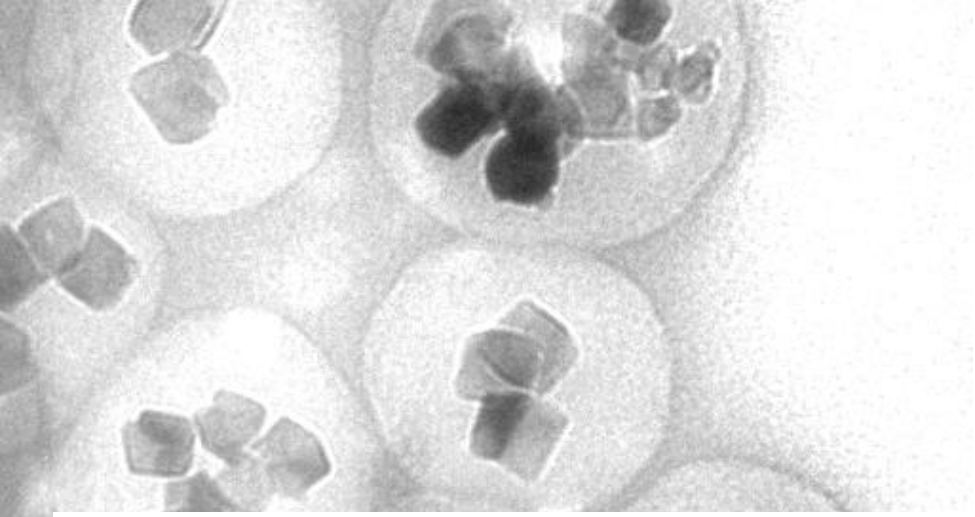
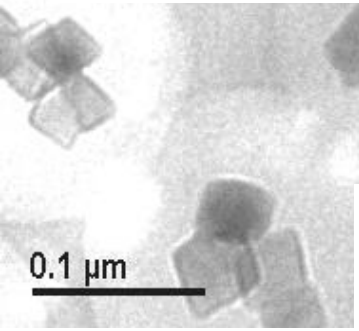
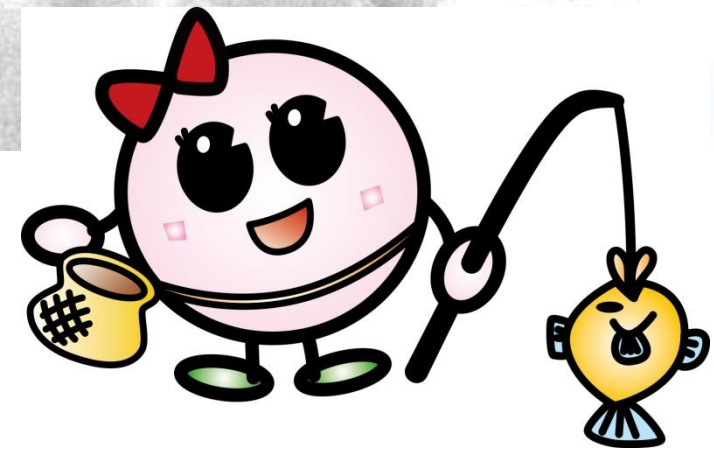
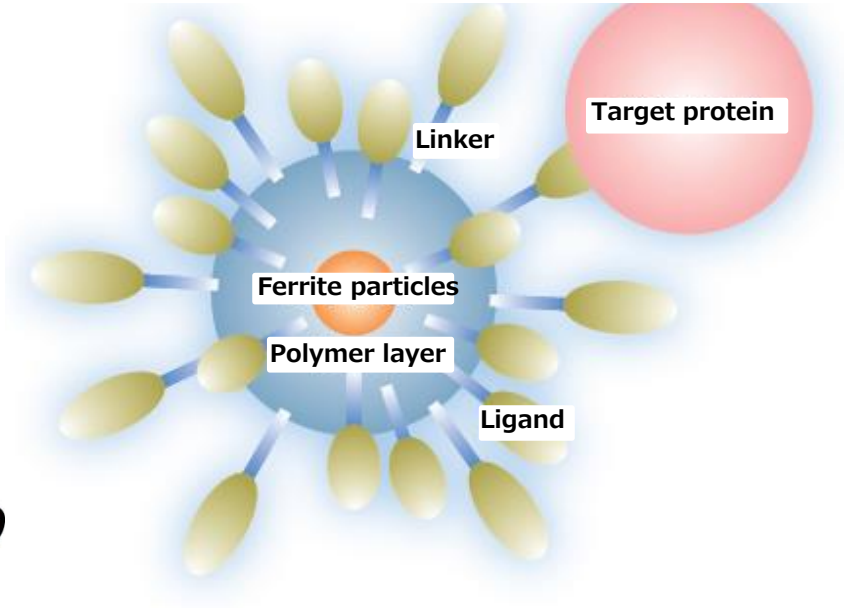


Method for Purification of Protein







TAMAGAWA SEIKI CO., LTD.

目次

Workflow for Affinity Purification

① Immobilization of ligand

- **Ligand Design**
- **Structure-Activity Relationship**
- **Control compound**

↓

- **Quantification of ligand amount (HPLC)**
- **Optimization of Ligand Immobilization Amount**

② Mix ligand immobilized beads and protein solution (Cell lysate)

- Centrifuging protein solution before use
- Method for preparing cell lysate (protocol 401, 402)

↓

③ Binding reaction・Washing・Elution (Salt/Boil elution)

- **Composition of Binding/washing Buffe**
- **Binding Reaction Time**
- **Specificity**
  (Competitive inhibition, Drug elution)
- **Precautions for affinity purification**
  (Avoiding keratin contamination)

↓

- Dispersion of beads (Scratch)
- Magnetic separation

④ Analysis

SDS-PAGE, Silver stain, Mass spectrometry(MS)

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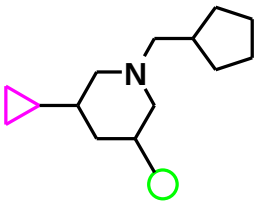
④ Analysis

SDS-PAGE, Silver stain, Mass spectrometry(MS)

Ligand Design/Structure-Activity Relationship

①Find out which part is important for activity（Structure-activity relationship）



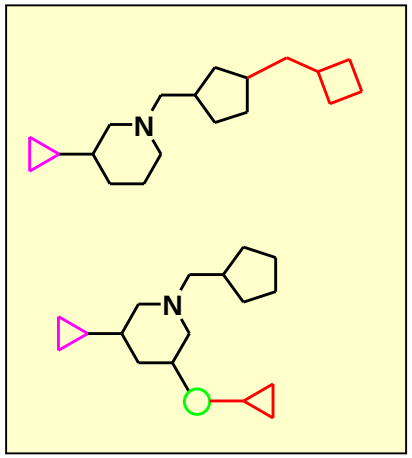
List of compounds with the same medicinal properties
Structure-activity relationship survey

⇨

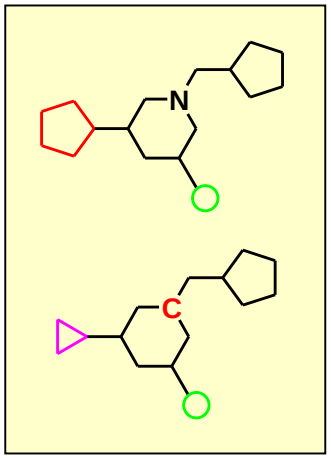
Pharmacophore prediction

Comparing the structures predicts the important partial structures / functional groups and non-affecting parts in the interaction with the target protein.

