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Importance of surface properties of affinity resin for capturing a target protein, Cyclooxygenase-1

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ABSTRACT

We have prepared affinity resins based on two kinds of solid phases, including a commercially available solid phase, to re-realize the importance of surface properties of affinity resins such as controlled ligand density as well as existential surroundings of the ligand. Affinity resins were prepared using non-steroidal anti-inflammatory drugs, such as Ketoprofen, Ibuprofen, and Aspirin, having different activities as ligands. The ligand density was controlled through two different strategies: one strategy was that the solid phases having different amino group densities (20, 60, 100, 125 μmol/ml) were utilized then, Ketoprofen was fully immobilized through condensation reaction to amino groups; another strategy was that a solid phase having amino group density (125 μmol/ml) was utilized then, each ligand was immobilized with controlled immobilization rate. In addition, a typical hydrophobic group, stearoyl group (C_{18} group), was immobilized on the affinity resin with controlled ligand immobilization rate to change the existential surroundings of the ligand. Affinity tests were performed for Cyclooxgenase-1 (COX-1) as it was the target protein in this work. The amount of captured COX-1 was evaluated utilizing each affinity resin. It was suggested that the density of surface ligand tends to relate to the amount of captured COX-1 on our solid phase-based affinity resins; however, several exceptions occurred according to the surface properties of affinity resins in the case of commercial one.

1. Introduction

Researches on the target proteins of bioactive substances utilizing affinity resin are receiving much attention.[1–8] It is simply because the experiment utilizing affinity resin (affinity test) is rather easy and quite effective. In the test, the affinity resin is stirred with protein lysate prepared from the target organs or even cells. The proteins interacting directly with the immobilized bioactive substances on affinity resins are captured and analyzed to assess the target proteins. These researches are obviously associated with novel drug discovery, therefore, development or improvement of techniques in these researches is related to the advancement of our health and living directly.

Since affinity resin is one of the most important tools to discover target proteins, several base solid phases have been developed through proprietary methods.[3,8–10] In fact, there are several commercial solid phases utilized for the preparation of the affinity resins, for example, Affigel™ and Toyopearl™.[11,12] These solid phases have been often utilized as base solid phases, but have several problems such as the possibility of chemical denature under the reaction condition of ligand immobilization and the adsorption of high level of non-specific binding proteins interfering with the analysis of specific binding proteins.

If we prepare affinity resin, bioactive substances should be immobilized on base solid phase as much as possible. It is a readily understandable concept that maximal interaction between proteins and immobilized bioactive substances is realized. On the other hand, Takahashi et al. reported that there were target proteins captured efficiently by affinity resin having lower or controlled density of immobilized bioactive substance.[5] Takahashi et al. also suggested in that report that the density of bioactive substance on affinity resin was important for accessibility of rather large target proteins and for interaction between proteins and the immobilized substance. This is an interesting suggestion because it will directly affect the achievement of affinity test and/or failure in capturing the possible target proteins. Therefore, the density of immobilized bioactive substance should be carefully controlled to optimize affinity test. In addition, Tanaka et al. reported quantitative analysis of non-specific binding proteins utilizing several compounds as ligands.[6] They have described that the amount of non-specific binding proteins is simply related to hydrophobic properties of ligands.

Based on the above-mentioned reports, we have recently developed a novel solid phase, namely Moli-gel (*Mo*no*li*thic affinity *gel*).[10] One of the advantages of Moli-gel is its highly hydrophilic property, which presumably prevents non-specific binding pro-

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teins. We have also shown that preparation of Moli-gel having controllable density of surface functional group, and in fact, ligand immobilization rate can be controlled on Moli-gel to prepare various types of affinity resins.

In this report, we wish to describe the preparation of affinity resins having several ligand densities based on the solid phase, Moli-gel and affinity tests for capturing Cyclooxygenase-1 (COX-1) as a target protein. Non-steroidal anti-inflammatory drugs, such as Ketoprofen (Ket), Ibuprofen (Ibu), and Aspirin (Asp), are utilized as ligands. This is because these drugs have different hydrophobic properties as well as different activities, while one of the target proteins is reported to be COX-1 in common.[13–15] Therefore, we can easily assess several affinity resins having different conditions in terms of hydrophobicity or the density of ligands, which definitely affects the capturing of COX-1. COX-1 is a membrane protein, and synthase of prostaglandin causes inflammation.[16] So, the protein stably exists on the membrane, which has a rather hydrophobic surrounding.

We prepared affinity resins having controlled ligand immobilization rate (directly related to ligand density), later, a typical hydrophobic group was immobilized on the residual functional groups on the solid support to construct hydrophobic surroundings on the affinity resins. Herewith, pseudo-membrane was reappeared by the introduction of hydrophobic group on the affinity resins where COX-1 may stably exist. We wish to discuss the advantage of interaction between ligand existing in the surroundings and COX-1 in solution. Then, the importance of surface property of controlling the existential surroundings of ligand will be discussed. This is also the reason why we selected and used these ligands and the target protein. These strategies are conceptually illustrated in Figure 1.

2. Experimental

2.1. Reagents

Solvents and reagents were utilized without further purification unless it was particularly mentioned. The details of monomers and porogen utilized for the preparation of Moli-gel were reported in our previous report, including the structures of those compounds.[12]

In the following experimental description, Nacalai Tesque, Inc. (Kyoto, Japan) Wako Pure Chemical Industries, Ltd (Osaka, Japan) Tokyo Chemical Industry Co., Ltd (Tokyo, Japan) and Bio-Rad Laboratories (Tokyo, Japan) have been abbreviated as Nacalai, Wako, TCI, and Bio-Rad, respectively.

Trifluoroacetic acid (TFA), sodium hydrogen carbonate, triethylamine ninhydrin, acetic anhydride, acetic acid, *N*,*N*-dimethylformamide (DMF), sucrose, 1 M Tris–HCl buffer solution (pH 7.6), sample buffer solution with 2-ME (2×) for SDS–PAGE including 4% (w/v)-SDS, 20% (v/v)-glycerol, 0.01% (w/v)-CBB, 10% (v/v)-2-mercaptoethanol, 0.125 M Tris–HCl, pH 6.8, running buffer solution (10×) for SDS–PAGE, Rapid Stain CBB Kit, protein Assay CBB solution (5×), and Blocking One were purchased from Nacalai. 2,2′-Oxydiethanol (DEG-p), 2,2′-azobis(2,4-dimethylvaleronitrile) (ADVN), acetonitrile, 2-(*p*-isobutylphenyl)propionic acid (Ibuprofen (Ibu)), 2-acetyloxybenzoic acid (Aspirin (Asp)), sodium *N*,*N*-diethyldithio carbamate trihydrate, methanol, dimethyl sulfoxide (DMSO), acetone, ethanol, and octanol were purchased from Wako. 2-(2-Methoxyethoxy) ethyl methacrylate (DEG-m) and 2-(3-benzoyl phenyl)-propionic acid (Ketoprofen) were purchased from TCI. Stearoyl chloride and Cyclooxgenase-1 (COX-1) were purchased from SIGMA–ALDRICH (Tokyo, Japan). *N*-Methyl-2-pyrrolidinone dehydrated (dry-NMP) was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (water-soluble carbodiimide: WSCD) and 1-hydroxybenzotriazole (HOBt) were purchased from PEPTIDE INS. (Osaka, Japan). *N*-*t*-Butoxy-17-amino-3,6,9,12,15-pentaoxaheptadecane-1-nyl methacrylate (Lig-m) was kindly donated by Reverse Proteomics Research Institute (Tokyo, Japan). Polyethylene glycol #400 dimethacrylate 9G NK ESTER (9G) was donated by Shin-Nakamura Chemical Co., Ltd (Wakayama, Japan). Toyopearl™; AF-Amino-650M (Toyopearl) was purchased from Tosoh Bioscience (Pennsylvania, USA). The certified surface density of amino groups is as much as 90 μmol/ml. Ready Gels J 7.5–15% 161J381V was purchased from Bio-Rad. Seventy-six percent w/w phenol/EtOH (phenol/EtOH) was purchased from Applied Biosystems (Tokyo, Japan). Anti-COX-1-Mouse-mono<Anti-Prostaglandin H Synthase-1> was purchased from Cayman Chemical (USA). Surfactant P20, Amersham ECL™ anti-mouse IgG, HRP Linked species-specific F (ab′)$_2$ fragment (from sheep), and ECL plus Western blotting detection system were purchased from Amersham Biosciences Corp. (Buckinghamshire, UK). Mark12™, NuPAGE Transfer Buffer (20×), PVDF membrane, MagicMark XP Western Standard, XCell SureLock™ Mini-Cell, and XCell II™ Blot Module were purchased from Invitrogen (CA, USA). PBS (phosphate buffered salts) Tablets was purchased from Takara Bio (Tokyo, Japan). Rat brain was purchased from Funakoshi (Tokyo, Japan). Albumin standard was purchased from Pierce Biotechnology, Inc., (Rockford IL, USA).

