-concentration potassium phosphate aqueous solution (Fig. 5).

To estimate the binding specificity of the Tris immobilized affinity column, LZM and other proteins that coexist in CEW were injected into the affinity column and eluted under elution conditions, respectively. The chromatograms are shown in Fig. 6. It was clear that except for LZM, these proteins were not retained on the column, indicating that only LZM specifically binds to the immobilized Tris. The screening result obtained by HPAC is well consistent with that obtained using the QCM biosensor, suggesting that the interaction between Tris and LZM is specific.

To monitor the interaction quantitatively, frontal analysis by analytical HPAC was used to determine the apparent dissociation constant [17]. LZM solutions of various concentrations, $[P]_0$, were injected continuously into the Tris immobilized affinity column at a flow rate of 0.5 mL min^{-1}, passing through the affinity column and being monitored by UV absorbance at 280 nm until a platform of maximum absorbance was observed (Fig. 7). At this time, saturation binding of LZM on the affinity column was achieved and the eluted solution had the same concentration as the initial applied mobile LZM solution. The variation in elution volume $\overline{V}$ was plotted according to the equation [24]

$$\frac{1}{\overline{V}-V_0}=\frac{K_D}{M_T}+\frac{1}{M_T}[P]_0 \quad (2)$$

where $\overline{V}$ (mL) is the affinity matrix half-saturated elution volume, V_0 (mL) is the void volume of the column, K_D (M) is the apparent dissociation constant of complex of the immobilized Tris and LZM, M_T (mol g^{-1}) is the amount of Tris immobilized on 1 g of silica, and $[P]_0$ (M) is the initial concentration of mobile LZM. From the plot of $\frac{1}{\overline{V}-V_0}$ versus $[P]_0$ (shown in Fig. 7), K_D was determined as K_D = intercept/slope = 6.7×10^{-5} M.

The K_D value obtained by analytical HPAC was in accord with that obtained in the SPR study. It showed that the interaction between LZM and immobilized Tris is weak but specific. Based on this result, the following phenomena can be easily understood: the binding of LZM to the Tris immobilized on a QCM biosensor chip was not so strong and could be desorbed in the loading buffer (50 mM PBS, pH 7.0); and on the HPAC column, LZM was eluted by 0.6 M NaCl in about 2 min, which indicated that the interaction

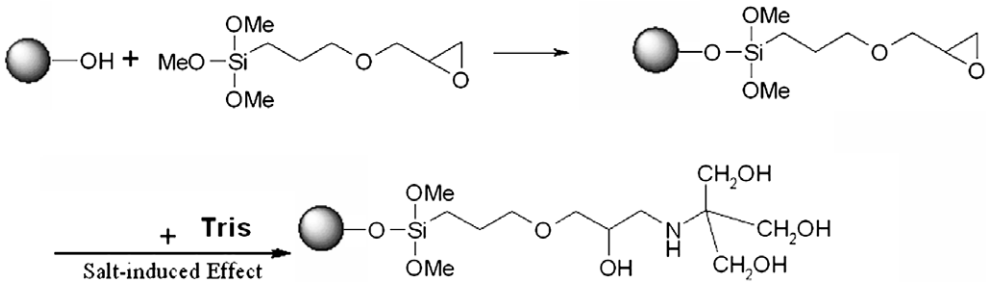

Fig. 5. Procedure for preparing Tris immobilized affinity packing.

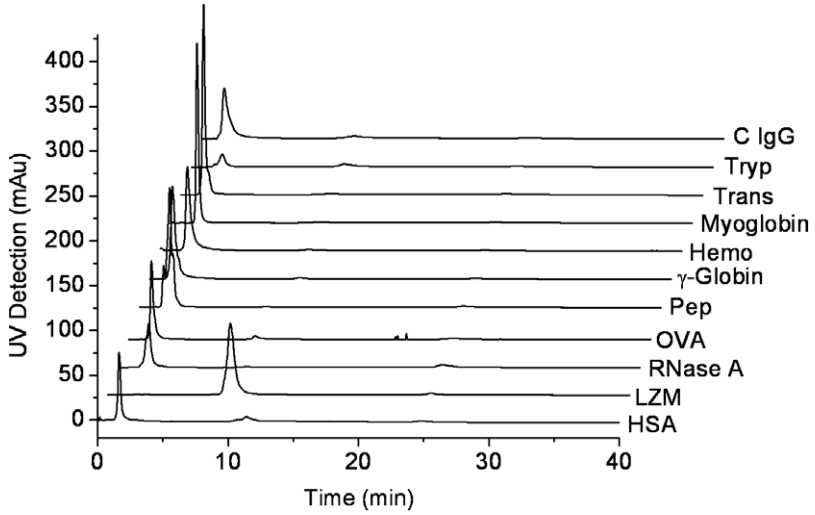

Fig. 6. Comparison of interactions of the Tris immobilized affinity column with various proteins (protein concentration = 0.5 mg mL^{-1}).

