1 **The identification of an anti-thrombin molecule** 2 **via the screening of semi-random DNA libraries**

3

4 Short Running Title: The identification of an anti-thrombin molecule

5

6 Fan Feng, Zijin Li, Zhenlang Chen, Le Wang, Jie Huang, Yulin Wan, Yunfan Shi and
7 Qiuyun Liu*

8

9 **Author affiliation**

10 ¹Biomedical Center, School of Life Sciences, Sun Yat-sen University, Guangzhou

11 510275, China.

12

13 **Corresponding author:** Qiuyun Liu, Ph.D, School of Life Sciences, Sun Yat-sen
14 University, Guangzhou 510275, China. Tel: +86 (020)84110296; E-mail:
15 lsslqy@mail.sysu.edu.cn

16

17

18 **Abstract:** Thrombosis remains one of the leading causes of mortality and morbidity in the world.
 19 Thrombin is a key enzyme involved in the blood clotting processes, which can be intervened by low
 20 concentrations of Hirudin. The C-terminal dodecapeptide of Hirudin was capable of inhibiting
 21 thrombosis. This peptide has been partially randomized in this report, and the coding sequences have
 22 been expressed in yeast as chimerical peptides for secretion into the culture media. Two other
 23 semi-random modules have been processed likewise. The supernatant was subsequently tested for
 24 anti-thrombin activities. DNA sequencing indicated that the putative positive clone encoded a single
 25 serine residue followed by a stop codon. The Ninhydrin assay of the culture supernatant of the positive
 26 clone indicated a high content of amino acid. Electrospray Mass Spectrometry showed a distinct peak
 27 at 430.5 when the expression products from *Pichia pastoris* were examined, suggesting that the
 28 compound may be a dimannosylated serine, as yeast possesses glycosylation at serine residues. The
 29 observed effects of α -Mannosidase treatments on the function of yeast induction products are consistent
 30 with this assumption. Partial randomization of peptides and proteins may accelerate directed evolution,
 31 yielding unprecedented number of variants for functional interrogation and drug development.

32

33 **Key words:** anti-thrombin • randomization • dimannosylated serine • yeast

34

35

Introduction

Thrombosis is one of the leading causes of thromboembolic diseases and strikes substantial number of people, especially the elderly. Thrombin is a key enzyme involved in the coagulation pathway, which processes fibrinogen into insoluble fibrin [1-4]. Thrombin also activates factor-XIII, consequently enhancing platelet aggregation. Different antithrombotics have been used to intervene different stages of the blood clotting processes. Anticoagulants prevent blood clotting or curb the propensity of the blood to clot.

Natural peptides are a vast reservoir of drug lead compounds given their huge combinatorial diversity. Oligopeptides are considered attractive chemical entities as their bioavailability is high. They can be also subjected to chemical modifications to enhance potency. *Saccharomyces cerevisiae* is a non-pathogenic organism and can be harnessed for secreted expression of many peptides and proteins. A semi-random DNA library approach would generate homologues of existing proteins or peptides for functional interrogation. Low concentrations of Hirudin could effectively prevent blood clotting. The C-terminal dodecapeptide NGDFEEIPEEYL of Hirudin is a sequence which was capable of inhibiting thrombosis [5]. This peptide was partially randomized here and the attendant coding sequences were expressed in yeast as chimerical peptides for secretion into the culture media. The supernatant was subsequently tested for anti-thrombin activities. Randomization of known peptides and proteins accelerates directed evolution, generating variants with potentially distinct features or enhanced functions from its predecessors.

Material and Methods

Primer Design

The oligonucleotides (oligo) 2, 3 and 4 encoding semi-random peptides and partially random Hirudin oligopeptide homologues are shown in Table 1. The peptide coding sequences come up immediately after the Kex2 cleavage site of *S. cerevisiae* α -factor secretion signal sequence.

Media and Culture Conditions

Luria Broth (LB) was prepared as described [6]. Ampicillin was added at a final concentration of 100 μ g/ml. The glucose/sorbitol selection plates contained 1.7 g Yeast Nitrogen Base (YNB, nitrogen free, Amresco, Inc., Solon, Ohio), 1 M sorbitol, 10 g glucose, 0.5 % $(\text{NH}_4)_2\text{SO}_4$, 10 ml 100 X (Ura⁻) amino acid concentrates, and 20 g agar per liter. Ampicillin was added at a final concentration of 100 μ g/ml to inhibit bacterial growth. The recipe for glucose selection plates was as above but free of sorbitol.

