Development of effective purification method for peptides and proteins by silica gel based reversed phase packing material

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Introduction

Reversed-phase HPLC is an invaluable tool 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.

We reported that optimum pore size of gel gave good peak shape and good separation in analytical and preparative/process scale. Based on these results, we attempted to purify insulin with cost effectively. Actually, in the production by chromatography, cost performance would be most important thing. This study, we show cost-effective purification of insulin by optimizing necessary factors.
Comparison of *preparative* and *analytical* chromatography

**Analytical**
- Peak shape
- Peak resolution
- Column choice
- Analytical time

**Preparative**
- Recovery
- Purity
- *Cost performance*
- Safety in production

*In preparative scale, factors to be considered are* easily recovery, cycle time, recovery, purity, loading amount, solvent, etc.
Preparative production cost includes:

- **Separation equipment**
- **Materials** e.g. packing material, column
- **Eluent, after-treatment of waste**
- **Time** e.g. separation, concentration
Flow chart of preparative chromatography

Set up separation conditions (eluent, time of interval, flow rate, etc)

Choosing **packing material** (functional group)

Choosing **particle size**

Optimization of **preparative conditions**

**Preparative chromatography**
Recommend combination of MW and pore size for peptides and proteins

<table>
<thead>
<tr>
<th>MW</th>
<th>C18</th>
<th>C8</th>
<th>C4</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>○</td>
<td>○</td>
<td>△</td>
</tr>
<tr>
<td>120 Å</td>
<td>△</td>
<td>○</td>
<td></td>
</tr>
<tr>
<td>200 Å</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>300 Å</td>
<td>△</td>
<td>○</td>
<td>○</td>
</tr>
</tbody>
</table>
Initial conditions for separation of insulin

Comparison of solvent, MeOH vs ACN

Methanol (10-90%B)

Acetonitrile (10-90%B)

Initial analytical conditions

- Column: YMC-Pack C8, S-5μm, 20nm (150mmX4.6mml.D.)
- Flow rate: 1.0mL/min
- Eluent: A) water / TFA (100/0.1)  
  
  B) _____ / TFA (100/0.1)
- Detection: UV at 220 nm
- Temperature: ambient
- Sample: Insulin (bovine/human=10/1)
Optimization of gradient condition with ACN

Appropriate optimization was not achieved in these analyses because of poor peak resolution between bovine and human.
Optimization of gradient condition with MeOH

By using methanol as eluent, better separation was obtained in condition of 55-60%B solution. Methanol was better than ACN because it gave good separation, cost effective and better safety.
Influences of additive type and concentration

Separations with 0.1% TFA and 0.05N HCl show appropriate retention time and good resolution.
Comparison of peak shape under high load

Using TFA additive shows broader than the case of HCl.
In terms of cost effective and peak shape, HCl was chosen as an additive.
Comparison of packing material/functional group

<table>
<thead>
<tr>
<th></th>
<th>C4</th>
<th>C8</th>
<th>C18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Durability for acids</td>
<td>○</td>
<td>◎</td>
<td>◎</td>
</tr>
<tr>
<td>Cost performance</td>
<td>△</td>
<td>◎</td>
<td>○</td>
</tr>
<tr>
<td>Peak resolution</td>
<td>○</td>
<td>◎</td>
<td>○</td>
</tr>
<tr>
<td>Peak shape</td>
<td>◎</td>
<td>◎</td>
<td>○</td>
</tr>
<tr>
<td>Retention time</td>
<td>◎</td>
<td>◎</td>
<td>△</td>
</tr>
</tbody>
</table>

© Excellent,  ○ Good, △ Moderate

C8 medium is best choice for the separation of insulin.
Optimization of analysis time and flow rate

1) Optimized initial conditions
   (No peak in 15-30 min)
   - Column: YMC-Pack C8, S-5μm, 20 nm (150mmX4.6mmI.D.)
   - Flow rate: 1.0mL/min ⇒ 0.5 mL/min
   - Eluent: A) water / HCl (100/0.05N)
     B) methanol / HCl (100/0.1)
   - Detection: UV at 220 nm
   - Temp.: ambient
   - Sample: Insulin (bovine/human=10/1)

2) Half time of 1cycle time
   Shorten analysis time.
   - 55-58%B (0-15min)

3) Fine optimization
   Flow rate : 0.5 mL/min, gradient curve
   - 57-60%B (0-15min)

Fine optimization of gradient curve and flow rate, shorten analysis time and reduce eluent amount were achieved.
Influence of particle size (C8, 20 nm)

In preparative, peak shape of each particle size are similar.

It is better to use larger particle size for cost effective production.
### Preprative conditions and cost performances

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Analytical</th>
<th>Preparative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle size</td>
<td>5 μm</td>
<td>15 μm</td>
</tr>
<tr>
<td>Packing material</td>
<td>C8, 20 nm</td>
<td>C8, 20 nm</td>
</tr>
<tr>
<td>Eluent</td>
<td>ACN / H$_2$O / TFA</td>
<td>MeOH / H$_2$O / HCl</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.0 mL/min</td>
<td>0.5 mL/min</td>
</tr>
</tbody>
</table>

#### Cost performance of each factor

- **Solvent (MeOH)**: 84% Decrease than ACN
- **Additive (HCl)**: 95% Decrease than TFA
- **Eluent (By reducing flow rate)**: 50% Decrease than initial condition
- **Preparative time of 1 cycle**: 50% Decrease than initial condition
- **Particle size (S-15 um)**: 95% Decrease than 5 μm
Preparative separation of insulin (1)