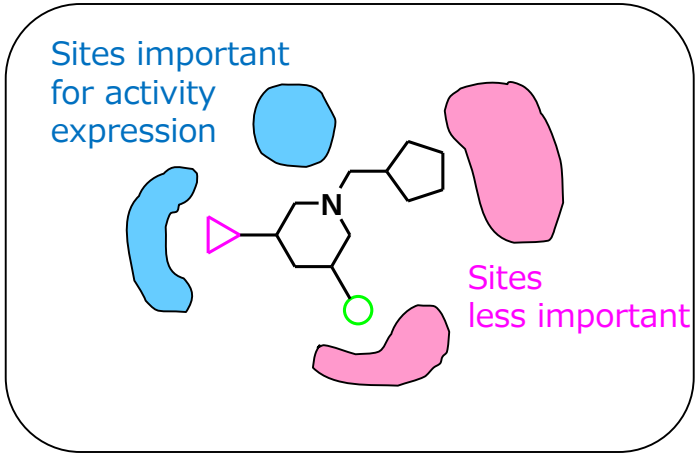
Active



Decreasing of activity



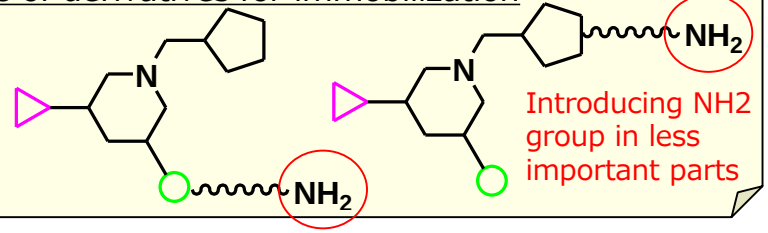
⇨



②Immobilization on beads at parts not important for activity expression

Immobilization on beads at important points of activity causes the ligand to lose activity and the target protein cannot be purified. If it does not have an effective group for immobilization, a derivative compound for immobilization is synthesized.

Synthesis of derivatives for immobilization



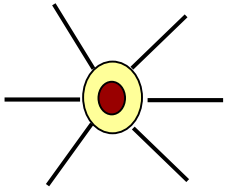
Selection of functional groups
from the viewpoint of reactivity

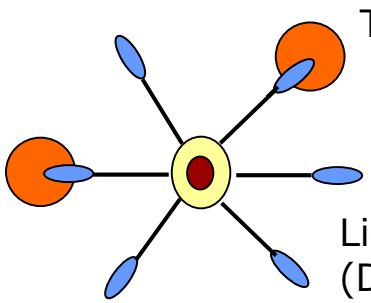
NH_2 group> Alkyne/Azide> Biotin

Experimental flow chart

Immobilization of ligand

Preparing ligand unfixed beads





Ligand unfixed beads
(For control)

Ligand immobilized beads

Mixing ligand-immobilized beads and protein solution

Binding reaction, washing and elution

– + : Ligand immobilized

SDS-PAGE

Mass spectrometry (Identification of binding proteins)

Binding specificity analysis

Preparation of recombinant protein

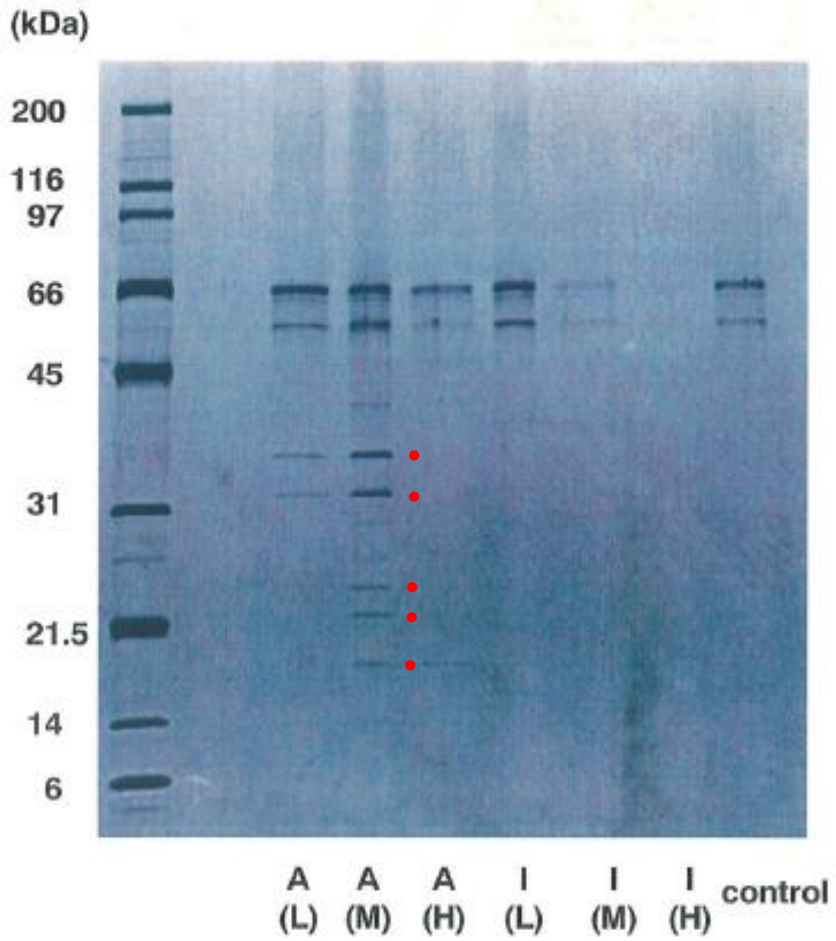
Analysis of binding domain

Functional analysis

Analyzing protein function from medicinal properties

(Biochemical and cell biological experiments)

Examples of target protein purification



Protein concentration : 1 mg/ml

Amount of protein : 250 µg

Multiple bands (red dots) of binding protein are confirmed around 20-40 kDa, and these proteins are expected to be specifically bind to active compound A.

Appropriate control compound makes it easier to find the target protein.

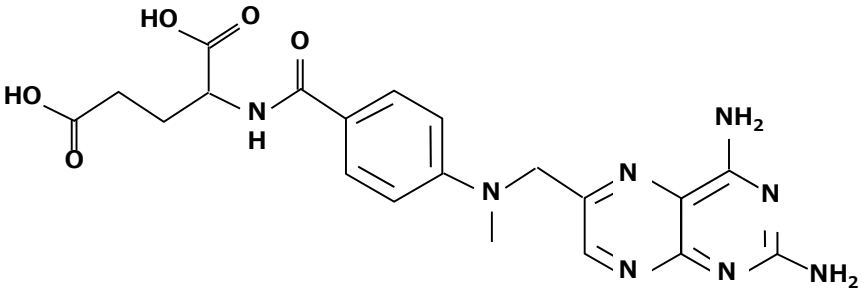
※ **A: Active compound**

I: Inactive compound

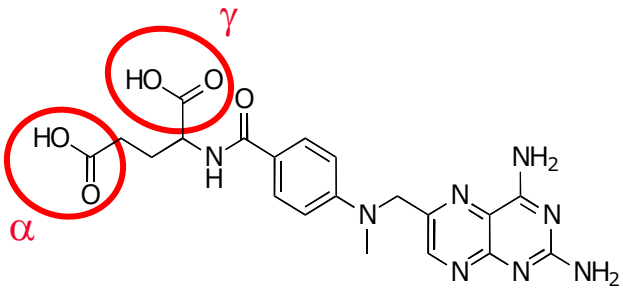
(L)(M)(H):Amount of compound immobilized Low/Medium/High

