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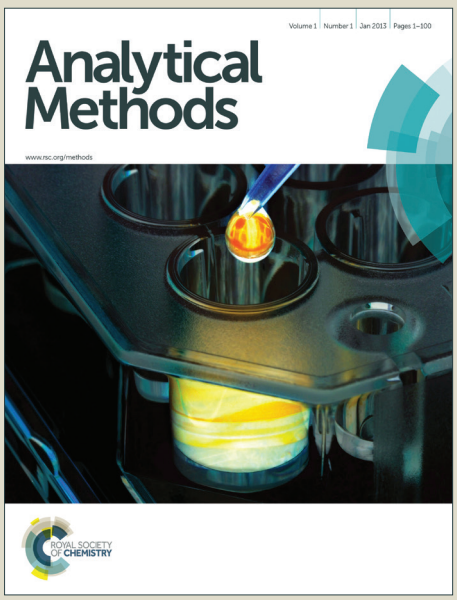

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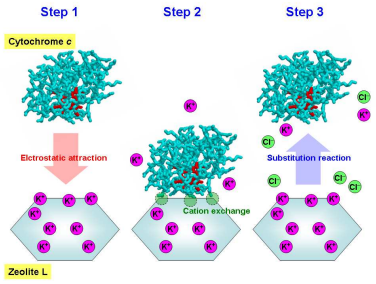

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COMMUNICATION

The protein recognition ability of zeolites: complete separation of cytochrome *c* from a ternary protein mixture by zeolite L†

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Zeolite L (LTL) can recognize cytochrome *c* (cyt *c*) in a model ternaly protein mixture and completely separate it with 100 % recovery from the mixture. LTL is a highly potential adsorbent for the purification of proteins.

Protein have been purified stepwise using conventional columns packed with adsorbents such as silica, alumina and organic polymers. However, the demand for new types of adsorbents taking the place of such traditional packing reagents dramatically increases with a rapid progress in the protein science and technology recently.[1,2] Zeolites[3,4] are crystalline aluminum silicates whose structures are performed by millions of tiny pores and channels allowing the movement of ions and molecules. These 'molecular sieves' can change their surface properties with their constituent ratios, SiO_2/Al_2O_3, for example, zeolites with low SiO_2/Al_2O_3 ratios are hydrophilic and can act as ion exchangers, on the other hand, with high ratios, are hydrophobic. Thus, zeolites have been widely used not only in industry but also in home as catalysts, separating materials, water softners, deciccants, deodorants, etc. Reflecting such wide uses, recently the adsorption of proteins on zeolites has been investigated. So far, it has been reported that zeolites A[1] and X[5] can separate γ-globulin with acceptable purity from a model ternary protein mixture of γ-globulin, transferrin and albumin, and ultra stable zeolite Y (USY)[6,7] can perform partial purification of lactate dehydrogenase, antibodies, peroxidase and lysozyme from an ox heart extract, hybridoma culture, horseradish extract and egg white, respectively, and at the same time, a protein binding to USY is highest in amount at or just below the isoelectric point (pI) of the protein, being dependent on the pH of the solution. In addition, the adsorption behaviors of bovine serum albumin (BSA)[8] and chytochrome *c* (cyt *c*)[9] on MFI-type zeolite were reported. We investigated the adsorption of pure proteins on different types of zeolites and found that, on the binding between a zeolite and a protein, electrostatic attraction is predominant in the pH region below pI and hydrophobic interaction becomes very important in the pH at and above pI.[2] These let us have a hope that under certain conditions, some zeolite can distinguish a particular protein in the mixture and isolate it. For example, zeolite beta (BEA) can separate proteins at their pI.[10] Based on the results, BEA was applied for the refolding of various proteins.[11] These findings suggest that zeolites are high potential for their application to the protein science and technology although only a limited number of materials (zeolite A,[1] X,[5] USY,[6,7] MFI[8,9] and BEA[10,11]) were tested in the previous works.

Selectivity of adsorbents, that is, recognition ability, is a significant factor for the purification of proteins. Of course, the selectivity of a zeolite as the adsorbent varies with proteins, depending their properties, and the purification degree of a protein changes with zeolites employed and conditions used. This means that more detailed investigation on the purification of proteins using various types of zeolites should be performed under different conditions. As mentioned above, the protein recognition ability of zeolites vary with the crystalline structure (type of zeolite), the SiO_2/Al_2O_3 ratio and pH of the buffer solution. In this study, we focused on the protein recognition of zeolites from the viewpoint of their cation exchange capacity and so selected zeolite L (LTL) which is one of the most popular zeolites with cation exchange property. Among zeolites tested in our previous work,[2] zeolite L (LTL) also showed excellent selectivity for the adsorption of basic proteins at physiological pH. In addition, LTL would release proteins under certain conditions (Fig. S1, ESI†). Based on these, here we investigate the recognition ability of LTL for both pure and mixed protein systems in two ways of batch adsorption and column chromatography. Using cyt *c*, which is well known as a

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† Electronic supplementary information (ESI) available: Experimental and other details. See DOI:

biomarker[12] of cancer therapy, liver disease and neuronal damage, namely one of the most important proteins, as the target protein, we show that LTL can recognize and separate it with 100 % recovery in a model ternary protein mixture.

