Efficient Peptide Purification by HPLC

- Effect of Pore size, Particle Size and Chemistry -

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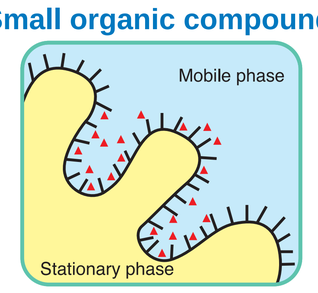
1. Introduction

Reversed-phase HPLC is an invaluable tool also for the analytical and preparative separation of peptides and proteins. Owing to the availability of different pore sizes and particle sizes, the alkyl-bonded silica gel products are economically the first choice for both analytical and preparative separations.

Although the surface area decreases with increasing pore size, large-pore silica gel products are popular for various separation purposes. A wrong pore size, however, gives poor chromatographic performance. Selection of an appropriate pore size in separation is important to assure high resolution and high yield. This study shows how a wrong pore size affects the resolution and performance of silica gel bonded with a ligand such as C18, C8, or C4.

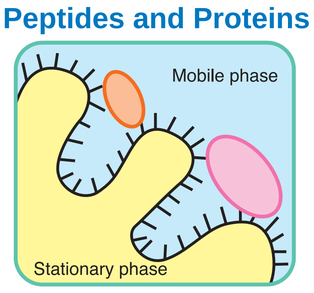
2. Retention Mechanism for Peptides and Proteins

Small organic compounds



The ▲*marks* represent small organic compounds. Small organic compounds easily enter the pores and interact with the ligands on the stationary phase.
They mobilize with distributing between the stationary phase and mobile phase.

Peptides and Proteins



Ovals represent peptides and proteins. Large molecules cannot enter the pores and merely interact with the ligands on the stationary phase surface.
Pore size plays a key role in determining resolution and loading amount in separation of peptides and proteins.