control:Beads without compound immobilization

Purification of proteins by different immobilization site



MTX

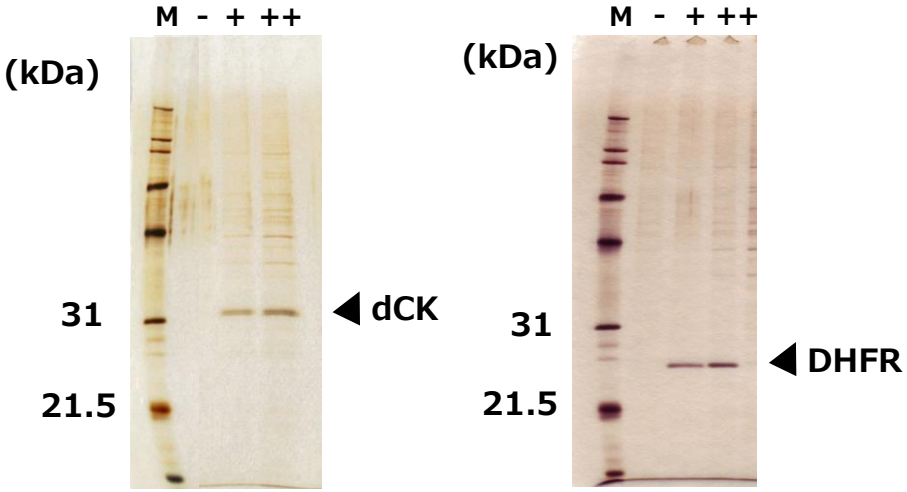


Immobilized at γ site

Immobilized at α site

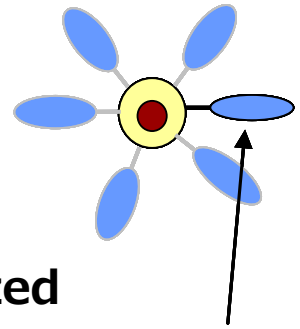
MTX reaction concentration

MTX derivative※ reaction concentration



OH beads

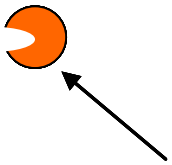
NHS beads



MTX

Immobilized FG beads®

MTX



dCK、DHFR

Target proteins

※MTX derivative were used to immobilize at the α site.

Workflow for Affinity Purification

① Immobilization of ligand

- Ligand Design
  Structure-Activity Relationship
  Control compound

↓

- Quantification of ligand amount (HPLC)
- Optimization of Ligand Immobilization Amount

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- Composition of Binding/washing Buffe
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- Specificity
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- Precautions for affinity purification
  (Avoiding keratin contamination)

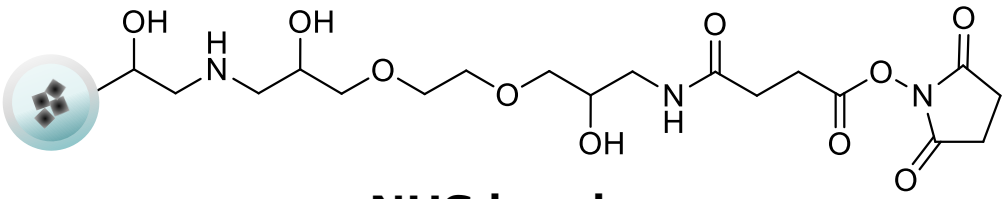
↓

- Dispersion of beads (Scratch)
- Magnetic separation

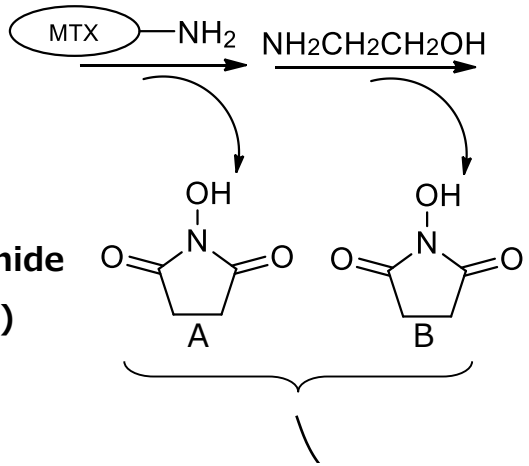
④ Analysis

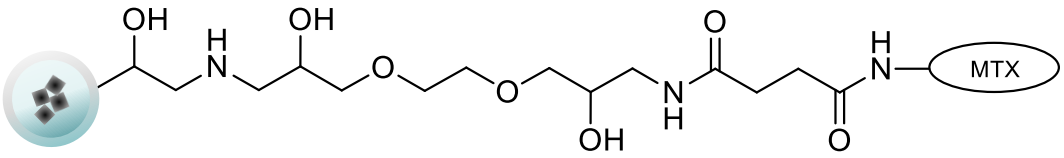
SDS-PAGE, Silver stain, Mass spectrometry(MS)

Quantification of the amount of MTX immobilized by HPLC

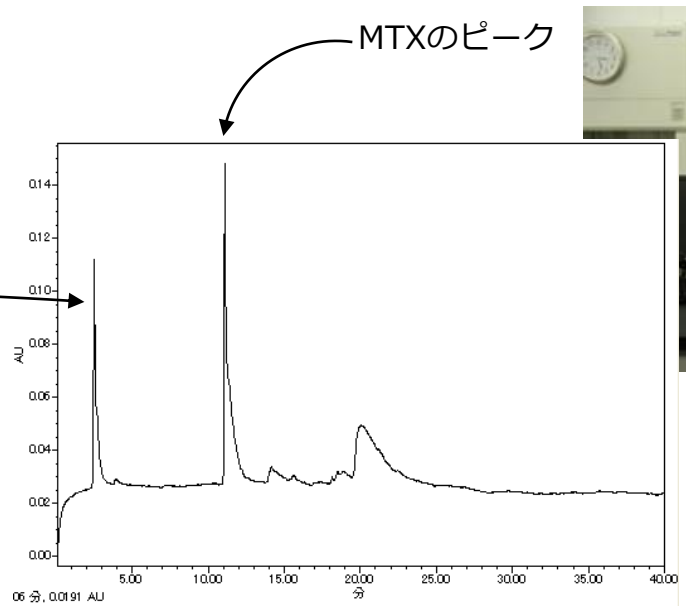


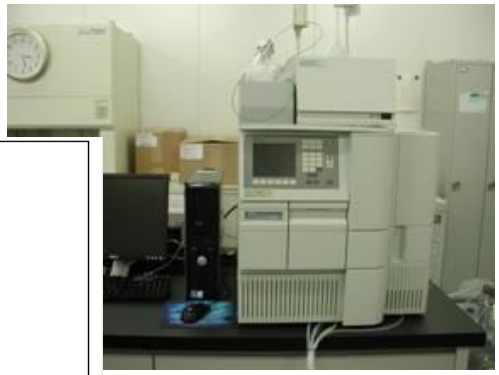
NHS beads





MTX amino derivative





The amount of ligand immobilized can be quantified.

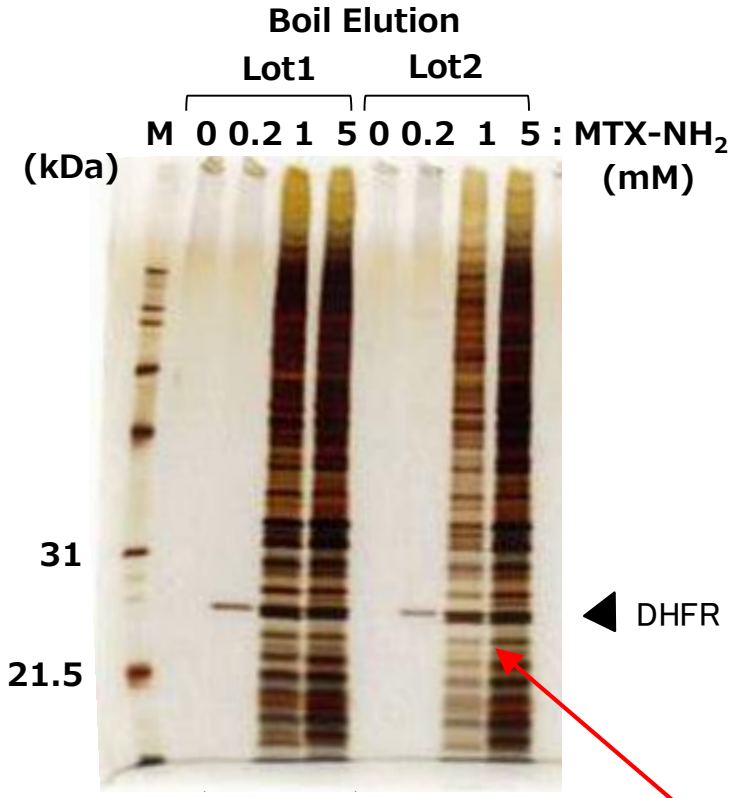
The same amount of NHS as the immobilized MTX is released in the reaction supernatant by the exchange reaction. (NHS-A)