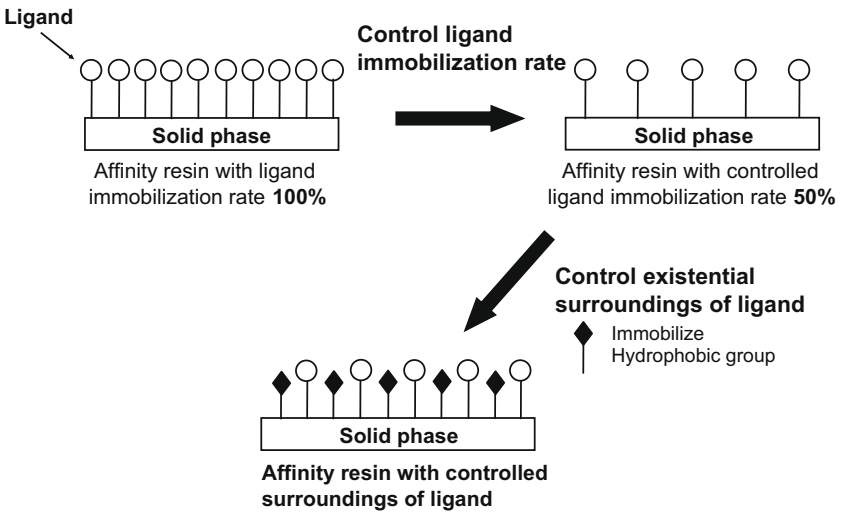


Figure 1. Schematic illustration of affinity test and affinity resins having controlled ligand immobilization rate, or the existential surroundings of ligand.

2.2. Apparatus

A Beckman centrifuge, Avanti J-25 was utilized with a JLA-10500. FT/IR 4200 Spectrometer and ATR PRO450-S (JASCO) were used for qualitative confirmation of the ligand immobilization, GS-800 Calibrated Imaging Densitometer, ChemiDoc XRS (BIO-RAD) recorded images of SDS–PAGE and Western blot analyses. SCL-10AVP, LC-10ADVP, DGU-14A, CTO-10AVP, and SPD-M10AVP (Shimadzu) were used for high performance liquid chromatographic (HPLC) evaluation.

2.3. Preparation of solid phase, Moli-gel

Preparation of solid phase, Moli-gel, was described in our previous paper.[12] The feed compositions of Moli-gel are summarized in Table 1. The density of amino group as surface functional group of Moli-gel was evaluated based on the theoretical value and ninhydrin method. Titration method was also utilized for further evaluation. Amino group densities of Moli-gels were controlled about 20, 60, 100, and 125 μmol/ml, and the symbols of each Moli-gel were M20, M60, M100, and M125, respectively, as shown in Table 1.

2.4. Preparation of affinity resin using Moli-gel and Toyopearl

2.4.1. Immobilization of ligands (Ket, Ibu, and Asp)

In this paper, the term 'affinity resin' means the solid phase having immobilized bioactive substance (ligand) on it, therefore, Moli-gel or Toyopearl is just base solid phase. After Moli-gel or Toyopearl was washed with acetonitrile and dry-NMP, the prepared ligand solution of Ket, Ibu, or Asp was added to the solid phase with 1.2 equiv of WSCD and/or HOBt. The equivalent of added ligand was calculated to be 0.5 or 4.0 equiv to the amount of total amino groups of solid phase. The reaction was usually carried out at ambient temperature for 24 h.

2.4.2. Immobilization of stearoyl chloride

The equivalent of added stearoyl chloride was 0.5 or 4.0 equiv to the amount of amino groups of solid phase. Triethylamine was added to stearoyl chloride with 1.2 equiv and dry-NMP as solvent. The reaction was usually carried out at ambient temperature for 24 h. Stearoyl chloride immobilized on the solid phase was described as C_{18} group or C_{18} groups.

2.4.3. Treatment after immobilization of ligand; acetyl capping

The residual amino groups on affinity resin after the immobilization with 0.5 equiv of ligand were fully capped with acetyl groups by the treatment of acetic anhydride in DMF. The resulting affinity resins were washed with dry-NMP, ethanol, 60%, and 20% ethanol.

Immobilization rate of affinity resins prepared utilizing each ligand is indicated in Table 2. Theoretical immobilization rate was 50%. These were evaluated by ninhydrin method. Toyopearl-based affinity resin could not be estimated by ninhydrin method.[12] The completion of each immobilization reaction was confirmed by ninhydrin method in the case of Moli-gel-based affinity resins.

Table 1
Feed composition of Moli-gels

Symbol	Lig-m (μl) (ratio of mole)	DEG-m (μl)	9G (μl)	DEG-p (μl)	ADVN (mg)
M20	34.5 (1)				
M60	68.9 (2)	15.8	390.8	750.0	10.0
M100	120.8 (3.5)				
M125	172.3 (5)				

2.5. Preparation of lysate

Unfreezed rat brain was homogenized in buffer A (1:10) (0.25 M sucrose, 0.3 mM *N*,*N*-diethyldithiocarbamate, 50 mM Tris–HCl, pH 7.5, 0.5% surfactant P20). The homogenate was sonicated for 2 min and centrifuged at 9000 rpm at 4 °C for 10 min. The obtained supernatant was used as the lysate and kept at −80 °C before use.[5] Total protein concentration was approximately 6 mg/ml. It was measured by staining with protein assay CBB solution and comparing its color with Albumin standard (2 mg/ml).

2.6. Affinity test

2.6.1. COX-1 capturing

Three types of concentrations (0.5, 5, and 50 mM) of free Ket, Ibu, and Asp/DMSO solutions were prepared as 'free ligand solutions'. Fifty microliters of each free ligand solution were admixed with 900 μl of lysate (prepared by buffer A) or only buffer A spiked with COX-1 (1.36 pmol) at 4 °C for 40 min before the affinity test. Then, final concentrations of free ligands added into lysate or buffer spiked with COX-1 were 0.026, 0.26, and 2.6 mM. Solvent effects were offset by the addition of 50 μl of DMSO instead of ligand/DMSO solution. The lysate or only buffer A spiked with COX-1 was gently stirred with affinity resin at 4 °C for 2 h to capture the binding protein.

After the affinity test, the affinity resins were centrifuged in a microcentrifuge at 12,000 rpm and then washed once with 1.0 ml of buffer A. The washed resins were resuspended in 45 μl of SDS-sample buffer solution, diluted 1.5 times with water, shaken 1000 rpm at 4 °C for 10 min, and then centrifuged for 3 min. The supernatant was subjected to SDS–PAGE (SDS–PAGE condition; 24 mA/acrylamide gel constant electric current, 1 h), and detected by CBB stain and Western blot analysis as described below.

2.6.2. Western blot analysis

Western blot analysis was carried out as follows: the proteins were subjected to SDS–PAGE followed by electroblotting onto a PVDF membrane using the XCell II blot module (transfer condition; 150 mA constant electric current, 1.5 h). After blocking with Blocking One overnight, the membrane was incubated with the COX-1-monoclonal antibody for 1 h at ambient temperature. After washing, the membrane was incubated with HRP-conjugated antibodies for 1 h at ambient temperature and washed again. The membrane was soaked for 5 min in the detection reagent ECL Plus.

2.7. HPLC analysis of Ibu, Ket, and Asp

A 50 μl of 50 mM Ibu, Ket, or Asp /DMSO was admixed with 900 μl of buffer A. HPLC evaluation was performed to assess hydrophobic properties of three ligands using typical hydrophobic stationary phase, C_{18} phase. Chromatographic conditions: column; Shim-pack VP-ODS 150 mm × 4.6 mm I.D. Shimadzu, mobile

Table 2
Immobilization rate (%)

Ligand	Immobilization rate (%)[a]	
	Moli-gel	Toyopearl
Ket	55.0	69.5
Ibu	46.1	63.5
Asp	78.3	87.6
C_{18}	41.3	

[a] In case 0.5 equiv of total amount of amino groups on the solid phase is utilized for immobilization reaction, theoretical immobilization rate would be 50%. These were calculated based on ninhydrin method.

phase; 0.05% TFA aq:acetonitrile = 60:40 (pH 2.5), flow rate; 1 ml/min., injection volume; 5 μl.