Potassium cation type of LTL (K-LTL) with the SiO_2/Al_2O_3 ratio of 6 was purchased from Tosoh Co. (Japan), and after pressed to shape into pellets of 250-420 μm mesh, was used for the batch adsorption and column chromatography. The proteins, cyt *c* (MW = 12.4 kDa, pI = 10.1), hemoglobin (Hb, MW = 64.75 kDa, pI = 6.8-7.0) and BSA (MW = 69 kDa, pI = 4.9) were purchased from Wako Pure Chem. Ind., Ltd. (Japan) and used as the typical examples of basic, neutral and acidic pure proteins, respectively. The buffer used here, Tirs-EDTA buffer [10 mM Tris-HCl (pH 7.5) / 0.1 mM EDTA] was prepared by dissolving tris(hydroxymethyl)aminomethane in water and adjusting the pH to 7.5 with HCl and then combining with a solution of EDTA dissolved in ultrapure water (18.2 MΩ·cm) in advance. KCl buffer solutions were prepared by dissolving appropriate amounts of KCl in the Tris-EDTA buffer. The proteins were dissolved in the buffers and used for the batch adsorption and column chromatography experiments.

In the batch adsorption, K-LTL (100 mg) was suspended in 1 ml of Tris-EDTA buffer, degassed for 10 min and centrifuged at 10,000 rpm. Then, 500 μl of the supernatant was removed and 500 μl of a protein solution (protein, 6 mg ml^{-1}) was added to give the protein solution of 3 mg ml^{-1}. Incubation was carried out for 1 h at room temperature, because the adsorption equilibrium for K-LTL was sufficiently reached within 1 h under this experimental condition (Fig. S2, ESI†). The supernatant was pipetted off after the centrifugation at 12,000 rpm, and this operation was repeated twice. The amount of a protein unadsorbed on K-LTL was obtained from the absorbance of the supernatant at 595 nm (Bradford method). The amount of protein bound to K-LTL was determined by subtracting the amount of unadsorbed protein from total amount of protein. As a result, K-LTL could catch cyt *c* enough, but not the other proteins at all. In the current buffer system contains EDTA which has very strong affinity towards the transitional metal iron ions. It is necessary to determine whether the adsorption of heme proteins such as cyt *c* and Hb on K-LTL is affected by EDTA or not. We confirmed that the effect of EDTA on adsorption of proteins was excluded by analysis of three-dimensional structure of cyt *c* and Hb (Fig. S3 and S4, ESI†). The detail on the adsorption of cyt *c* is shown in Fig. 1. In the pure cyt *c* buffer solution, about 95 % of the original cyt *c* amount was adsorbed on K-LTL and about 5 % of that remained in the solution. In the 0.5 and 1.0 M KCl buffer solutions, K-LTL caught about 41 % of the cyt *c* and did not catch it at all, respectively. These indicate that the amount of K-LTL used (100 mg) was a bit short to catch all cyt *c* (3 mg) contained in the solution, in other words, 100 mg of K-LTL is completely saturated with 2.85 mg of cyt *c*, and both the 0.5 and 1.0 M KCl buffer solutions would elute the cyt *c* adsorbed on K-LTL. It should be noted that the saturated adsorption amount of cyt *c* obtained by the adsorption isotherm analysis is approximately 40 mg g^{-1} (Fig. S5, ESI†). The performance of

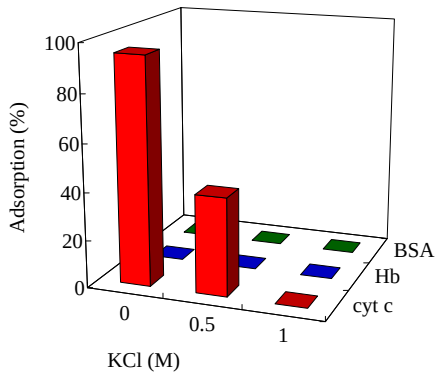

Fig. 1 Adsorption of cyt *c*, Hb and BSA on K-LTL in the absence and presence of KCl.