The same amount of NHS as the masked aminoethanol is released in the masking supernatant by the exchange reaction. (NHS-B)

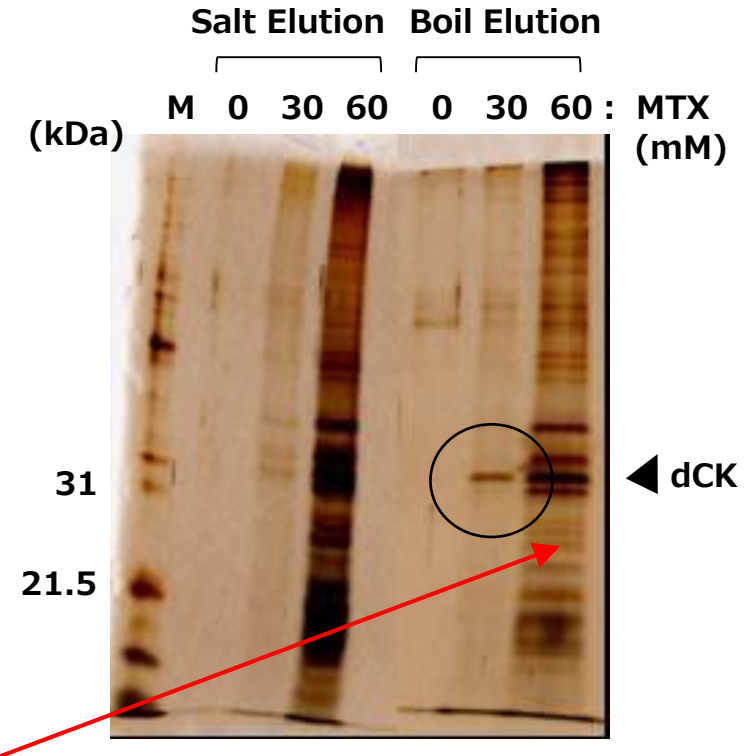
The amount of MTX immobilization (NHS-A) + masking amount (NHS-B) = functional group (NHS) amount of beads

Optimization of ligand immobilization amount

①COOH beads



②OH beads



Too much ligand immobilization increases nonspecific protein adsorption.

Affinity purification	: Based on protocol 001
Bead amount	: 0.5 mg/1 condition
Protein solution	: ①3 mg/ml, 200 µl ②1 mg/ml, 200 µl
Protein	: HeLa cell lysate
Binding reaction time	: 2 hours

Amount of Ligand Immobilized and protein binding

Immobilization of compounds with phenolic OH group (Protocol 003)

Linker beads 2.5mg (×4)
Compound 0 mM (DMF 500 µl)
2 mM (50 mM 20 µl + DMF 480µl)
10 mM (50 mM 100 µl + DMF 400µl)
50 mM (50 mM 500 µl)
K_2CO_3 10 times the mole of the compound

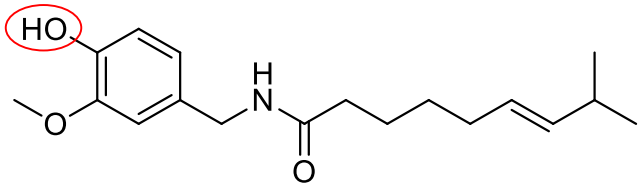
↓ 60℃, 24hours

Compound immobilized beads

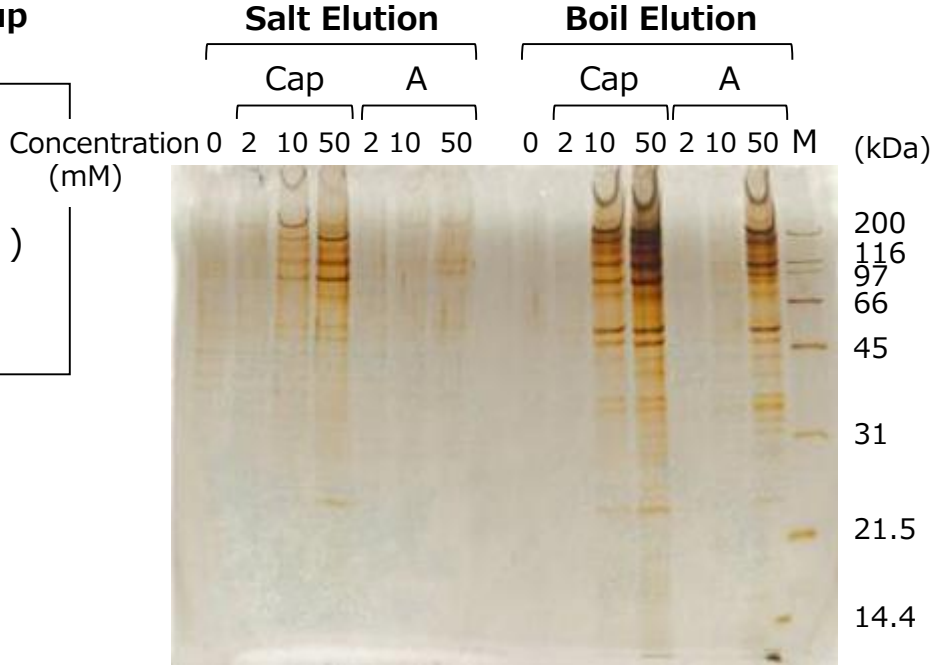
↓

Affinity purification

(Protocol 001)



Capsaicin (Cap)



Based on Protocol 001

beads	: Capsaicin immobilized beads Compound A immobilized beads
Reaction concentration	: 0, 2, 10, 50 mM
bead amount	: 0.5 mg
Binding/Washing buffer	: 100 mM KCl buffer (200 µl)
Elution buffer	: ①1M KCl buffer (30 µl) (Salt Elution) ②Sample buffer (40 µl) (Boil Elution)
Protein	: HeLa cell lysate
Protein concentration	: 3 mg/ml (200 µl)
Reaction time	: 4 hours

The protein is hydrophobically and non-specifically binded to the ligand as the concentration of the ligand is increased, and the amount of protein bound increases ,when the ligand is a hydrophobic compound. If the amount of binding protein increased in this way, it can be determined that the ligand is immobilized on the beads.