2.8. Dispersion of affinity resin

Ket, Ibu, or Asp was immobilized on Moli-gel that is classified below 50 μm in diameter and Toyopearl. The ligand immobilization rate of the affinity resin prepared based on Moli-gel was 100%. That based on Toyopearl was 50% or 100%, after following the method mentioned in Section 5.1. These affinity resins were washed with dry-NMP and ethanol, and dried in vacuo overnight. Dried affinity resins were dispersed in a glass tube containing 5 ml of octanol and water. The tube was turned around vigorously several times.

3. Result and discussion

3.1. Qualitative evidence of immobilization of ligand

As demonstrated in Figure 2, FT/IR measurements clearly indicate immobilized ligand on Moli-gels. The arrow in the figure indicates the adsorption attributed to phenyl ring of the ligands, while base solid phases included no phenyl ring. In another way, we have had quantitative evidence for the immobilized ligand, which will be published elsewhere.[17]

3.2. Moli-gel- and Toyopearl-based affinity resins varying ligand density of Ketoprofen

First of all, we wish to discuss how the difference in ligand density on affinity resins affects the capturing of COX-1. As in Table 2, a titration method confirmed that the immobilization rates on Toyopearl accorded with those obtained theoretically, while ninhydrin method was not able to afford correct immobilization rate on Toyopearl.[17] Therefore, we described that the ligand densities on Toyopearl-based affinity resins were 45 and 90 μmol/ml, respectively. Affinity test using the lysate, COX-1 was not captured enough. Therefore, we could not assess the amount of captured COX-1. This is one reason why COX-1 was added to the lysate for further affinity tests.

As shown in Figure 3, COX-1 is captured on Moli-gel-based affinity resins having different ligand densities, while the amount of non-specific binding proteins is effectively reduced, compared with that of Toyopearl-based affinity resin having immobilization rate of 100%. Interestingly, the amount of captured COX-1 increased proportionately as the ligand density increased on Moli-

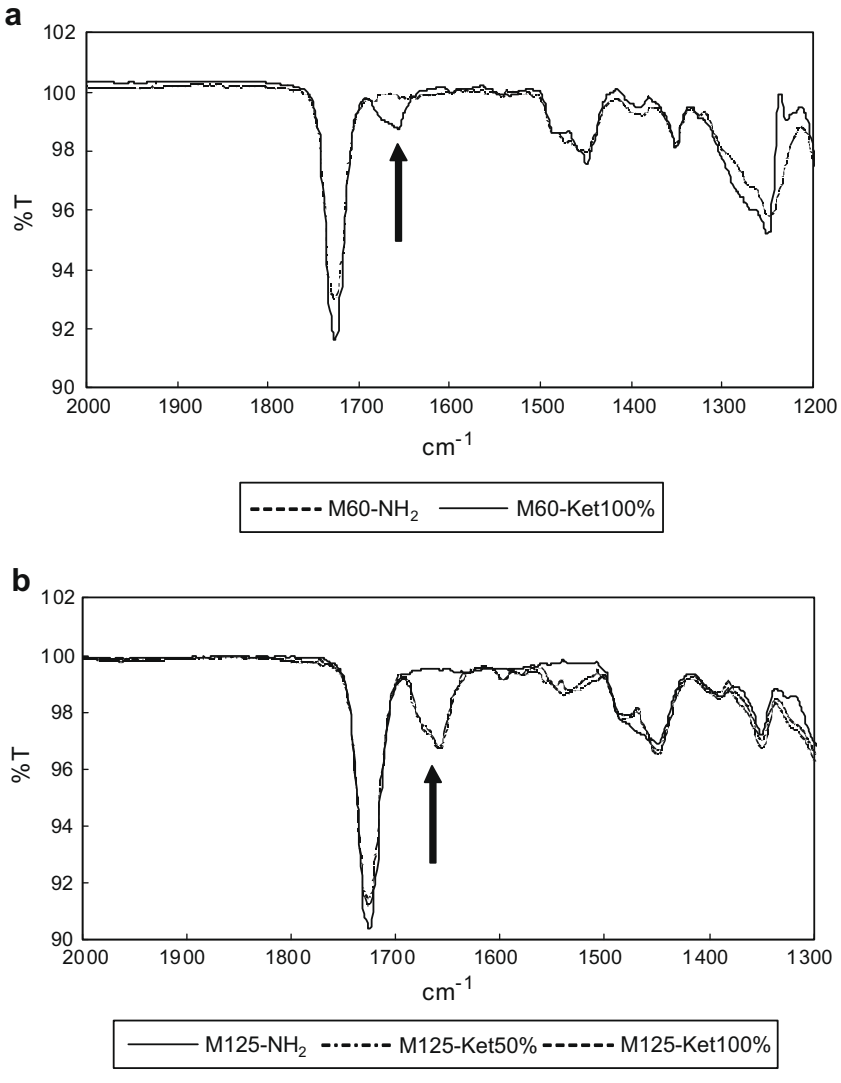


Figure 2. Qualitative investigation of affinity resins by FT/IR-ATR. Absorption of 1660 cm^{-1} (indicated by an arrow) is attributed to aromatic group of Ket on Moli-gel. (a) Moli-gel having amino group density of 60 μmol/ml and immobilizing Ket (immobilization rate of 100%), (b) Moli-gel having amino group density of 125 μmol/ml and immobilizing Ket (immobilization rate of 50% or 100%).

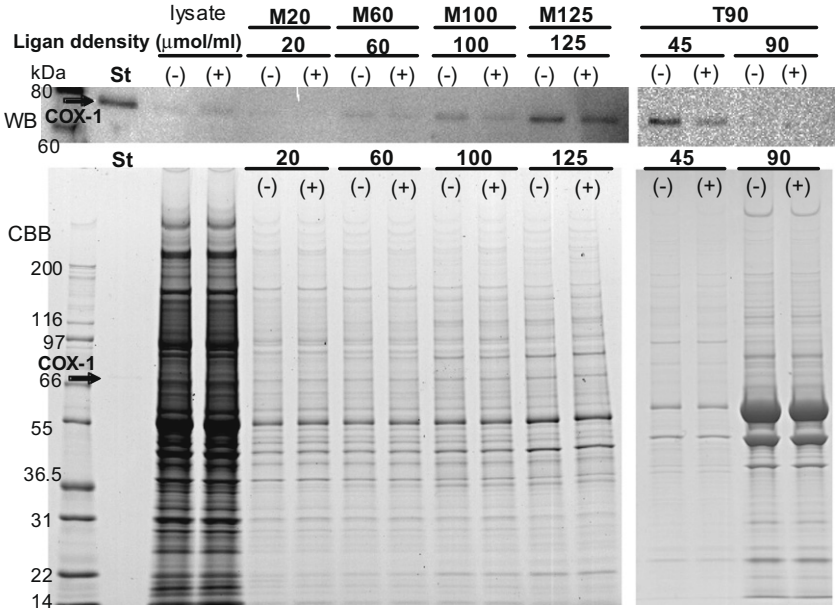


Figure 3. Affinity tests using Moli-gel- and Toyopearl-based affinity resin immobilizing Ket and lysate spiked with COX-1. The Moli-gel-based affinity resin having several ligand densities (20, 60, 100, and 125 μmol/ml). Toyopearl-based affinity resin having ligand densities of 45 and 90 μmol/ml (St: COX-1 standard).

gel-based affinity resins. These findings revealed that higher ligand density on Moli-gel-based affinity resin made the capturing of a greater amount of COX-1 possible. This is in fact a reasonable and expected result.