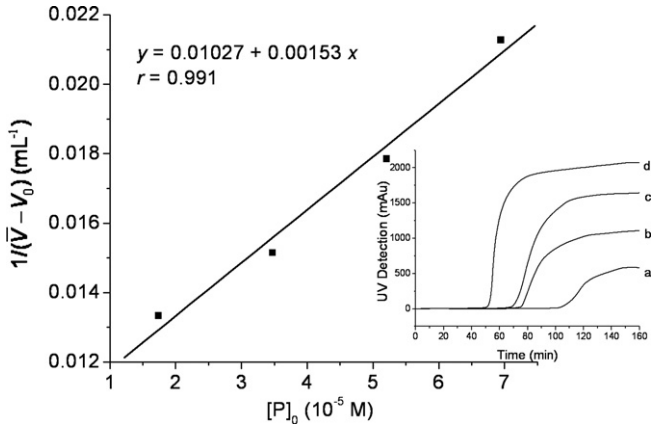

Fig. 7. Analytical HPAC experiment performed with LZM on the Tris immobilized affinity column. The concentrations of LZM were (a) 1.74×10^{-5}, (b) 3.47×10^{-5}, (c) 5.21×10^{-5}, and (d) 6.94×10^{-5} M.

between Tris and LZM could be easily interrupted by medium ionic strength in the mobile phase. The apparent dissociation constant of the interaction between LZM and Tris indicates that Tris can be safely used as a buffer solution when the ligand under study has strong binding ability to LZM. However, when the ligand can bind only weakly to LZM, the binding of Tris to LZM must be considered. This has been observed in previous studies, for example, the longer retention time of LZM than other proteins on the Tris column (used as a control) of affinity chromatography [13].

The binding mode simulation

The result of blind docking in molecular docking showed that the lowest binding energy conformation of Tris linked to a binding arm (shown in Fig. 8) with LZM lay in the substrate binding site of LZM, and refined molecular docking was performed based on the identified site. The lowest binding energy conformation is shown in Fig. 9. The three hydroxy groups of the Tris molecule formed H bonds with Asp52, Glu35, and Ala107 in LZM. The estimated binding free energy was −6.34 kcal mol^{-1}, corresponding to a K_D of 2.3×10^{-5} M, which was in good accord with the apparent dissociation constant obtained using the SPR biosensor and frontal analysis by HPAC. This binding model further confirmed that immobilized Tris does interact specifically with LZM.

Conclusion

We have demonstrated that there is a specific interaction between lysozyme and an extensively used buffer, Tris, with a dissociation constant on the order of 10^{-5} M, using QCM-FIA, the SPR biosensor, frontal analysis by HPAC, and molecular docking. Tris molecules can bind to the substrate binding pocket in lysozyme and, thus, might compete with substrate binding. This study shows that in quantitative binding studies, buffers should be carefully chosen to avoid possible complexity resulting from buffer molecule binding. In the case of lysozyme ligand binding studies, it is better to avoid using Tris when the ligands are weak. As we have shown that the Tris immobilized affinity column can specifically bind to lysozyme, this type of column could be used for the separation and purification of lysozyme directly from CEW.

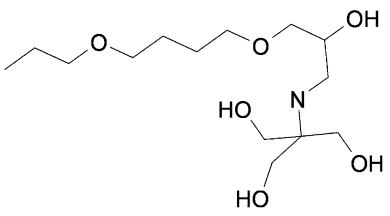

Fig. 8. Tris and binding arm used in molecular docking.

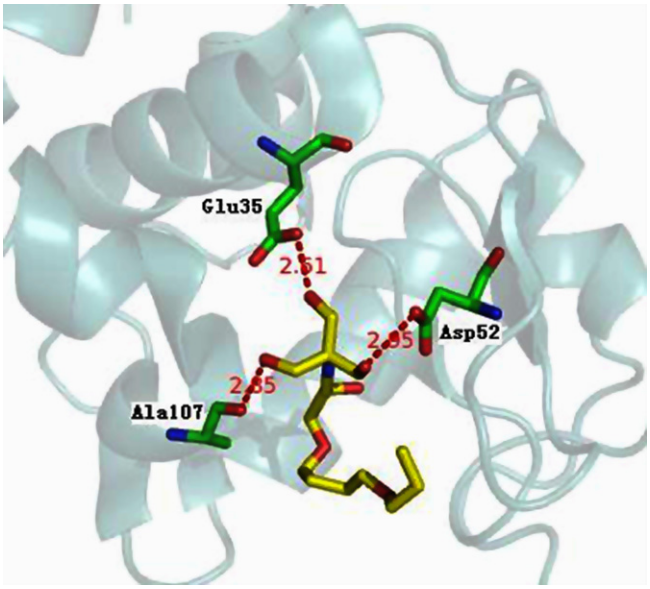

Fig. 9. Modes of binding of LZM with Tris linked with a binding arm.

Acknowledgments

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