3. Impact of Pore Size on Efficiency

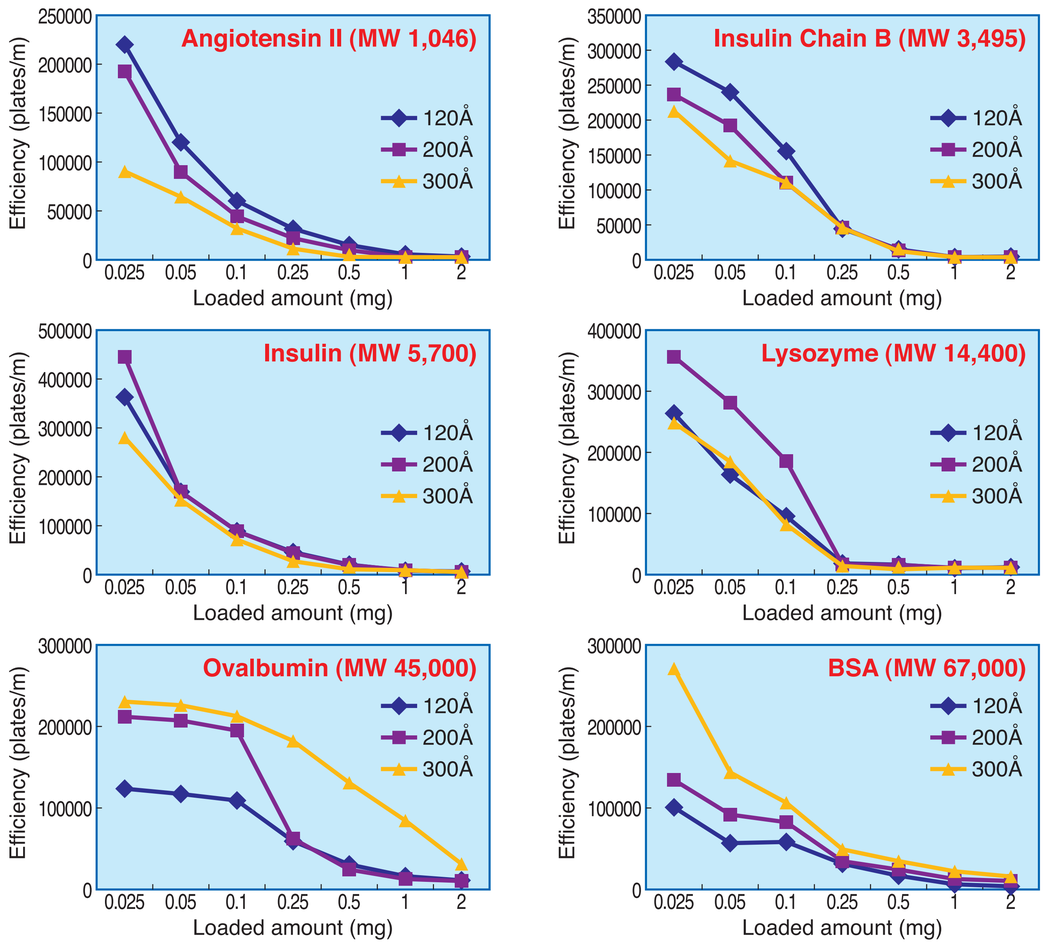
HPLC conditions

Stationary phase	: C4
Pore size	: 120, 200, 300 Å
Particle size	: 5µm
Column size	: 150 × 4.6 mmI.D.
Flow rate	: 1.0 mL/min
Temperature	: 37 ℃
Detection	: UV at 220 nm
Eluent	: A) water / TFA (100/0.1) B) acetonitrile / TFA (100/0.1) 10-90%B(0-20min), 90%B(20-25min)

Peptides and proteins in this study

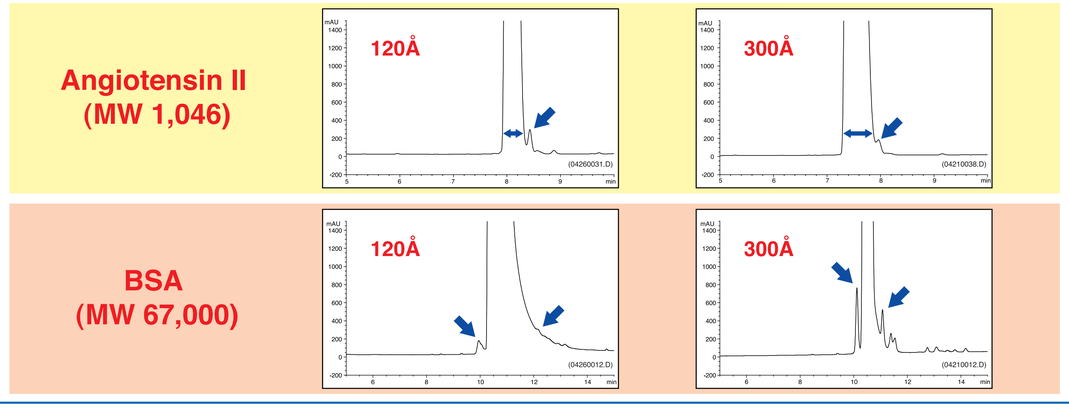
- Angiotensin II, Human — MW 1,046
- Insulin Chain B, Oxidized, from Bovine Pancreas — MW 3,495
- Insulin, from Bovine Pancreas — MW 5,700
- Lysozyme, from Egg White — MW 14,400
- Ovalbumin (Albumin, from Chicken Egg) — MW 45,000
- BSA — MW 67,000

Effect of sample load on efficiency of C4 columns with 120, 200, and 300Å pore sizes



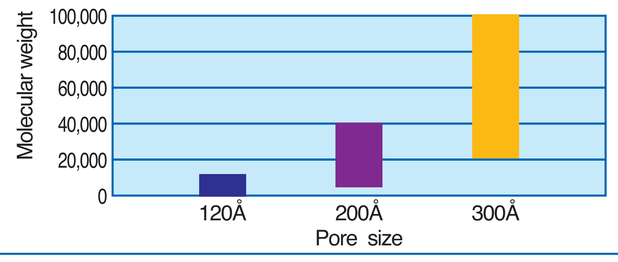
In the case of low molecular weight peptides up to MW 3,500, 120 Å pore size (◆) is most efficient at all the loading levels. Large peptides and small proteins such as Insulin and Lysozyme are adapted to 200 Å pore size (■) at relatively low loading levels. In separation of proteins larger than MW 40,000, 300 Å pore size (▲) gives the highest efficiency. 120 Å and 200 Å pore sizes would be too small to give a good peak shape and resolution.