Amount of Ligand Immobilized and protein binding

Immobilization of compounds with COOH group
(Protocol 005)

Compound	7 μmol
Succinimide	7 μmol
EDC	7 μmol (Final 10mM)

2 hours | 0.7ml (DMF)

NH_2 beads 2.5mg (×4)
Acttivated compound
0 mM (DMF 500 μl)
0.4 mM (10 mM 20 μl + DMF 480 μl)
2 mM (10 mM 100 μl + DMF 400 μl)
10 mM (10 mM 500 μl)

Over night | 500 μl (DMF)

Masking

Triethylamine※1 50 μl
Acetic anhydride 20 μl

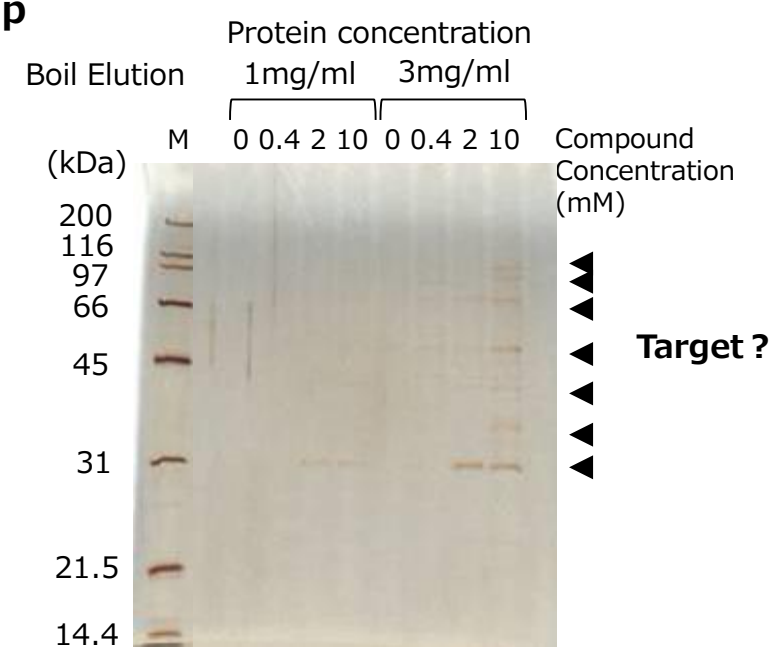
2 hours | 500 μl (DMF)

Compound immobilized beads

Affinity purification

(Protocol 001)

※1) No triethylamine added in current protocol.



Based on Protocol 001	
beads	: NH_2 beads (S250PSSNENH2)
beads amount	: 0.5 mg
Binding/Washing buffer	: 100 mM KCl buffer (200μl)
Elution buffer	: ①1M KCl buffer (30μl) ②Sample buffer (40 μl) (Boil Elution)
Protein	: HeLa cell lysate
Protein concentration	: 1 mg/ml, 3 mg/ml (200μl)
Reaction time	: 4 hours

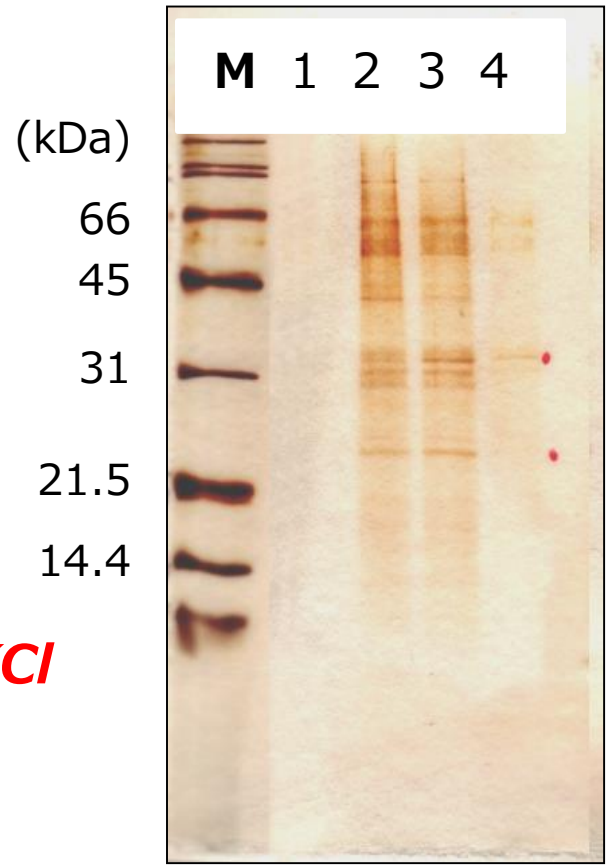
The bound protein was purified at a compound concentration of 2,10 mM, but the amount of purified protein was low. When the abundance of the target protein in the protein solution is low, even if the ligand is immobilized on the beads, the amount of the target protein purified by affinity purification is low, which is insufficient for protein identification and evaluation. In this case, it is necessary to consider increasing the concentration and volume of the protein solution.

Purification of Capsaicin Target Protein

【First Review】

○Capsaicin was immobilized on Linker beads at 4 concentrations.

	1	2	3	4
Conc. (mM)	**0**	**0.19**	**0.38**	**0.75**
Et_3N (μl)	0	2	2	2



○Affinity Purification Conditions

- **Beads : 0.5 mg**
- **Binding/Washing buffer : *50 mM KCl***
- **Lysate : NB4 cell lysate 1 mg/ml (500μl)**

Based on the band pattern of the binding protein, we thought that there was an optimal capsaicin concentration between Lane 1 and Lane 2, and proceeded to the second study.

Purification of Capsaicin Target Protein

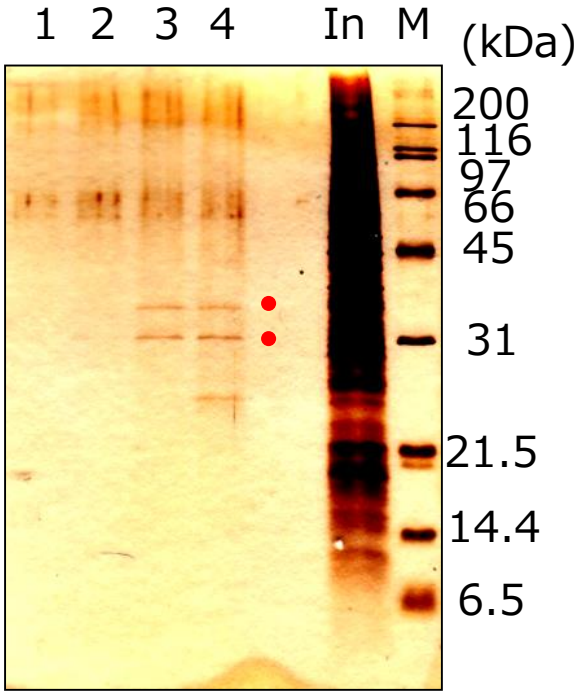
【Second Review】

○Capsaicin was immobilized again at 4 concentrations.

	1	2	3	4
Conc. (mM)	**0**	**0.05**	**0.10**	**0.20**
Et_3N (μl)	0	2	2	2

○Affinity Purification Conditions

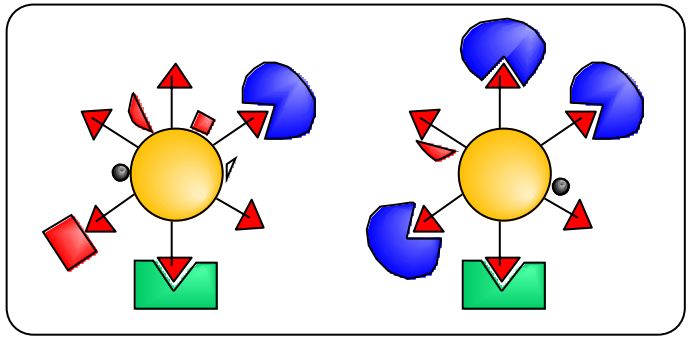
- Beads : 0.5 mg
- Binding/Washing buffer : ***150 mM KCl***
- Lysate : NB4 cell lysate 1 mg/ml (500μl)

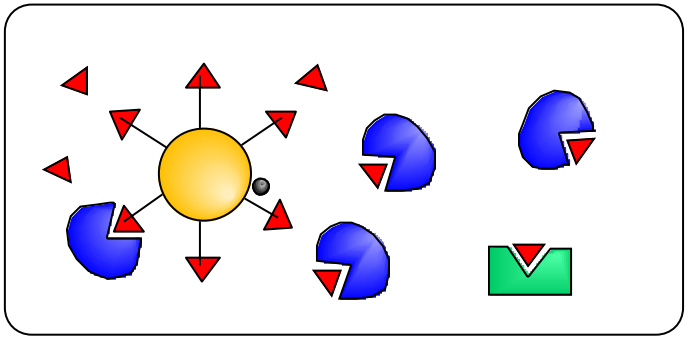


Capsaicin was immobilized again and affinity purification was performed. There was little non-specific adsorption of the protein, and the target protein could be purified with high purity. At the same time, the salt concentration of the binding / washing buffer was changed from 50 mM to 150 mM in order to suppress non-specific adsorption of proteins.

