Recombinant-RGD-Hirudin \( \text{r-RGD-Hirudin} \) cDNA \( \text{cDNA} \) RGD-Hirudin-\( \text{pPIC9K} \)（\( \text{RGD-Hirudin-pPIC9K} \)）

1. 材料与方法
1.1 菌种和培养基
1.1.1 5L BioFlo 3000 \( \text{5L BioFlo 3000} \) NBS \( \text{NBS} \) FPLC \( \text{FPLC} \) Waters prep 4000 \( \text{Waters prep 4000} \) Sephadex-G50 \( \text{Sephadex-G50} \) Pharmacia \( \text{Pharmacia} \)

2. 实验动物

3. 主要设备、化学试剂和实验动物

4. 结果与讨论

5. 结论

关键词：重组双功能水蛭素、分泌型高效表达、纯化、鉴定

中图分类号：U23

基金项目：上海市现代生物与新药发展基金（沪科基预[2003]65-66）

收稿日期：2003-07-17

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关键词

重组双功能水蛭素

中图分类号

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接种：从深低温冰箱中取出种子菌，室温下解冻，划平板，温箱培养。从平板上挑取单菌落，接种于培养液中，培养过夜，此为一级种子液。再将一级种子液加入培养液中，培养24小时，直至发酵。

发酵：在全自动发酵罐内加入发酵基础培养基，于37℃下灭菌，冷却到30℃加入2mol/L，加入2mol/L，氨水调节酸度为7.4，接入二级种子液进行发酵，控制溶氧为0.25，搅拌速度为30rpm，搅拌速度由溶氧控制，温度为20℃。每隔4个小时测定OD_600，当基础培养基中的甘油耗尽后，适当加入甘油补料，直至OD_600为150，再将一级种子液加入培养液中，培养24小时，此为二级种子液。再将一级种子液加入培养液中，培养24小时，此为三级种子液。

超滤浓缩和凝胶过滤：收集酵母培养液上清，以截留分子量为200000的超滤膜超滤、浓缩至10ml/l，除盐，收集有抗凝血酶活力的组分。

离子交换层析：用Q-Sepharose-F.F.柱，凝胶过滤后收集的抗凝血酶活力部分以20mol/L，线性梯度洗脱，收集有抗凝血酶活力部分，分装，冷冻干燥，保存。

抗凝血酶活力分析：参照的方法进行，取人血浆，在缺少或存在不同量的水蛭素的情况下用凝血酶滴定血浆，由凝血酶的消耗量换算得到水蛭素的单位数。一个凝血酶单位相当于一个抗凝血酶单位。

抗血小板聚集作用分析：取大鼠，戊巴比妥钠腹腔麻醉，腹主动脉取血，枸橼酸钠抗凝，分离富血小板血浆，并用贫血小板血浆稀释，血小板计数为2×10^9，分别加入和野生型水蛭素，终浓度0.1U/ml，孵育20min，以后一直维持此速度。每隔4个小时测定OD_600和发酵液上清的抗凝血酶活性。诱导5天后，下罐。

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质谱分析：由上海市计量研究所测定。

实验结果

发酵诱导前，酵母菌一般需要增殖。加入甲醇诱导后，培养液上清中目的蛋白的表达和抗凝血酶活性的变化情况，酵母菌密度的变化如图2、3所示。

图2：甲醇诱导后，Q1'E38？R+89的表达(以培养液上清中，单位体积抗凝血酶活性变化表示)。经过36小时甲醇诱导，培养液上清中，单位体积抗凝血酶活性可达150U/ml，随着时间延长，抗凝活性增长缓慢或停滞。