The galactose/raffinose selection plates included 1.7 g YNB, 1 M sorbitol, 30 g galactose, 10 g raffinose, 0.5 % $(\text{NH}_4)_2\text{SO}_4$, 10 ml 100 X (Ura⁻) amino acid concentrates, and 20 g agar per liter. Ampicillin was added at a final concentration of 100 μ g/ml to inhibit bacterial growth.

100 X amino acid concentrates (Ura⁻) included 0.5 % adenine, arginine, cysteine, leucine, lysine, threonine and tryptophan; 0.25 % aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine and tyrosine. It was used after autoclaving for 20 min.

Construction of Chimeric DNA Fragments

Chimeric DNA fragments encoding *S. cerevisiae* α -factor secretion signal and oligopeptides were

generated from PCR amplifications of 50 ng/ml pPICZ α A (Invitrogen, Carlsbad, California) using oligos 2, 3 and 4 respectively with the 5' forward primer 1 (Fig. 1). Pfu DNA Polymerase was adopted for the reactions. PCR mixture was heated at 94 °C for 5 min, followed by 30 cycles of incubation at 94 °C for 30 s, 46 °C for 30 s and 72 °C for 1 min, and a final extension at 72 °C for 5 min. The 0.26 kb PCR amplicons were visualized on 2 % agarose gel. PCR products were ethanol precipitated, and blow dried with a hair dryer. Overnight double digestion with EcoR I and Xba I (Takara, Dalian, China) was performed followed by heat inactivation at 60 °C for 15 min.

Construction of pYES2/CT- semi-random Libraries

pYES2/CT plasmid DNA was triple digested with EcoR I, Xba I and Xho I in the presence of Calf Alkaline Intestinal Phosphotase (Takara, Dalian, China) [7-8]. The digested plasmid and inserts were ligated overnight at 1:3 molar ratios at 16 °C overnight. The reaction mixture was purified with QIAquick PCR Purification Kit (QIAGEN, GmbH, Hilden, Germany) and eluted with deionized water. Electroporations to *Escherichia coli* XL1 strain (Promega, Madison, WI) were conducted as described [9]. Plasmid DNA was then extracted from pools of transformants.

Transformations of Plasmid Libraries into *S. cerevisiae*

The electroporations of above plasmid DNA pool to *S. cerevisiae* strain S150-2B 2 μ ⁺ were performed as described [10]. Genuine yeast transformants were formed on Ura⁻ glucose/sorbitol selection plates after incubating 2 to 3 days at 30 °C. The colonies were inoculated to 1 ml galactose/raffinose media for induction at 30 °C. Caps were open every 48 h to allow fresh air in in the laminal hood. After 7 days of induction, cultures were precipitated at 13,000g for 1 min. 30 μ l supernatant was mixed with 0.5 μ l thrombin from bovine plasma (0.5 U/ μ l in 50 mM Tris-HCl, pH 7.5, stored at -70°C freezer; Sigma-Aldrich, St. Louis, MO) and incubated for 1 min. It was then mixed with 50 μ l fibrinogen from bovine plasma (5 mg/ml; Sigma-Aldrich, St. Louis, MO). The generation of the precipitates was then visualized.

The preparation of PCR templates from positive transformants was as described [11], which was then amplified using Pfu DNA Polymerase with PCR primers 5 and 6 (Table 1). 0.34 kb PCR amplicons were subjected to DNA sequencing by Shanghai BioAsia Bio-technology Co. Ltd with sequencing primers 7 and 8.

Ninhydrin Assays

The Ninhydrin Amino Acid Indicator solution included the following reagents: 0.5 g ninhydrin, 0.3 g fructose, 10 g Na₂HPO₄·10H₂O, 6 g KH₂PO₄ in a total volume of 100 ml.

The pH of the supernatant from the induction media was adjusted to 5.5 with 1 M Tris-HCl (pH 7.5). 20 μ l was then withdrawn and added to 2.00 ml distilled water followed by the addition of 1.00 ml Ninhydrin Amino Acid Indicator solution. Boiling for 15 min was performed after mixing well.

Paper Chromatography of the Supernatant [12]

The serine standard solution contained 5 μ g/ml serine. After 1 h equilibrium in the equilibrium solvent of 12 % aqueous ammonia, paper chromatography with Xinhua No. 1 filter paper (Hangzhou Xinhua Paper Industry Co., Ltd, Hangzhou, China) was conducted, with the use of chromatography solvent

(n-butyl alcohol:12 % aqueous ammonia:95 % ethanol = 13:3:3 (volume)). After air drying, filter paper was sprayed with acetone solution of 0.5 % Ninhydrin Amino Acid Indicator, and incubated for 30 min at 65 °C. R_f values were calculated subsequently.