Unexpectedly, COX-1 was clearly captured on even (+) lane of 125 μmol/ml of Moli-gel-based affinity resin, while the dark hue was found to be more diminished than that on the (−) lane. Other interesting phenomena are that COX-1 was captured on 45 μmol/ml of Toyopearl-based affinity resin, but 90 μmol/ml of Toyopearl-based affinity resin did not capture COX-1 at all. Similar to Moli-gel-based affinity resin, COX-1 was captured on even (+) lane of 45 μmol/ml of Toyopearl-based affinity resin. Interestingly, there were fewer non-specific binding proteins on 45 μmol/ml of Toyopearl-based affinity resin, but high level of non-specific binding proteins was observed on 90 μmol/ml of Toyopearl-based affinity resin. These unexpected phenomena might suggest that 125 μmol/ml of Moli-gel-based affinity resin as well as 45 μmol/ml of Toyopearl-based affinity resins might have favorable 'surface properties' for the capturing of COX-1 compared with other affinity resins, therefore, COX-1 was captured even on the (+) lane where free ligand existed in the solution. At this moment, the reason why even (+) lane had capture of COX-1, but, one of the possibilities was that the equilibrium somehow moved favorably to the immobilized ligand on affinity resins compared with the free ligand in liquid phase.

Next, the affinity tests were carried out in a simple system, buffer solution spiked with COX-1, because, in the above-mentioned experiments, we did not cancel the influence of other proteins in the utilized lysate. As shown in Figure 4a and b, the results obtained are the same as those obtained in the lysate described above. The amount of captured COX-1 increased proportionately as the ligand density on Moli-gel-based affinity resin increased. In a similar way, COX-1 was captured on 45 μmol/ml of Toyopearl-based affinity resin, but 90 μmol/ml Toyopearl-based affinity resin did not capture COX-1 even in the buffer solution. This is quite interesting, because other proteins in lysate did not interfere with the capturing of COX-1 by the immobilized ligand.

For easy comparison, the relationship between the amount of COX-1 captured and the ligand density of affinity resins is drawn in Figure 4c. As mentioned before, again, the captured COX-1 increased proportionately as the ligand density on Moli-gel-based affinity resin increased. These findings suggest that Moli-gel has comparable surface properties for capturing COX-1 without relation to the ligand densities. In the case of Toyopearl-based affinity resin having immobilization rate of 100% might change the surface properties to be unable to capture COX-1. These findings will raise an alarm over traditional affinity tests, where higher ligand density was favored to capture unknown target proteins with greater amount.

The most important point is that Moli-gel-based affinity resin is one of the ideal affinity resins, because a variety of affinity resins can be prepared based on a solid phase, Moli-gel, to afford the above-mentioned reasonable results. On the other hand, Toyopearl has presumably heterogeneous surface properties varied with the density of ligand immobilized on it.

3.3. Effects of affinity resins with controlled ligand immobilization rate on the capturing of COX-1

Other affinity resins were prepared using Moli-gel having amino group density of 60 μmol/ml in addition to 125 μmol/ml. Herewith, we would discuss the effects of Moli-gel-based affinity resins having different ligand densities on the capturing of COX-1. Ket was immobilized on Moli-gel having amino group density of 60 μmol/ml. Immobilization rate of the affinity resin was 100% to that of solid phase; therefore, in other words, the ligand density of the affinity resin was 60 μmol/ml. This affinity resin was abbreviated as M60-Ket100%. Ket was also immobilized on Moli-gel having amino group density of 125 μmol/ml. In this case, immobilization rates of the affinity resins were 50% and 100%. Abbreviations for these affinity resins are M125-Ket50% and M125-Ket100%, respectively. Accordingly, M60-Ket100% has theoretically equal density (amount) of surface ligand to that of M125-Ket50%. The affinity test was performed using these affinity resins. Additionally, the concentration of free Ket for competition experiments was 10 times higher to reduce the amount of captured COX-1 on (+) lane in the affinity tests.

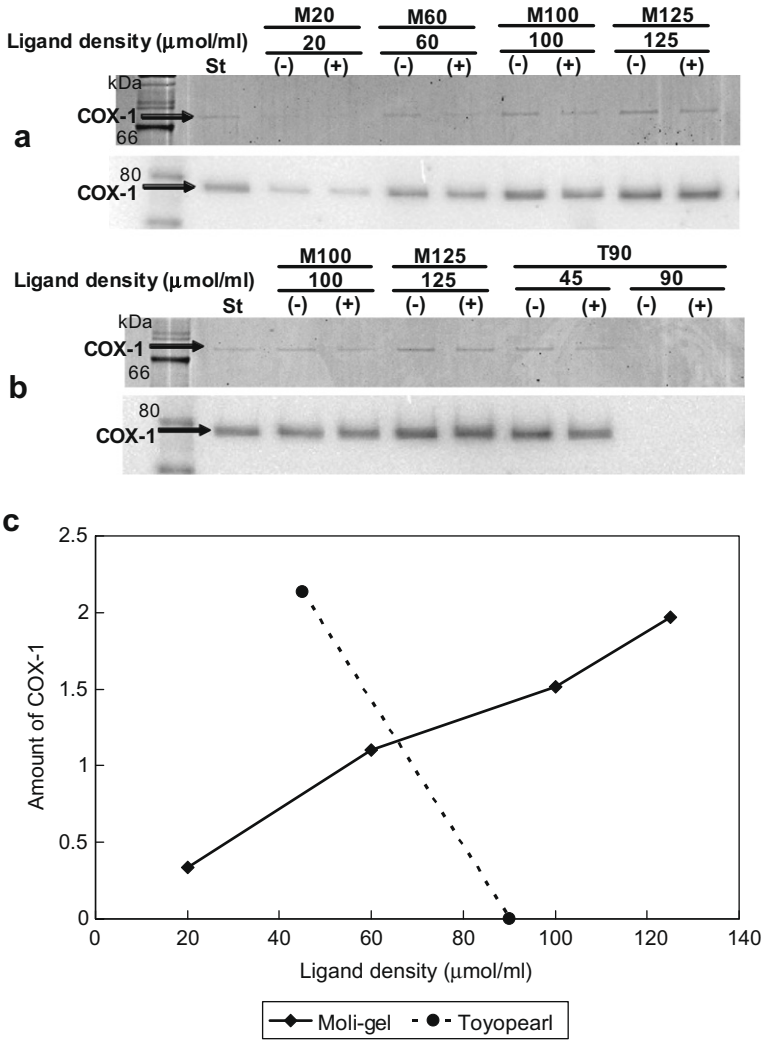


Figure 4. Affinity tests using Moli-gel- and Toyopearl-based affinity resin immobilizing Ket and buffer spiked with COX-1. Concentration of free Ket of competition was 0.26 mM (final concentration) (St: COX-1 standard). (a) Comparison of Moli-gel-based affinity resin (ligand densities of 20, 60, 100, and 125 μmol/ml). (b) Comparison of Moli-gel-based affinity resin (ligand densities of 100, 125 μmol/ml) and Toyopearl-based affinity resin (ligand densities of 45 and 90 μmol/ml). (c) Relationship between ligand density on each affinity resin and the amount of captured COX-1.

Reduction in the amount of proteins on (+) lane indicates one of the evidences that captured proteins on (−) lane are possibly specific binding proteins of the ligand. Contrarily, no reduction in the amount of proteins on (+) lane traditionally indicates that captured proteins on (−) lane are non-specific binding proteins. This is one reason why reduction in the amount of COX-1 on (+) lane was discussed and 'percentage of captured COX-1 (+)/(−) (%)' was calculated.

Figure 5a reveals that COX-1 was captured on the (−) lane of M125-Ket50%, while it was not found on the (+) lane of M125-Ket50%. For clearer evaluation, the percentage of captured COX-1 (+)/(−) (%) was calculated as shown in Figure 5b. Comparison of the results on M60-Ket100% and M125-Ket50% revealed different capturing results, nonetheless the density of surface ligand on both affinity resins was theoretically equal (Fig. 5a). M125-Ket50% afforded an ideal affinity test with a reduced amount of captured COX-1 on the (+) lane, while M60-Ket100% showed a result similar to that of M125-Ket100%. These results are not understandable at this moment, but one of the possible reasons is that different properties of surface amino groups on the base solid phases produced through their co-polymerization processes.

Basically, polymer-based solid phase has limited surface area; therefore, the introduction of higher functional group through a typical co-polymerization method with a majority of cross-linking monomers presumably results in 'block polymerization type' introduction of the monomers having functional groups. This result might realize overlapped or close functional groups on the surface of cross-linked solid phase compared with an ideal distance based on feed ratio of cross-linking monomers and the functional monomers. These differences might afford the above-mentioned findings in the affinity tests utilizing M60-Ket100% and M125-Ket50%, in which theoretically equal ligands were involved. Further experiments are now under progress.