K-LTL as the adsorbent for cyt *c* is superior to that of other types of adsorbents for cyt *c*, such as metal chelate affinity adsorbent (32 mg g^{-1})[13] and MCM-48 (11.8 mg g^{-1}).[14]

In the column chromatography, K-LTL (300 mg for pure proteins, 300 mg for a ternary mixture) was suspended in an appropriate amount of Tris-EDTA buffer, degassed for 10 min and then packed into a glass column (diameter 10 mm, length 50 mm, Bio-rad, USA). Three ml of a pure protein solution or 3 ml of a mixture which was composed of three kinds of 1 mg ml^{-1} protein solutions was poured onto the column, and incubated for 1 h at room temperature. Then, the fractionation was carried out by collecting every ten drops which passed through spontaneously, were washed out with 5 ml of Tris-EDTA buffer and were eluted with respective 5 ml of the 0.5 M and 1.0 M KCl buffer solutions. The presence of protein in each fractions was monitored by Bradford method. Consisting with the results in the batch experiments, in the K- LTL column adsorptions, two respective proteins of Hb and BSA spontaneously passed through the column, and only cyt *c* was entirely trapped on the column. As expected, both the 0.5 and 1.0 M KCl solutions could elute the cyt *c* adsorbed, as shown in Fig. 2.

For a ternary protein mixture, the protein recognition ability of K-LTL was evaluated only in the column adsorption. For the mixture of cyt *c*, Hb and BSA, the fractionation by the K-LTL column showed two peaks as shown in Fig. 3. The first peak consists of 20 fractions that flowed through the column spontaneously, and the second peak was obtained by eluting the thing that adsorbed on the column with the 0.5 and 1.0 M KCl buffer solutions.

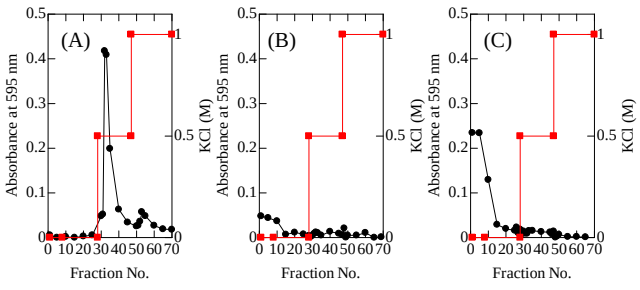

Fig. 2 Fractionation of pure proteins by the K-LTL column; cyt *c* (A), Hb (B) and BSA (C). ■: KCl buffer.

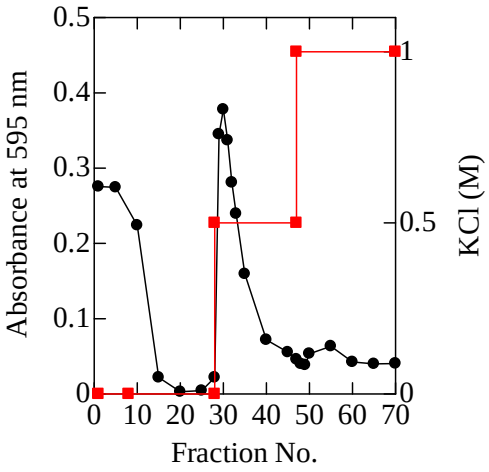

Fig. 3 Fractionation for a ternary mixture of cyt *c*, Hb and BSA by the K-LTL column. ■: KCl buffer.

The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and native PAGE (PAGE in the absence of SDS) analysis of these two peaks, the protein mixture and the respective pure proteins are shown in Fig. 4. Fraction No. 1, which belongs to the peak 1, and washing after the elution of the peak 1 show quite similar patterns to each other, and these patterns are found to be overlapping of the pure BSA and Hb or cyt *c*. On the other hand, four fractions Nos. 29, 30, 48 and 49 that belong to the peak 2 are similar in the pattern to one another, and also, to each pure protein of cyt *c* and Hb. Thus, based on the SDS-PAGE analysis, it can be easily concluded that BSA is contained in the peak 1 and flows through the column completely, and the peak 1 consists of more than one protein. However, both Hb and cyt *c* give bands around 10 kDa and 25 kDa (possibly came from the impurities in both Hb and cyt *c* purchased as the pure proteins), moreover, it has been known[15,16] that in the presence of SDS, Hb turns down to monomers whose molecular weight is almost equal to that of cyt *c*, although it normally takes tetrameric structure. Accordingly, it is hard to say to which peak Hb belongs and whether the peak 2 contains only cyt *c* as the protein or not. For these questions, the native PAGE analysis could give answers. Under the conditions of the native PAGE analysis, Hb keeps its tetrameric structure,[15,16] on the other hand, cyt *c* is charged plus. Positively charged cyt *c* is repelled and cannot enter into the gel. Accordingly, in the native PAGE analysis, cyt *c* is not detectable, but Hb can be definitely detected. It is found that both fraction No. 1 and the washing show bands due to Hb together with those arisen from BSA, and all four fractions, Nos. 29, 30, 48 and 49, which belong to the peak 2, show no bands, indicating no Hb in the peak 2. Combining the results of the SDS-PAGE and native PAGE analyses, it is clear that the peak 1, which flows through the K-LTL column smoothly, contains both Hb and BSA, and the peak 2 which is trapped in the K-LTL column contains only cyt *c*. The recovery of cyt *c* from a ternary mixture by K-LTL was detected as follows. The concentration of recovered cyt *c* in each fraction was