It is important to consider buffer conditions in addition to optimizing the amount of ligand immobilization.

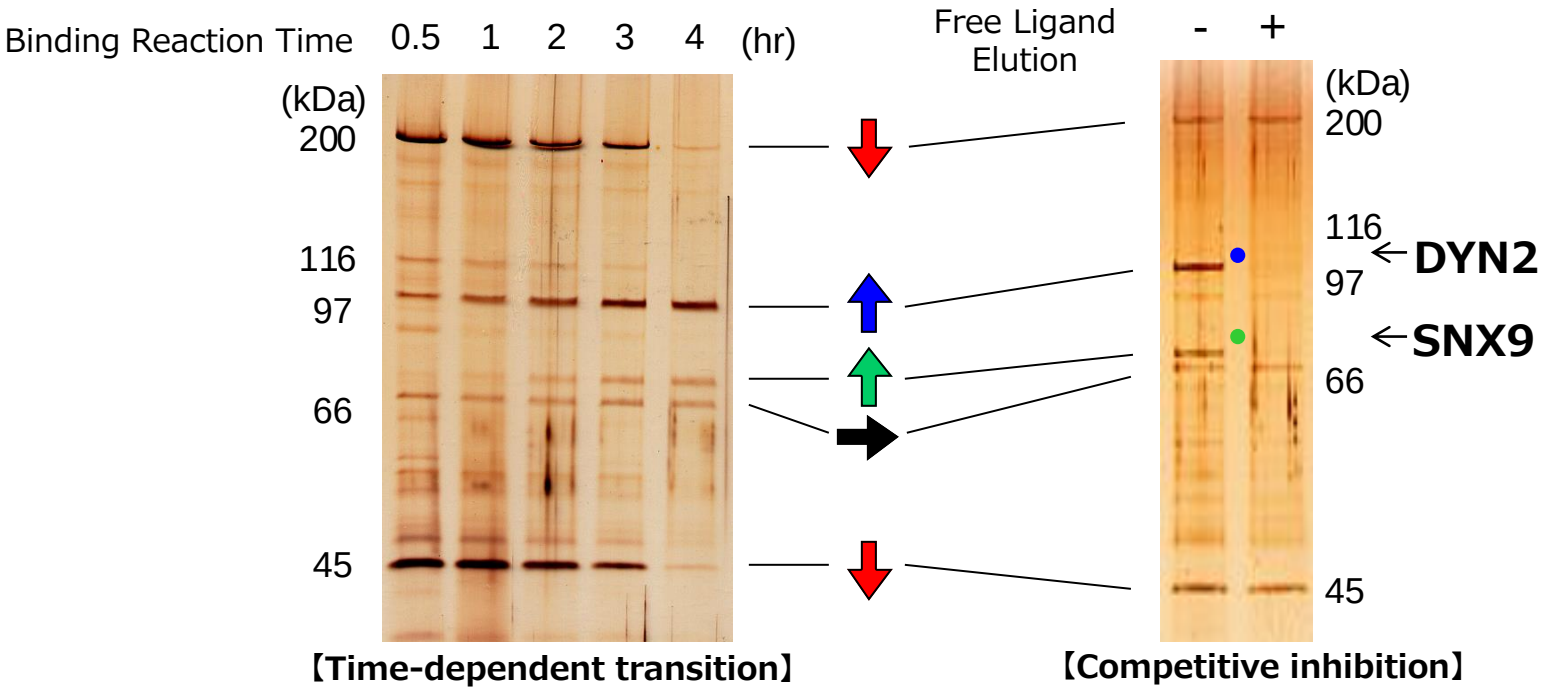
Binding Reaction Time
Time-dependent enrichment of bound proteins





: Target proteins

: Non-specific proteins



Some proteins are bound at a binding reaction time of 0.5 hours, but the binding amount of two known target proteins (blue and green) is increased at 4 hours. It is thought that the target protein that binds more strongly to the ligand over time is replaced with the weakly bound protein, and the amount of binding of the target protein increases.

Workflow for Affinity Purification

① Immobilization of ligand

・Ligand Design

Structure-Activity Relationship

Control compound

↓ ・Quantification of ligand amount (HPLC)

・Optimization of Ligand Immobilization Amount

② Mix ligand immobilized beads and protein solution (Cell lysate)

・Centrifuging protein solution before use

↓ ・Method for preparing cell lysate (protocol 401, 402)

③ **Binding reaction・Washing・Elution (Salt/Boil elution)**

・Composition of Binding/washing Buffer

Binding Reaction Time

↓ ・Dispersion of beads (Scratch)

・Magnetic separation

・**Specificity**

(Competitive inhibition, Drug elution)

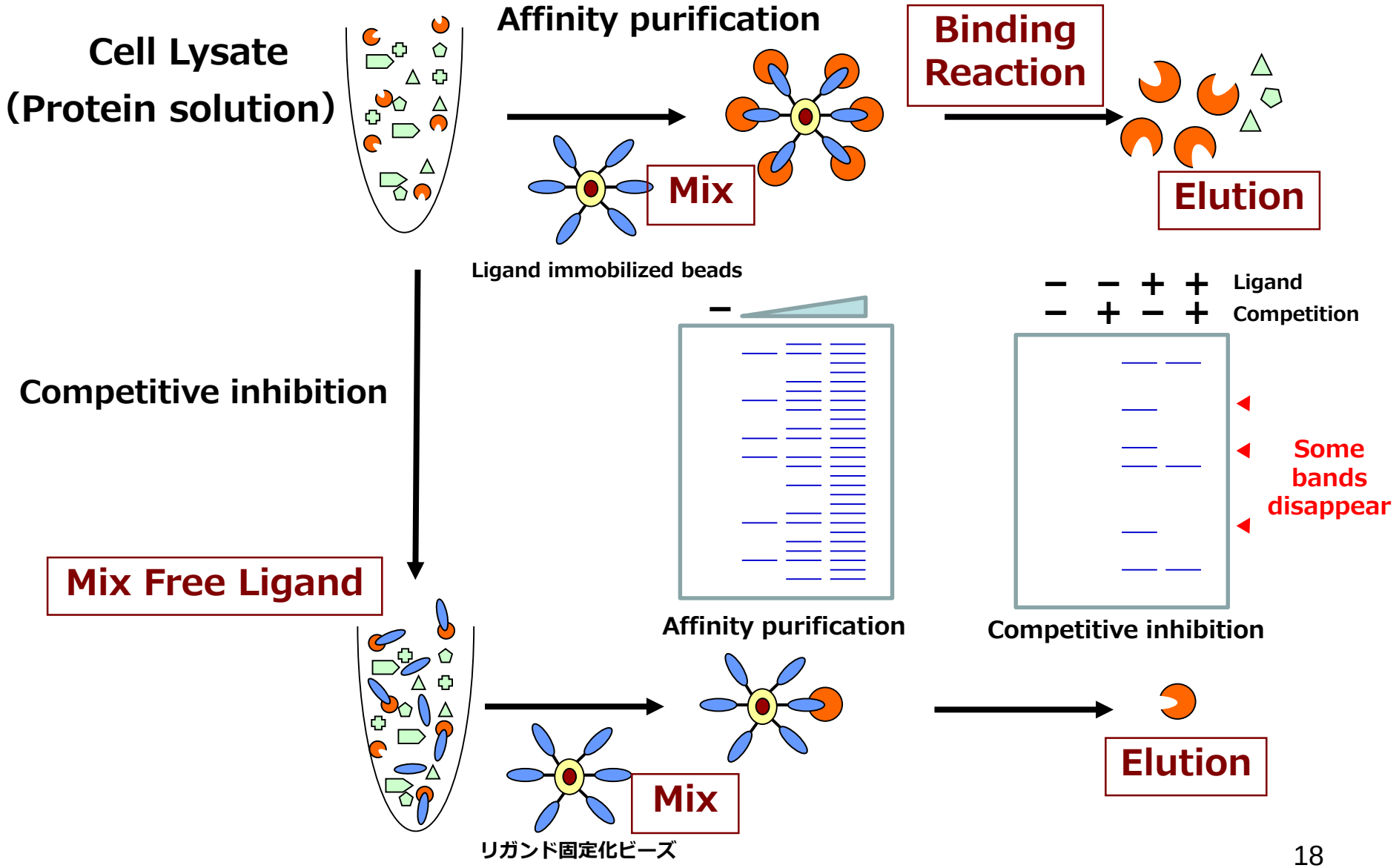
・Precautions for affinity purification

(Avoiding keratin contamination)