Control of ligand immobilization rate on Moli-gel having an amino group density gave the different phenomena in the amount of captured COX-1 on (−) and (+) lanes. For example, using affinity resins immobilizing a ligand immobilization rate of 100%, we performed affinity test. Once the same amount of protein was captured on both (−) and (+) lanes, we were able to retry the affinity test using different affinity resins with controlled immobilization rate. If the different amounts of captured proteins on (−) and (+) lanes were observed, we were able to determine specific binding protein. In fact, our observation might suggest that optimal affinity resin depends on the combination of ligand and its target proteins. Therefore, control of ligand density on affinity resin was an important method.

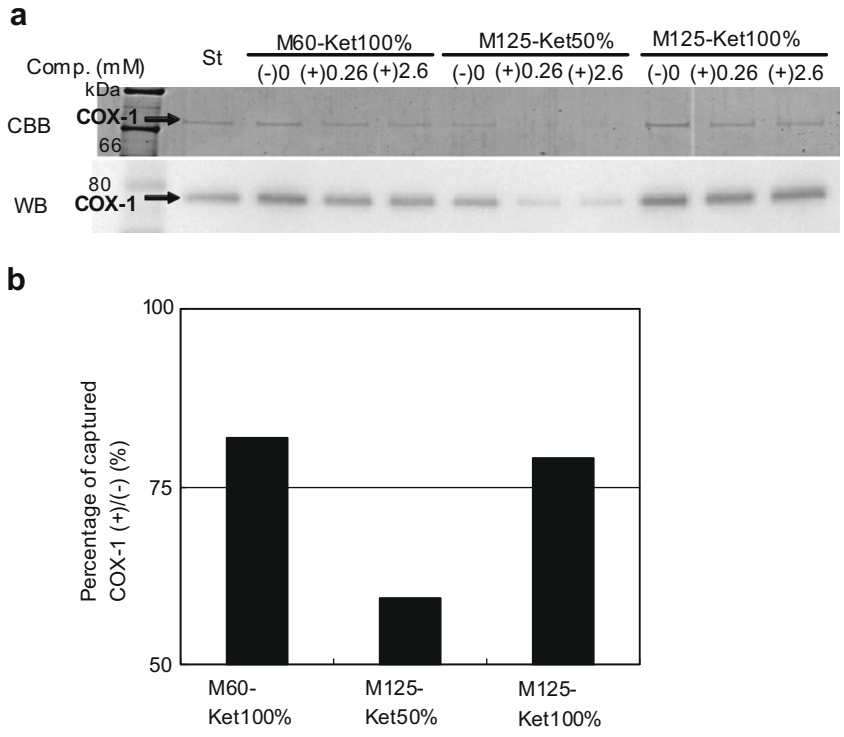


Figure 5. (a) Captured COX-1 using Moli-gel-based affinity resin immobilizing Ket with each ligand density. (St: COX-1 standard, Comp.: concentration of competition). (b) Percentage of captured COX-1 (+)2.6/(−)0. The values were calculated based on the intensity of COX-1 (−) and (+). They were detected by the resulting CBB stained gel and densitometer.

3.4. Capturing of COX-1 using Moli-gel- and Toyopearl-based affinity resins having Ibuprofen, Ketoprofen, and Aspirin

The ligands (Ibu, Asp, and Ket) have different hydrophobic properties as well as activities, so it will be possible to discuss the effects on the capturing of COX-1 by each ligand, in other words, the relationship between hydrophobic properties of ligand and the amount of captured COX-1. Hydrophobic properties of these ligands were evaluated by calculating *C*Log*P*, k', and Log k'. HPLC analysis was performed to calculate k' (Log k'). These results are summarized in Table 3. Ibu has the highest hydrophobicity among the ligands, while Asp showed much hydrophilic property.

Immobilization rate of all affinity resins was 100%, where Moli-gel having amino group density of 125 μmol/ml was utilized as a base solid phase. The abbreviations of all the affinity resins are M125-Ibu100%, M125-Ket100%, M125-Asp100% (for Moli-gel) or T90-Ibu100%, T90-Ket100%, T90-Asp100% (for Toyopearl), respectively. The affinity tests were performed utilizing these affinity resins with the buffer spiked with COX-1.

Figure 6a gives us the most interesting result, Moli-gel- and Toyopearl-based affinity resins afforded completely contrast results. COX-1 was captured on M125-Ibu100% and M125-Ket100%, but not on T90-Ibu100% and T90-Ket100%. Contrarily, much higher amount of COX-1 was captured on T90-Asp100%, but less amount of COX-1 was captured on M125-Asp100%. The amount of captured COX-1 on M125-Ibu100% was higher, and that of captured COX-1 on M125-Asp100% was less. Again, we show the percentage of captured COX-1 (+)/(−) (%) in Figure 6b. It was suggested that the amount of captured COX-1 on (−) lane (including on (+) lane, too) of each Moli-gel-based affinity resin could relate to hydrophobic properties of each ligand (Fig. 6c). While no relationship was observed between the amount of captured COX-1 and hydrophobic properties of ligands on Toyopearl-based affinity resin, because only Toyopearl-based affinity resin immobilizing rather hydrophilic Aspirin could capture COX-1.

A qualitative discussion on the surface properties of ligand immobilized affinity resins can be made with the help of the following experiments. The completely dried affinity resins were dispersed in two immiscible phase systems containing octanol phase and water phase. For this discussion, new Toyopearl-based affinity resins having immobilization rate of 50% were prepared using Ibu and Asp as ligands.

Figure 7 demonstrates an example on the dispersion phenomena of affinity resins immobilizing Ket. Firstly, majority of base solid phases, Moli-gel and Toyopearl (ligand immobilization rate was 0%), dispersed in water phase. Moli-gel dispersed in octanol phase partly. It showed adequacy of Moli-gel because it has been prepared using PEG (poly-ethylene glycol) type monomer, so it dispersed mainly in hydrophilic phase as well as hydrophobic phase.

Secondly, Moli-gel-based affinity resin having ligand immobilization rate of 100% dispersed in both phases. Also, Toyopearl-based affinity resin having ligand immobilization rate of 50% dispersed in both phases. What is interesting is that Toyopearl-based

Table 3
*C*Log*P*, k', and Log k' of Aspirin, Ketoprofen, and Ibuprofen

	Aspirin	Ketoprofen	Ibuprofen
*C*Log*P*[a]	1.024	2.761	3.679
k'[b]	0.273	3.31	12.3
Log k'[c]	−0.564	0.520	1.09

[a] *C*Log*P* is calculated by ChemDraw Ultra 10.0.
[b] k' is $(t_R - t_0)/t_0$. t_R = retention time, $t_0 = L/v$ (L: column length, v: mobile phase flow rate), retention times of Aspirin, Ketoprofen, and Ibuprofen are 3.17, 10.74, and 33.28 min, respectively.
[c] Log k' is calculated as a common logarithm of k'.