Scale up study (150mmX50mm I.D., DAU-50-700)

**Analytical conditions**
- **Column**: YMC-Pack C8, 5 μm, 20nm
- **Flow rate**: 0.5mL/min
- **Eluent**: A) water / HCl (100/0.05N)
  - B) methanol / HCl (100/0.05N)
  - 57-60%B (0-15min, linear)
- **Detection**: UV at 220nm
- **Temperature**: ambient

**Preparative conditions**
- **Column**: YMC * GEL C8, 15 μm, 20nm
  - 150mmX50mm I.D., (DAU-50-700)
- **Flow rate**: 60mL/min
- **Eluent**: A) water / HCl (100/0.05N)
  - B) methanol / HCl (100/0.05N)
  - 57-60%B (0-15min, linear)
- **Detection**: UV at 220nm
- **Temperature**: ambient

Load: 700 mg

**Purity**: 99%
**Recovered amount**: 620 mg
**Recovery**: 88%
**Preparative separation of insulin (2)**

**Loadability of insulin**

<table>
<thead>
<tr>
<th>Loaded amount</th>
<th>200 mg</th>
<th>700 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purity</td>
<td>99%</td>
<td>99%</td>
</tr>
<tr>
<td>Recovered amount</td>
<td>190 mg</td>
<td>620 mg</td>
</tr>
<tr>
<td>Recovery</td>
<td>95%</td>
<td>88%</td>
</tr>
</tbody>
</table>

The separation in 200 mg loaded shows high recovery, but low recovered amount. In production scale, higher loaded amount would give high recovery.
## Choice of column size

### 3000 kg insulin purification per year

<table>
<thead>
<tr>
<th>Column size →</th>
<th>50 mml.D.</th>
<th>200 mml.D.</th>
<th>600 mml.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loaded amount / day*</td>
<td>68 g</td>
<td>1056 g</td>
<td>4896 g</td>
</tr>
<tr>
<td>Product amount / year</td>
<td>24 kg</td>
<td>360 kg</td>
<td><strong>3560 kg</strong></td>
</tr>
<tr>
<td>Cost of packing material (ratio)</td>
<td>1.0</td>
<td>16</td>
<td>144</td>
</tr>
</tbody>
</table>

* Cycle time: 15 min, Loading was performed 96 times per day.

To achieve expected production amount, it is necessary to use 600 mml.D. column.
# Improvement of cost performance (1)

## Comparison of cost performance in different conditions

<table>
<thead>
<tr>
<th></th>
<th>Example conditions</th>
<th>Optimized conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 cycle time</td>
<td>30 min (48 times/day)</td>
<td>15 min (96 times/day)</td>
</tr>
<tr>
<td>Eluent</td>
<td>Acetonitrile</td>
<td>Methanol</td>
</tr>
<tr>
<td>Flow rate*</td>
<td>17 L</td>
<td>8.6 L</td>
</tr>
<tr>
<td>Packing material</td>
<td>C18, 10μm</td>
<td>C8, 15μm</td>
</tr>
</tbody>
</table>

- **Eluent cost**: 95% Decrease
- **Cost of gel**: 90% Decrease
- **Production efficiency**: 100% Increase

*By using 600 mm I.D. column.

High flow rate and high column pressure cause not only increasing eluent amount but also increasing equipment cost.
### Improvement of cost performance (2)

#### Example conditions

<table>
<thead>
<tr>
<th>Cost Category</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cost</td>
<td>30,550,000 US$</td>
</tr>
<tr>
<td>Product amount/year</td>
<td>1,780 kg</td>
</tr>
<tr>
<td>Total cost / Product 1 kg</td>
<td>17,162 $ / kg</td>
</tr>
<tr>
<td>Initial cost / Product 1 kg</td>
<td>566 $ / kg</td>
</tr>
<tr>
<td>Labor cost / Product 1 kg</td>
<td>309 $ / kg</td>
</tr>
<tr>
<td>Gel cost / Product 1 kg</td>
<td>1132 $ / kg</td>
</tr>
<tr>
<td>Eluent cost / Product 1 kg</td>
<td>15,102 $ / kg</td>
</tr>
</tbody>
</table>

#### Optimized conditions

<table>
<thead>
<tr>
<th>Cost Category</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cost</td>
<td>3,810,000 US$</td>
</tr>
<tr>
<td>Product amount/year</td>
<td>3,560 kg</td>
</tr>
<tr>
<td>Total cost / Product 1 kg</td>
<td>1,070 $ / kg</td>
</tr>
<tr>
<td>Initial cost / Product 1 kg</td>
<td>278 $ / kg</td>
</tr>
<tr>
<td>Labor cost / Product 1 kg</td>
<td>149 $ / kg</td>
</tr>
<tr>
<td>Gel cost / Product 1 kg</td>
<td>107 $ / kg</td>
</tr>
<tr>
<td>Eluent cost / Product 1 kg</td>
<td>631 $ / kg</td>
</tr>
</tbody>
</table>

By optimizing conditions, total cost is extremely lower than the example case.
Summary

• In preparative production, by choosing optimum conditions as we shown this study, you can make purification effectively.

• It is important to choose best combination of pore size, functional group and particle size for the preparative purification of peptides and proteins.

• YMC can offer not only various packing materials but also preparative products (e.g. dynamic axial column, process equipments). We also offer the information of every stage of purification process.