④ Analysis

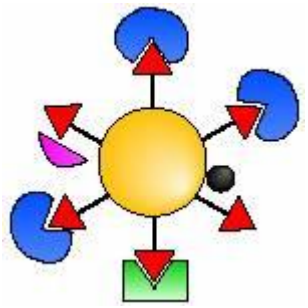
SDS-PAGE, Silver stain, Mass spectrometry(MS)

Competitive Inhibition

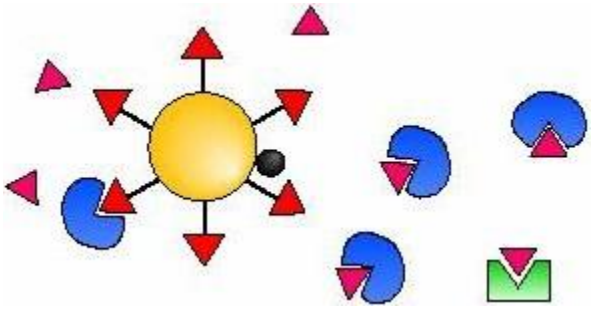


If the band disappears, it is very likely that it is the target protein.

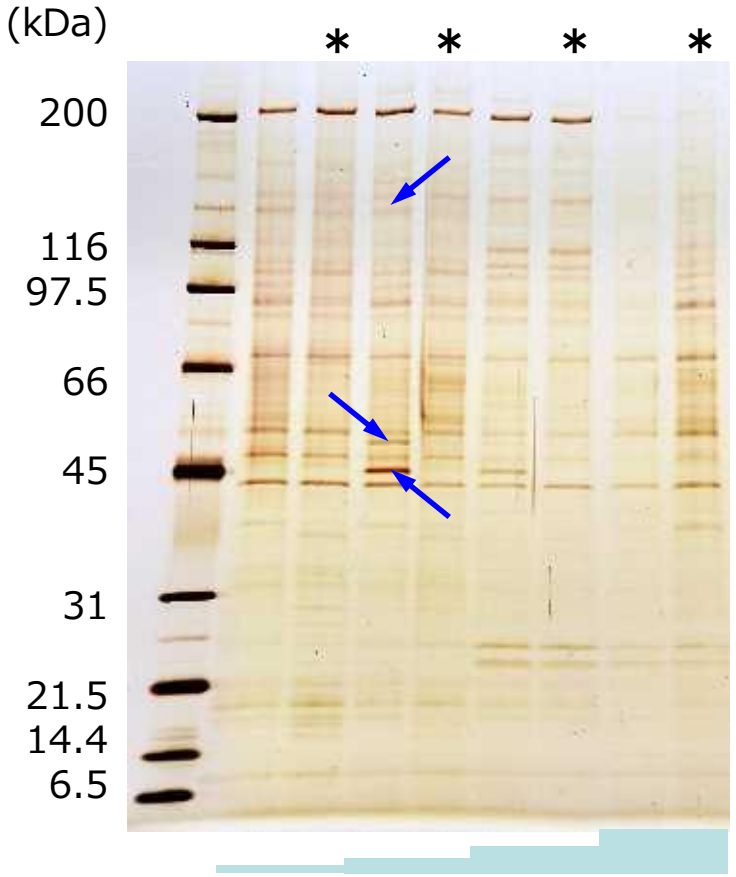
Examples of competitive inhibition



Binding reaction after addition of free ligand



: Target protein

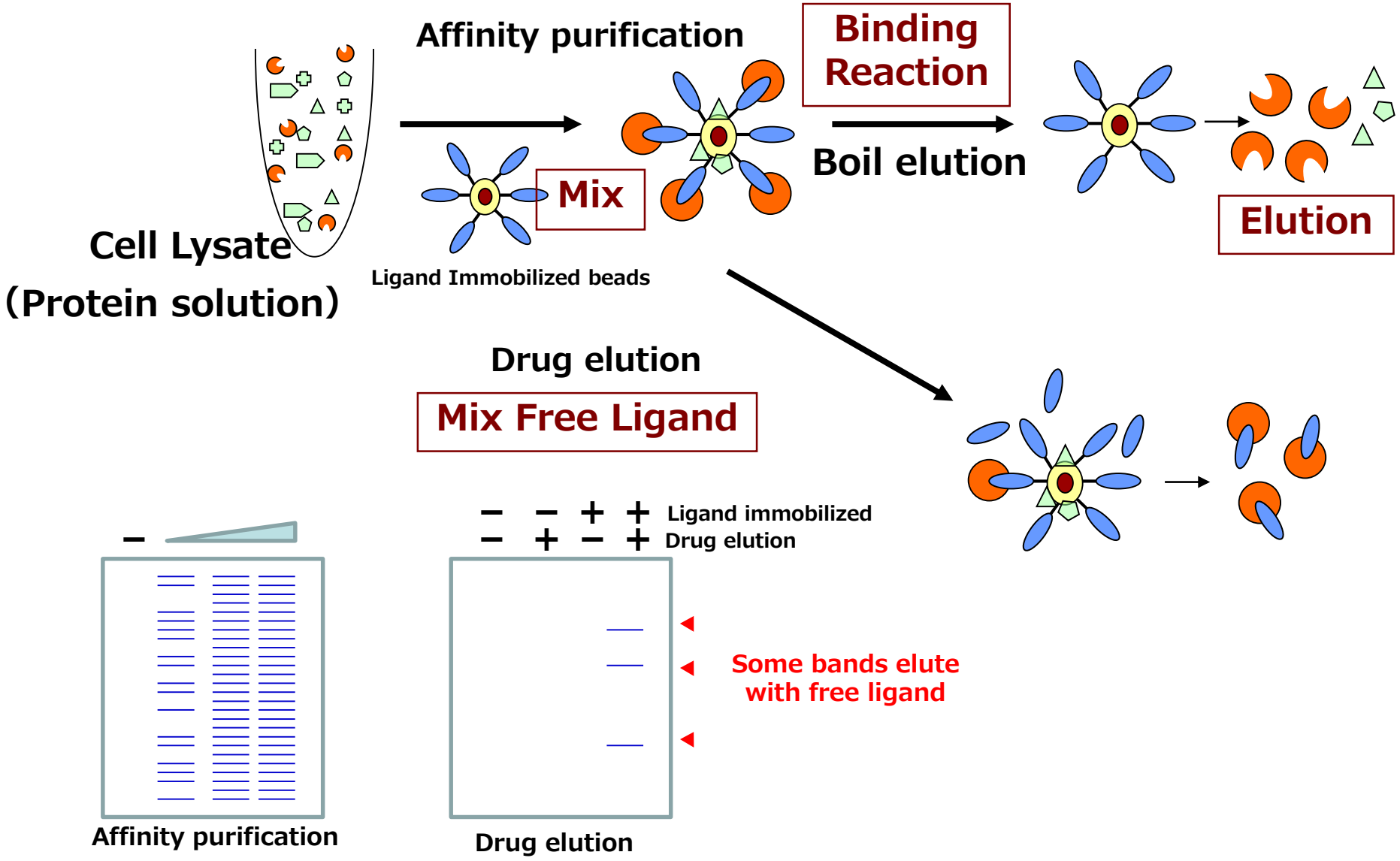


Immobilized amount of ligand

＊ : Addition of ligand（Competition）

The bands disappeared. They are likely target proteins.