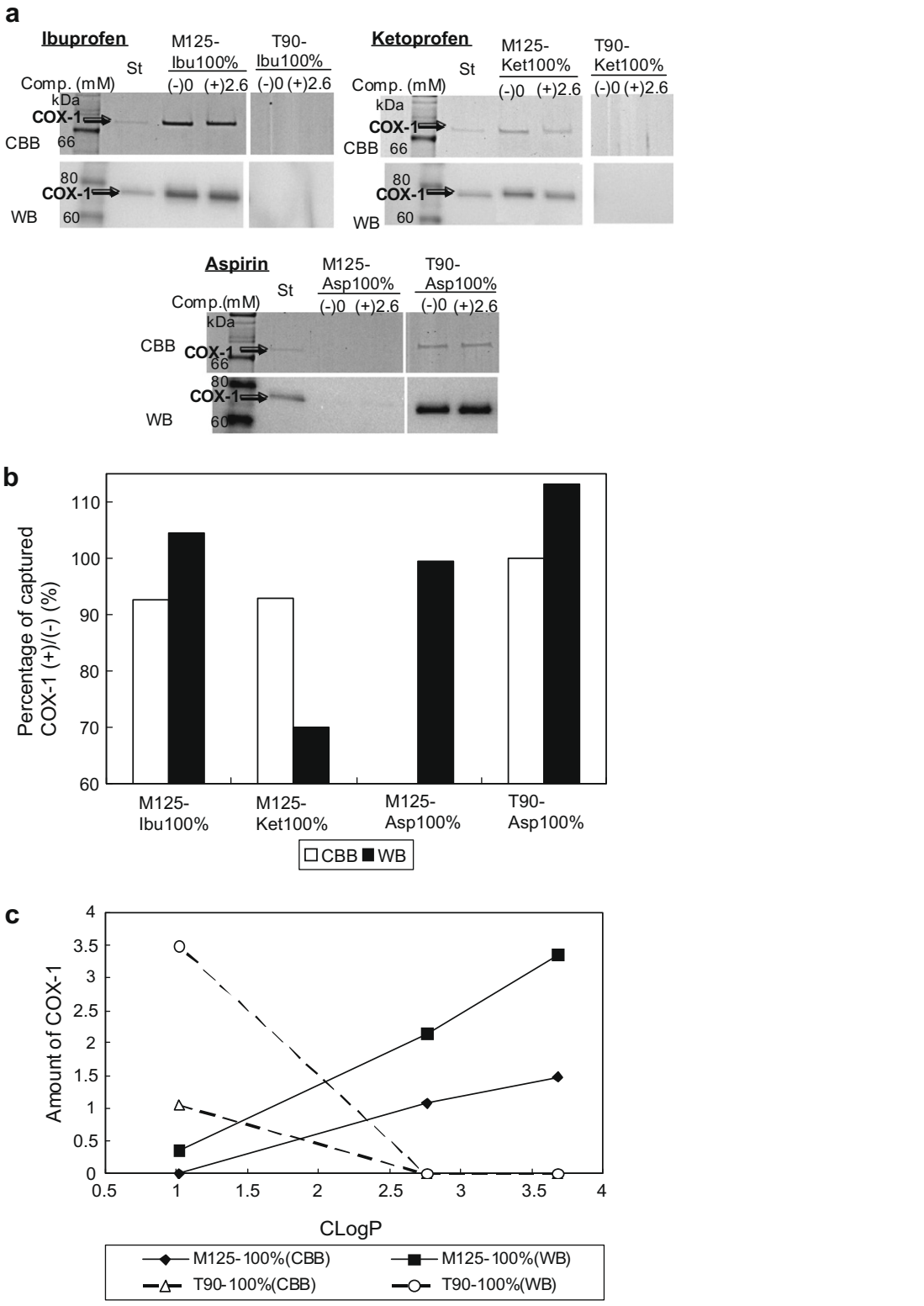
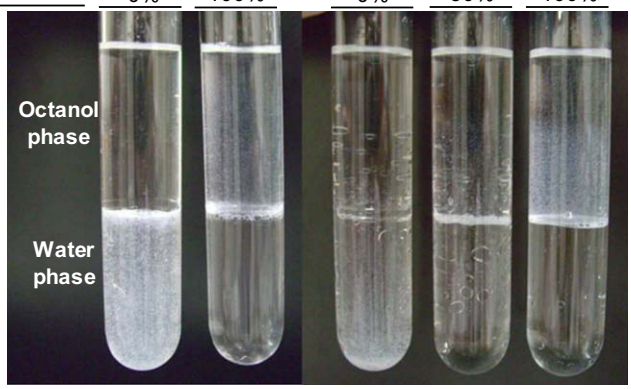


Figure 6. (a) Captured COX-1 using Moli-gel- and Toyopearl-based affinity resin immobilizing Ibu, Ket, or Asp, with immobilization rate of 100% of each affinity resin. (St: COX-1 standard, Comp.: concentration of competition). (b) Percentage of captured COX-1 (+)/(−). They were detected by the resulting CBB stained gel, Western blotting film and Densitometer. (c) Relationship between *CLog P* and the amount of COX-1 captured by affinity resin immobilizing Asp, Ket, Ibu. *CLog P* of each ligand; Asp 1.024, Ket 2.761, Ibu 3.679.

affinity resin having immobilization rate of 100% dispersed in octanol phase dominantly. From the findings on the dispersion phenomena, it can be noted that both the base solid phases, Moli-gel and Toyopearl, have enough hydrophilic properties. In spite of Ket immobilized Moli-gel and Toyopearl, Moli-gel-based affinity resin having ligand immobilization rate of 100% and Toyopearl-based affinity resin having immobilization rate of 50% retained the hydrophilic property. At the same time, Toyopearl-based affinity resins having ligand immobilization rate of 100% did not retain the hydrophilic property of base Toyopearl. These simple findings

a



b

	Moli-gel (125 μmol/ml)		Toyopearl (90 μmol/ml)		
Ketoprofen immobilization rate	0%	100%	0%	50%	100%
Octanol phase	△	◎	×	○	◎
Water phase	◎	○	◎	△	×

Figure 7. Dispersion of Moli-gel- and Toyopearl-based affinity resin immobilizing Ket in octanol/water phases. Ket immobilization rate on Moli-gel is 0% or 100%. Ket immobilization rates on Toyopearl are 0%, 50%, and 100%. (a) Shows the picture of dispersion, (b) shows the degree of the dispersions pointed at four levels (◎ great dispersion: 10, ○ good dispersion: 7, △ moderate dispersion: 3, and × no dispersion: 1).

strongly suggested that surface property changed drastically once full quantitative immobilization with a relatively hydrophobic ligand was done on Toyopearl. The balance of hydrophilic/hydrophobic and ligand immobilization rate is quite important to determine the surface properties on the affinity resin, and finally it relates to the ability of capturing COX-1. In order to demonstrate these findings more clearly, degree of the dispersions was pointed using four levels (◎ great: 10, ○ good: 7, △ so: 3, × no dispersion: 1) for descriptive purpose. Based on these values, a modified Log*P* was calculated. Calculation formula 1 is shown below:

$$\text{Modified Log}P = \text{Log}\,(\text{number of dispersion levels in octanol}/\text{number of dispersion levels in water}) \quad (1)$$

Modified Log*P* of Moli-gel and Toyopearl (no ligand) were determined as standard values, each modified Log*P* of Moli-gel- and Toyopearl-based affinity resins (with ligand) was divided by the standard values to calculate relative modified Log*P*. Calculation formula 2 is shown below:

$$\text{Relative Modified Log}P = \text{each modified Log}P\text{ of the affinity resins}/\text{modified Log}P\text{ of Moli-gel or Toyopearl} \times (-1) \quad (2)$$

The values are schematically illustrated in Figure 8. In addition, the relationship between *C*Log*P* and Log *k*′ is also provided in the figure. The results indicate that the curve of Log *k*′ was calculated quantitatively and the curves of relative modified Log*P* about Toyopearl-50% and Moli-gel-100% were nearly parallel to each other. Based on the findings, relative modified Log*P* had an adequacy on the distribution of the affinity resins into octanol/water phases. If the value is higher, the affinity resin has more hydrophobic property. If the value is lower, it has more hydrophilic property. It is suggested that affinity resins having balanced properties of hydrophilicity and hydrophobicity made it possible to capture COX-1.

3.5. COX-1 captured by Moli-gel- and Toyopearl-based affinity resins immobilizing Ibuprofen with controlled ligand immobilization rate

The abbreviations of affinity resins prepared, including new resins, are M125-Ibu50%, M125-Ibu100%, and T90-Ibu50%, respectively. M60-Ibu100% indicated that Ibu was immobilized on Moli-gel having an amino group density, 60 μmol/ml, where the immobilization rate was 100%.

Figure 9a and b shows that the amount of captured COX-1 and the percentage of captured COX-1 (+)/(−) (%) on M125-Ibu50% and M125-Ibu100% afforded almost the same results. Therefore, in the case of Ibu as the ligand, the affinity resin based on Moli-gel having different amino group densities with controlled immobilization rate of ligand did not affect the amount of captured COX-1. The results found for Ibu were different from those found for Ket (Fig. 5a). In contrast, the amount of captured COX-1 on M60-Ibu100% was less. The percentage of captured COX-1 (+)/(−) (%) indicated the minimal value (Fig. 9b). Difference in the ligand densities on the same base Moli-gel did not affect the amount of captured COX-1, and the same ligand densities on different base Moli-gel affected the amount of captured COX-1 in the case of Ibu ligand. If we observe the results of Toyopearl-based affinity resins, it can be found that COX-1 was captured on T90-Ibu50%, although it was not captured on T90-Ibu100%. Therefore, the difference in ligand density on Toyopearl had affected the amount of captured COX-1. But the percentage of captured COX-1 (+)/(−) (%) on Toyopearl was 100%, so COX-1 might adsorb like a non-specific binding protein.