图3：甲醇诱导后，酵母菌密度变化趋势。我们采用高密度发酵，甲醇诱导前，酵母菌密度为2×10^9，经过诱导，可达150U/ml。
1 RGD-Hirudin

Table 1 Expression and purification of RGD-Hirudin

<table>
<thead>
<tr>
<th></th>
<th>Fermentation supernatant</th>
<th>After ultra-filtration</th>
<th>After gel filtration chromatography</th>
<th>After anion exchange chromatography</th>
</tr>
</thead>
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<tr>
<td>Total volume/L</td>
<td>4.00</td>
<td>0.25</td>
<td>0.5</td>
<td>0.25</td>
</tr>
<tr>
<td>Activity ATU/mL</td>
<td>2700</td>
<td>14000</td>
<td>12800</td>
<td>24000</td>
</tr>
<tr>
<td>Total antithrombin activity/10^6 ATU</td>
<td>800</td>
<td>700</td>
<td>640</td>
<td>600</td>
</tr>
<tr>
<td>Purity</td>
<td>&gt; 75%</td>
<td>&gt; 80%</td>
<td>&gt; 95%</td>
<td>&gt; 97%</td>
</tr>
<tr>
<td>Recovery yield</td>
<td>87.5%</td>
<td>91.4%</td>
<td>94%</td>
<td></td>
</tr>
</tbody>
</table>

2.3 RGD-hirudin

Fig. 4 The effect of RGD-Hirudin on ADP-induced platelet aggregation

2.4 LC/MS RGD-hirudin

Fig. 5 LC/MS analysis of purified RGD-Hirudin

2.5 RGD-hirudin vs. wt-Hirudin

Fig. 6 pH analysis of purified RGD-Hirudin and wt-Hirudin

3 Discussion

"The expression system has successfully expressed various exogenous proteins, such as human serum albumin, tumor necrosis factor, and human interleukin A, with expression levels up to the level of the kilogram [14]. Therefore, the expression level of RGD-Hirudin requires improvement. Our yeast expression system is a secretory expression vector system, which makes downstream purification very convenient, especially suitable for the purification of proteins with disulfide bonds.

In the fermentation process, the time when methanol is added to induce is very important. After screening, we obtained a high-expression Q2/V type clone, so that the induction time can be determined."

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一方面杂蛋白中的蛋白酶容易降解目的产物。体蛋白释放出来，一方面增加了杂蛋白，给纯化带来困难，另
们研制的水蛭素衍生物从理论上讲既具有抗凝血酶的作用，原与之结合，达到抑制血小板聚集的目的。利酵母菌的继续生长，反而会产生大量的死菌，菌体破裂，菌
浓缩是纯化过程中最为关键的一步。我们采用超滤浓缩的方法，可以去除小分子的色素，再经过凝胶过滤和阴离子交
控制甲醇终浓度为9-8%，并最后达到所需的终浓度。最初
测定，分子量约为98kD，诱导时间控制在6-9h，诱导相当长的时间后，菌体密度已经很高，不
通过血小板聚集试验，证明了
浓缩的蛋白质晶体，行
的超滤膜超滤，收集滤出液，再经第二步超滤(上述方

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**Fermentation, Purification and Identification of Recombinant RGD-Hirudin**

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**Abstract** Recombinant RGD-Hirudin r-RGD-Hirudin has double functions anti-thrombin activity and anti-platelet aggregation activity. To identify these functions the expression plasmid RGD-Hirudin-pPIC9K was constructed by inserting cDNA of RGD-hirudin in yeast expression vector pPIC9K. The high expression clone was gained after screening. This clone was fermented for 3 days. The r-RGD-hirudin was secreted into the culture. It was ultra-filtrated from culture supernatant then after gel filtration chromatography and anion exchange chromatography the purified r-RGD-hirudin was gained. Its purity was larger than 97% and its specific activity was 12 000 ATU/ng. The yield per liter culture of purified r-RGD-hirudin was 1 g and overal recovery yield was more than 75%. The purified r-RGD-hirudin was identified by reductive SDS-PAGE anti-thrombin activity assay anti-platelet aggregation assay LC/MS and isoelectrofocusing assay. It is proved that r-RGD-Hirudin is ramification of wt-Hirudin and it has anti-thrombin activity and anti-platelet aggregation activity.

**Key words** r-RGD-hirudin, high expression, fermentation, purification, identification

Received 07-17-2003

This work was supported by Shanghai Modern Biotechnology and R&D of Novel Medicine Fund No. 20014319202.

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