Comparison of peaks on C4 with 120Å and 300Å pore sizes



It is important to choose an appropriate pore size for achieving a good peak shape. An overly small or overly large pore size results in peak broadening and poor resolution.

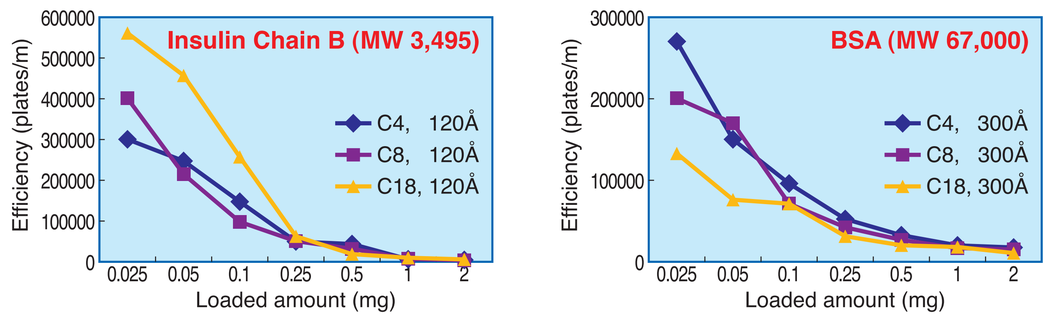
Optimum pore size for peptides and proteins



The 120 Å pore size is ideal for peptides with a MW ranging from 200 to 10,000. The 200 Å pore size is ideal for large peptides and small proteins with a MW ranging from 5,000 to 40,000. The 300 Å pore size is suitable to proteins with a MW of ≧ 20, 000.

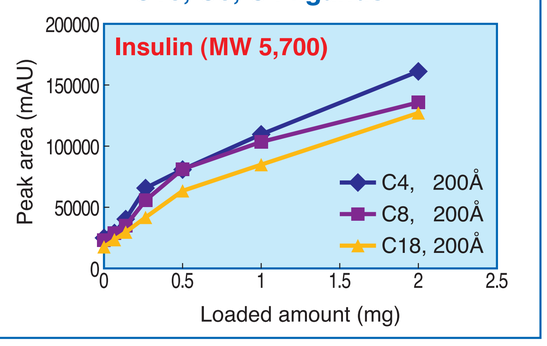
4. Impact of ligand on separation

Comparison of C18, C8, C4 ligands on gel with optimum pore size for sample MW



The alkyl chain length creates a difference in the efficiency of separation of peptides and proteins. In separation of Insulin chain B (MW 3,495) with 120 Å pore size, C18 alkyl chain is most efficient at all loading levels. For low-MW peptides, the combination of a small pore size and a hydrophobic alkyl chain would be favorable. For separation of BSA (MW 67,000) with 300 Å pore size, C4 ligand shows good efficiency at almost all the loading levels. For separation of proteins, the combination of a large pore size and a short alkyl chain would be the best choice.