3.6. COX-1 captured by Moli-gel- and Toyopearl-based affinity resins immobilizing Aspirin with controlled ligand immobilization rate and existential surroundings of ligand

Figures 7 and 8 show that the surface properties of affinity resins affect the capturing of COX-1. The immobilization rate of M125-Asp had been changed down to 50%, and additional immobi-

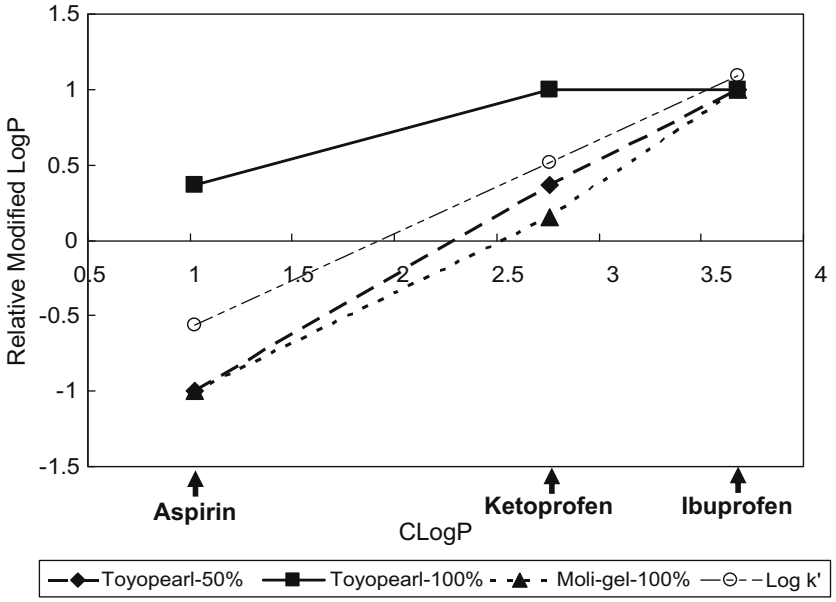


Figure 8. Relative modified Log*P*. Ligand immobilization rate of 50% shows Toyopearl-50%. Ligand immobilization rate of 100% shows Toyopearl-100% or Moli-gel-100%.

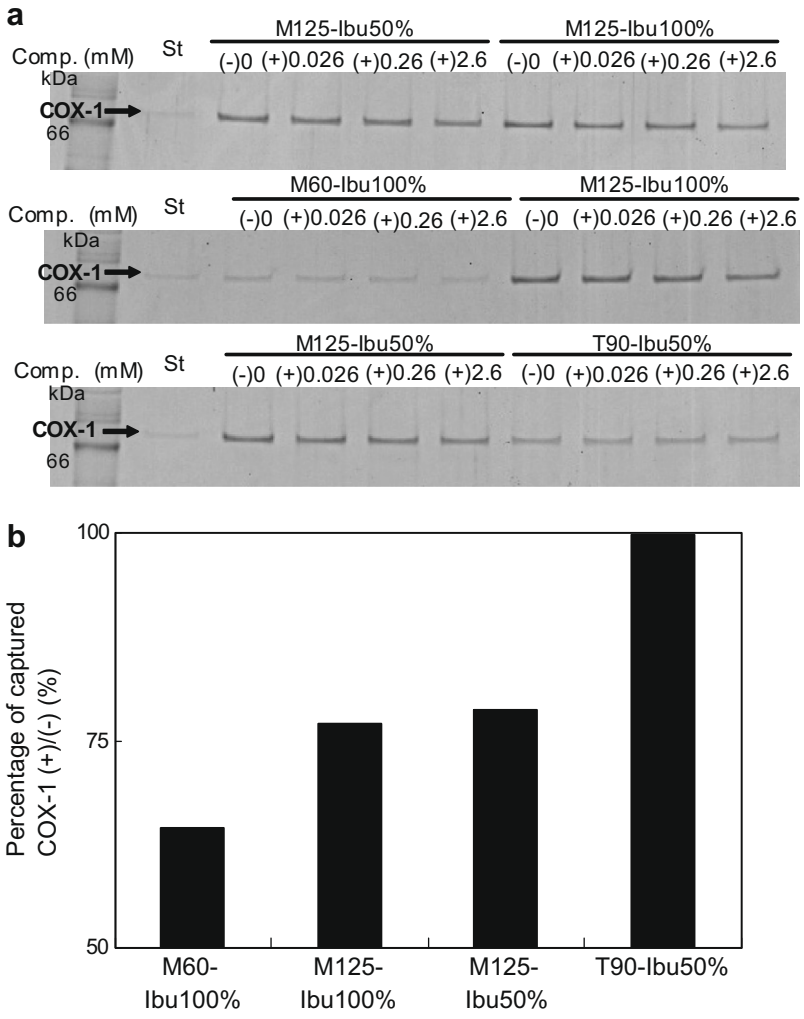


Figure 9. (a) Captured COX-1 using Moli-gel- and Toyopearl-based affinity resin immobilizing Ibu (St: COX-1 standard, Comp.: concentration of competition). (b) Percentage of captured COX-1 (+)2.6/(−)0.

lization of a typical hydrophobic group, C_{18} group, took place to control the existential surroundings of the ligand.

For Toyopearl, the immobilization rate of T90-Asp100% was also changed down to 50% from 100%. Similar approaches were discussed for M125-Ket, too.

In these cases, relatively hydrophilic ligand, Asp, was immobilized on Moli-gel having an amino group density of 125 μmol/ml and on Toyopearl having an amino group density of 90 μmol/ml with a controlled immobilization rate of 50% or 100%. The abbreviations are M125-Asp100%, T90-Asp100%, and T90-Asp50%, respectively. Especially, the immobilization rate on Moli-gel with Asp was found to be as much as 80%. C_{18} group was immobilized on the affinity resin after the introduction of Asp to control the existential surroundings of the ligand. In this case, the abbreviation is M125-Asp80% + C_{18}20%. Moli-gel immobilizing only C_{18} group (immobilization rate of 50%) was prepared, too. It was shown as M125-C_{18}50%. Moli-gel having an amino group density of 60 μmol/ml was not used because it would not be able to capture COX-1, as expected from that mentioned in Sections 10.2–10.5.

It can be seen from Figure 10a that the amount of COX-1 increased on M125-Asp80% + C_{18}20%, contrarily, less amount of COX-1 was captured on M125-Asp100%. The amount of captured COX-1 on M125-C_{18}50% was much higher than that captured on M125-Asp80% + C_{18}20%, therefore, it could not be decided that