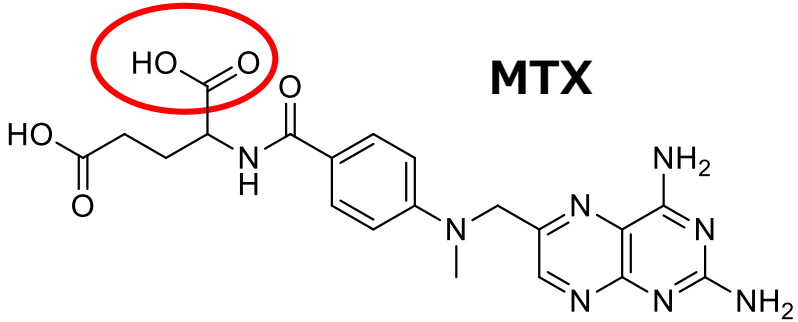
Drug Elution



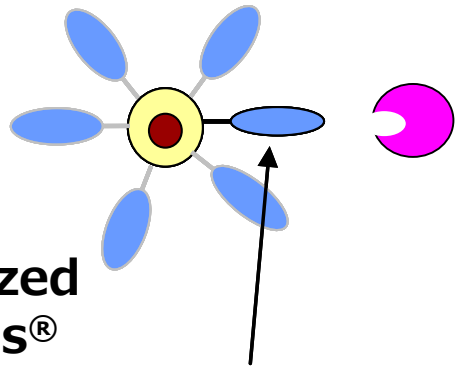
If the band eluted with a free ligand, it is very likely to be a target protein.

Competitive inhibitor and Drug elution

Immobilization site



MTX



MTX immobilized FG beads®

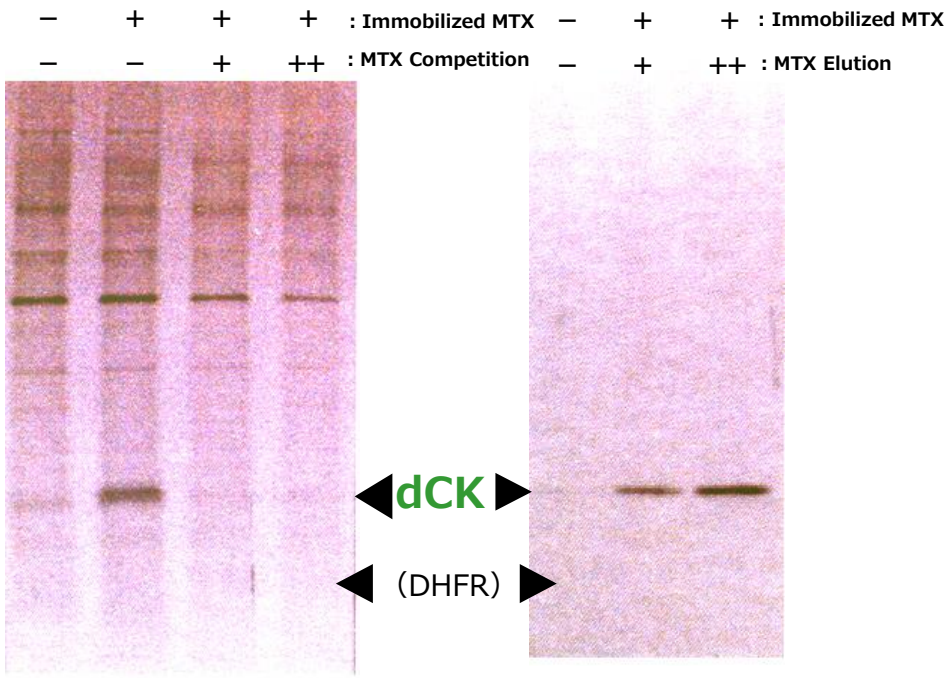
MTX

dCK

Target protein

Competitive inhibition

Drug Elution



dCK : Deoxycytidine kinase

In competitive inhibition, the addition of MTX reduced the protein of the target protein, dCK, and in drug elution, the dCK band was eluted with MTX. The amount of dCK eluted increased by increasing the MTX concentration of the drug elution.

Workflow for Affinity Purification

① Immobilization of ligand

- Ligand Design
  Structure-Activity Relationship
  Control compound

↓

- Quantification of ligand amount (HPLC)
- Optimization of Ligand Immobilization Amount

② Mix ligand immobilized beads and protein solution (Cell lysate)

- Centrifuging protein solution before use
- Method for preparing cell lysate (protocol 401, 402)

↓

③ **Binding reaction・Washing・Elution (Salt/Boil elution)**

- Composition of Binding/washing Buffer
  Binding Reaction Time
- Specificity
  (Competitive inhibition, Drug elution)

↓

- Dispersion of beads (Scratch)
- Magnetic separation

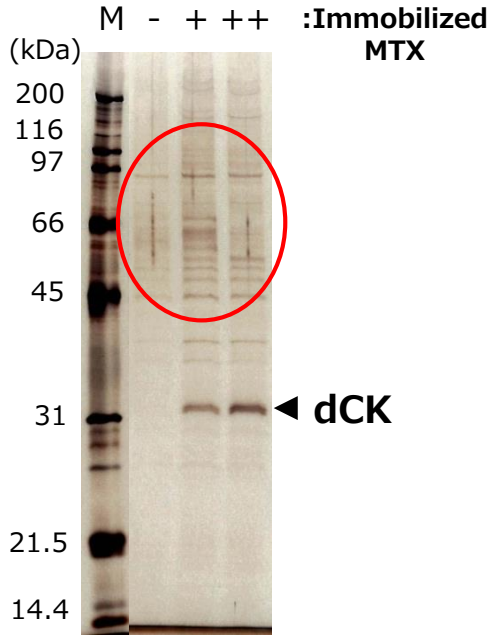
・Precautions for affinity purification
(Avoiding keratin contamination)

④ Analysis

SDS-PAGE, Silver stain, Mass spectrometry(MS)

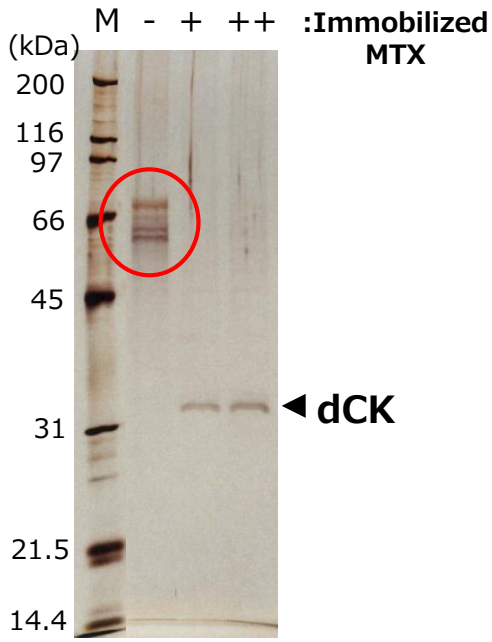
Precautions for affinity purification

Example 1



High background

Example 2



Human Keratin contamination

- Disperse the beads properly.
- Wear gloves when affinity purification.
- Do not touch the inside of the tube lid.