Comparison of sample recoveries with C18, C8, C4 ligands



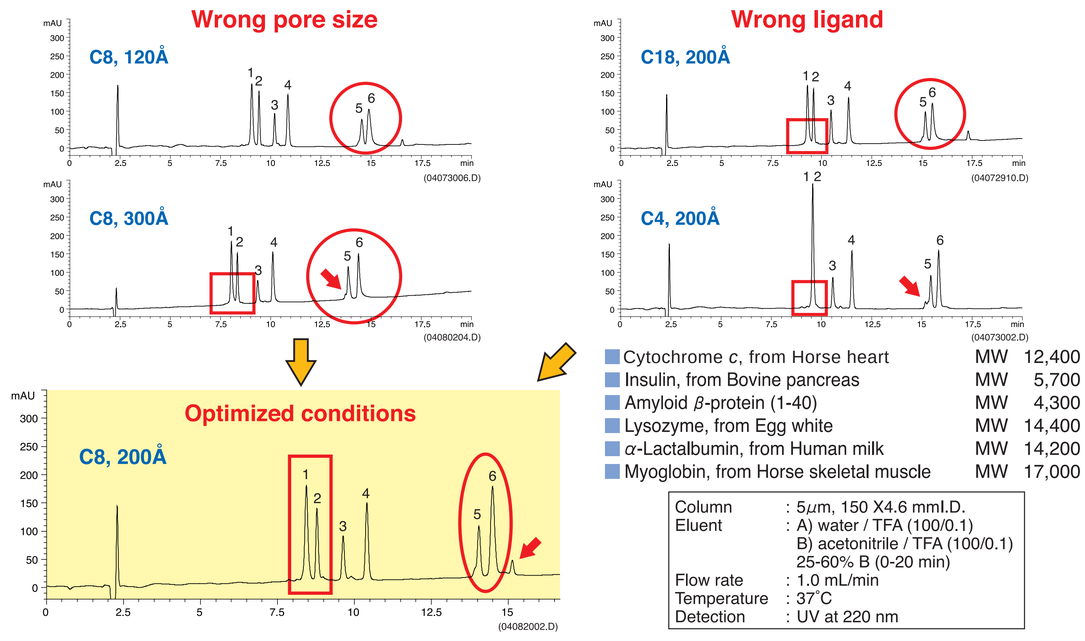
Effects of ligand on separation

Ligand	C18	C8	Ph	C4	CN
Hydrophobicity of gel	high ←				→ low
Suitable MW of sample	low →				← high
Recovery of sample	low →				← high
Column durability	good ←				→ poor

The higher the molecular weight of sample, the less hydrophobic the favorable gel is. However, the less hydrophobic ligand results in shorter column-life. Meanwhile, the higher the hydrophobicity of the stationary phase, the lower the sample recovery is. For efficient preparative separation of peptides and proteins, it is necessary to select not only an appropriate pore size but also an appropriate ligand.

5. Combination of Appropriate Pore size and Ligand

Separation of peptide and protein mixtures on columns of different pore sizes and ligands



- Cytochrome *c*, from Horse heart — MW 12,400
- Insulin, from Bovine pancreas — MW 5,700
- Amyloid β-protein (1-40) — MW 4,300
- Lysozyme, from Egg white — MW 14,400
- α-Lactalbumin, from Human milk — MW 14,200
- Myoglobin, from Horse skeletal muscle — MW 17,000

Column	: 5µm, 150 X4.6 mmI.D.
Eluent	: A) water / TFA (100/0.1) B) acetonitrile / TFA (100/0.1) 25-60% B (0-20 min)
Flow rate	: 1.0 mL/min
Temperature	: 37°C
Detection	: UV at 220 nm

These chromatograms show an optimization of separation of large peptide and small protein mixtures (MW 4,300 ~ 17,000) on the columns of different pore sizes and ligands. The combination of appropriate pore size and hydrophobicity of bonding chemistry, 200 Å C8, yields the best resolutions and peak shapes.

Optimized stationary phase for separation

MW	C18	C8	C4
120Å (5,000)	◎	○	△
200Å (20,000)	○	◎	○
300Å (100,000)	△	○	◎

The C18 column with 120 Å pore size is generally suitable for small peptides up to MW 5000 similar to the analyses of small non-peptidic molecules. In the case of large peptides or small proteins up to MW 20000, the C8 column with 200 Å pore size often gives the best column efficiency. Furthermore, most of proteins are eluted effectively by the C4 column with 300 Å.

◎ : excellent ○ : good △ : moderate

6. Conclusions

- It is important to choose the appropriate pore size to achieve optimal separation of peptides or proteins. An overly small or overly large pore size gives poor resolution.
- The Ligand on the gel also plays an important role in achieving efficient separation. Appropriate hydrophobicity of the gel is essential for efficient separation.
- It is necessary to combine appropriate pore size, hydrophobicity, particle size and column size to achieve higher recoveries or higher resolutions of peptides and proteins.