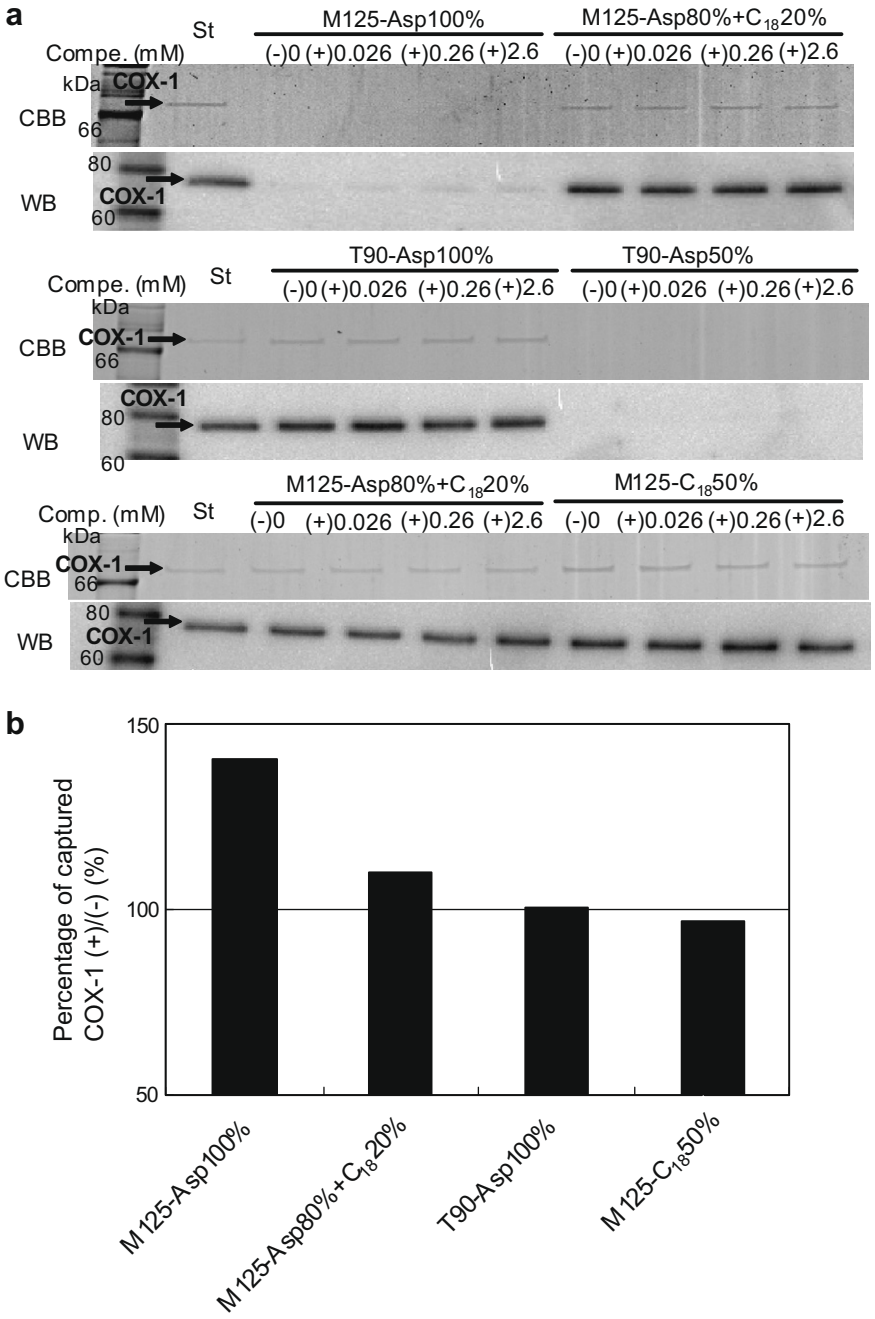


Figure 10. (a) Captured COX-1 using Moli-gel- and Toyopearl-based affinity resin immobilizing Asp (St: COX-1 standard, Comp.: concentration of competition). (b) Percentage of captured COX-1 (+)2.6/(−)0.

C_{18} group with Asp on Moli-gel-based affinity resin increased the amount of captured COX-1. For Asp, we expected that there might be more effective groups to control the existential surroundings of the ligand. At this point, the amount of captured COX-1 on M125-Asp100% increased gradually and proportionately with higher concentration of free Aspirin. The reasons are not understandable, but it might be suggested that there are some interactions or balances between COX-1, free Asp, and immobilized Asp on Moli-gel-based affinity resins.

It can be seen from Figure 10b that COX-1 was not captured on T90-Asp50% at all. The reason might be lower ligand density. Although COX-1 was captured on T90-Asp100%, the percentage of captured COX-1 (+)/(−) (%) was nearly 100%, so it might be adsorbed non-specifically.

3.7. COX-1 captured by Moli-gel-based affinity resins immobilizing Ketoprofen with controlled ligand immobilization rate and the existential surroundings of ligand

Ket or C_{18} group was immobilized on Moli-gel having an amino group density of 125 μmol/ml and on Toyopearl having an amino group density of 90 μmol/ml with a controlled immobilization rate of 50% or 100%. We have abbreviated these as M125-Ket50% + C_{18}50%, M125-C_{18}50%, M125-C_{18}100%, and T90-C_{18}100%, respectively.

In the case of Asp, C_{18} group was not suitable for increasing the amount of captured COX-1. But in the case of Ket, it was suggested that C_{18} group on affinity resin with Ket appeared effective for capturing COX-1 (Fig. 11a). The effect is that the amount of COX-1 on (−) lane of M125-Ket50% + C_{18}50% was much higher than that on (−) lane of M125-Ket50%. The percentage of captured COX-1 (+)/(−) (%) on M125-Ket50% + C_{18}50% was smaller than that on M125-C_{18}50%. (Fig. 11b) In addition, the amount of COX-1 on M125-Ket50% + C_{18}50% was the same as that on M125-Ket100%. (Fig. 11a) These findings suggested that C_{18} group was suitable to construct favorable existential surroundings of the ligand for superior interaction between COX-1 and Ket immobilized on the affinity resins.

The affinity resin immobilizing only C_{18} group also captured COX-1. On M125-C_{18}100%, the amount of COX-1 was reduced gradually, it indicated that free Ket was able to bind with COX-1 normally. But the amount of captured COX-1 was constant on T90-C_{18}100%. It might be because of non-specific adsorption. These results showed difference in the surface condition between Moli-gel- and Toyopearl-based affinity resins.

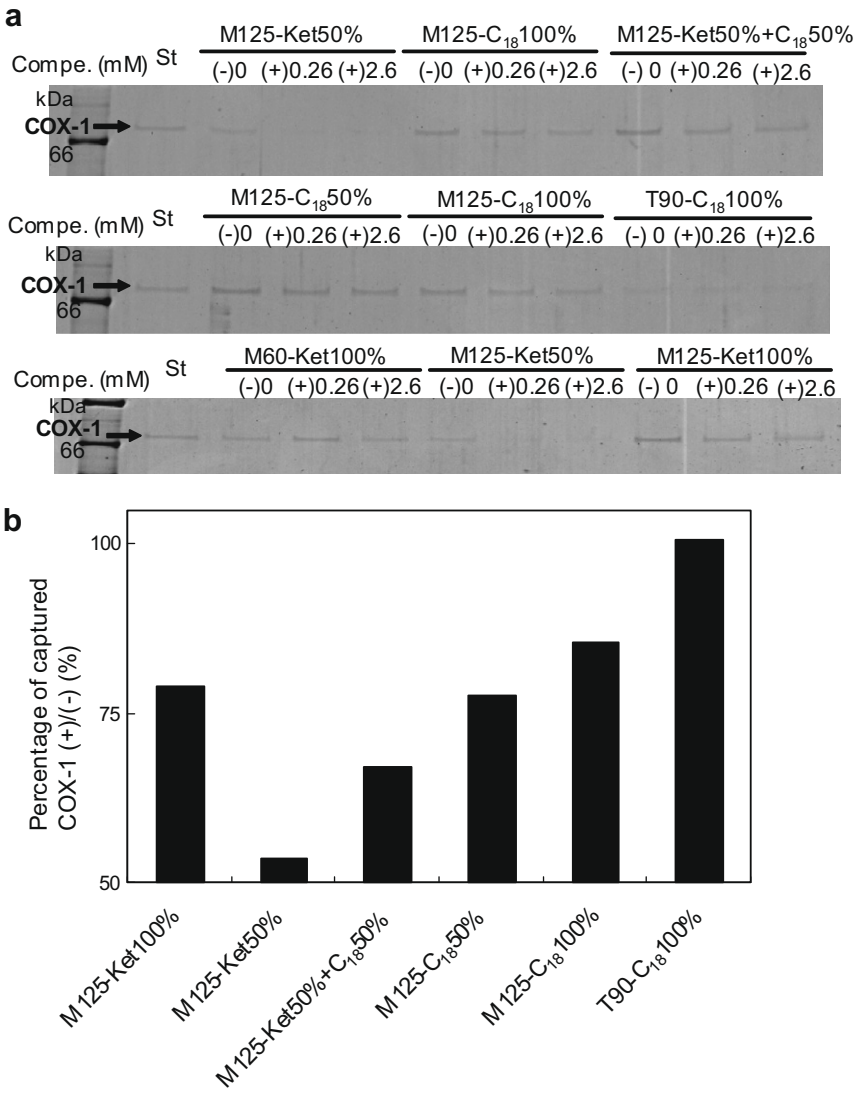


Figure 11. (a) Captured COX-1 using Moli-gel- and Toyopearl-based affinity resin immobilizing Ket and C_{18} group. (b) Percentage of captured COX-1 (+)2.6/(−)0.

4. Conclusion

- Peculiar results were observed for affinity resins immobilizing different ligands.
- Hydrophobic property of ligand and hydrophilic and hydrophobic properties of affinity resin affected the amount of captured COX-1 as well as the capturing of COX-1.
- Controlled ligand density and existential surroundings of ligand gave effects on the capturing of COX-1.
- Affinity resins with controlled existential surroundings of ligand by the immobilization of C_{18} group afforded some advantage for capturing COX-1.
- Control of the existential surroundings of ligand was found to be a useful method to control the interaction between ligand and proteins, although further consideration of selection of functional groups will be required.
- These results will predict that when the research of target proteins by affinity resins is proposed, it is necessary to discuss the phenomena very carefully using several affinity resins having controlled ligand density, ligand immobilization rate, and existential surroundings of the ligand.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.12.066.

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