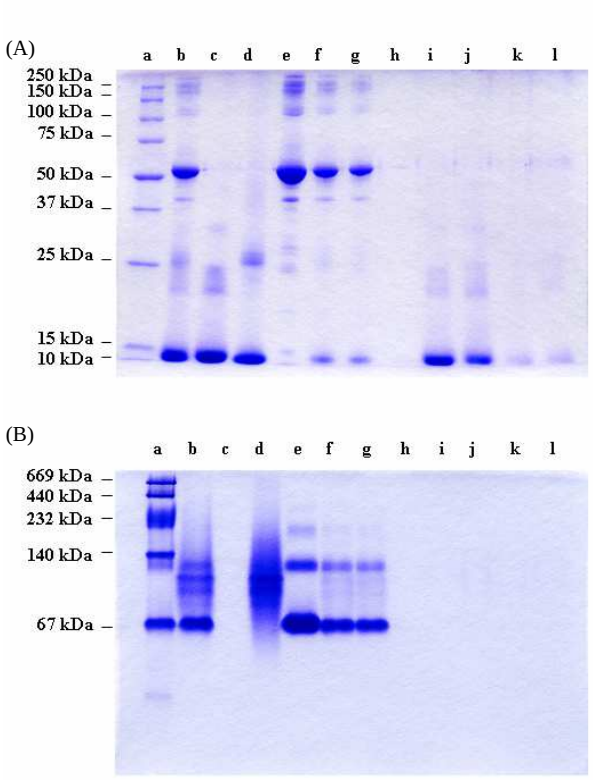

Fig. 4 SDS-PAGE (A) and native PAGE (B) analysis of pure proteins, a protein mixture (cyt *c*, Hb and BSA) and the fractions shown in Fig. 3. Maker (a), mixture (b), cyt *c* (c), Hb (d), BSA (e), and fractions No. 1 (f: passed through spontaneously), Nos. 10 and 20 (g and h: eluted with Tris-EDTA buffer, or washings), Nos. 29 and 30 (i and j: eluted with 0.5 M KCl buffer), Nos. 48 and 49 (k and l: eluted with 1.0 M KCl buffer).

determined by the Bradford method using standard curves of cyt *c* which containing 0.5 M KCl for fraction Nos. 29 to 47 and 1 M KCl for fraction Nos. 48 to 70, respectively (Fig. 3). Every one fraction contains approximately 0.22 to 0.26 ml of solution. The amount of eluted cyt *c* (mg) in each fraction was calculated by multiplying the concentration of cyt *c* (mg ml^{-1}) by the volume of solution (ml). The total recovery amount of cyt *c* was calculated as the sum of the amount of cyt *c* in each fraction and found to be 107 %, which indicate complete recovery of cyt *c* from a ternary protein mixture.

From the above, it is concluded that K-LTL can recognize cyt *c* in the protein mixture and completely isolate it. We have already stated[2,10] that zeolites recognize proteins through ion exchange, electrostatic attraction, hydrophobic interaction, ligand exchange on the Lewis acid site, etc. In this work, we would like to say that K-LTL recognizes cyt *c* in the mixture through mainly electrostatic attraction and ion exchange between functional groups of a protein and the potassium ions near the surface of K-LTL, because only cyt *c* among the three proteins employed here is considered to be charged positively under the experimental conditions. At last it should be emphasized that zeolites are very interesting materials for the purification of proteins. Because zeolites have much higher mechanical strength, thermal and chemical resistance than the conventional adsorbents, moreover, according to the

properties of a target protein and the conditions of the solution containing it, a proper zeolite can be chosen from a wide variety of the types, and the factors affecting the protein recognition ability, or ion exchange capacity, hydrophobicity, meso pore size, etc. can be adjusted and optimized by changing the ratio of SiO_2/Al_2O_3 and the preparation conditions.

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