125

126 Construction of Expression Vector pICZser of *P. pastoris*

127 pPICZ α A vector DNA was double digested with Xho I and Xba I, and ligated with oligos
128 TCGAGAAAAGATCTTGAT and CTAGATCAAGATCTTTTC at a molar ratio of 1:3 at 5 °C
129 overnight. The two oligos encoding a single serine would anneal and form cohesive ends for the
130 ligation. The mixture was ethanol precipitated, and blow dried with a hair dryer. Electroporations to *E.*
131 *coli* DH5 α strain (Promega, Madison, WI) were conducted as described, with zeocin used as selection
132 for the recombinant clones. Positive clone pICZser was confirmed by sequencing. The plasmid was
133 linearized with ScaI, and electroporated to *P. pastoris* GS115 strain (Invitrogen, San Diego, CA) [10],
134 and selected on YPDS/ZeocinTM (Manual, Invitrogen, San Diego, CA). After incubating at 30 °C for 2
135 to 3 days, transformants were inoculated to YPD media and grew at 30 °C. PCR characterizations were
136 performed subsequently with Taq DNA Polymerase and the following primers: 1 *AOX1*/primer:
137 GACTGGTTCCAATTGACAAGC; 2 *AOX1*/primer: GCAAATGGCATTCTGACATCC. PCR mixture
138 was heated at 94 °C for 2 min, followed by 35 cycles of incubation at 94 °C for 1 min, 55 °C for 1 min
139 and 72 °C for 1 min, and a final extension at 72 °C for 7 min. The 0.516 bp PCR amplicons were
140 visualized on 2 % agarose gel.

141 The colonies were inoculated to 25 ml BMGH media (Manual, Invitrogen, San Diego, CA) for
142 growth at 28-30 °C with shaking at 250 to 300 rpm, reaching OD₆₀₀ to 2-6 (approximately 16 to 18 h).
143 Cells were harvested at 1,500-3,000g for 5min, and supernatant was discarded. Cells were then
144 resuspended in 100-200ml BMMH to allow OD₆₀₀ to be 1.0. The cells were cultured at 28-30 °C with
145 shaking at 250 to 300 rpm in 1 L flask with 2 layers of cheesecloth.

146 100 % methanol was added every 24 h to allow the final concentration to be 0.5 %. After 7 days,
147 supernatant was collected after precipitation at 13,000g for 1 min and stored at -80 °C.

148

149 Purification of Expression products via Ethanol Precipitation

150 30 ml of absolute ethanol was mixed with 10 ml of supernatant from the induction culture, and
151 incubated at room temperature for 30 min. Centrifugation at 10,600g was performed at 4 °C for 10 min,
152 and supernatant was discarded. Precipitates were added with 80 % ethanol, and spun at 10,600g at 4 °C
153 for 7 min, and supernatant was discarded. The precipitates were air dried, and dissolved in 4 ml 10 mM
154 Tris-Cl (pH7.0).

155

156 Extraction of Expression Products with Organic Solvents

157 300 μ l purified products using ethanol were mixed well with 300 μ l n-butanol, and placed at room
158 temperature for 30 min. The water phase was transferred, and mixed well with 300 μ l isoamyl alcohol,
159 and the above steps were repeated. Extraction with ethyl acetate was then performed. The water phase
160 was subjected to ES-MS. 50 μ l of the above products was mixed with 1 μ l thrombin and incubated for
161 1 min 30 s. It was then mixed with 50 μ l fibrinogen (5 mg/ml). The formation of insoluble fibrin
162 precipitates was then visualized. 10 mM Tris-Cl (pH 7.0) buffer was used as another negative control
163 and treated likewise.

164

165 α -Mannosidase treatments of induction products and synthesis of mannosylated serine

166 Two milliliter culture of yeast INVSc1 (Invitrogen, Carlsbad, California) transformants was centrifuged
167 at 10,600 g after 105 h induction, and the supernatant was ethanol precipitated as above. The
168 precipitates were dissolved in 1.2 ml sterile water. Fifty three microliters were mixed with 6 μ l 200 mM
169 10X NaAc buffer (pH 5.5) [13] and 1 μ l 7.5mU/ μ l α -Mannosidase from *Canavalia ensiformis*
170 (Sigma-Aldrich, St. Louis, MO). After incubating for 20 min at room temperature (about 25 °C), 0.5
171 μ l 0.5 U/ μ l thrombin was added and mixed well, followed by addition 50 μ l of 5 mg/ml fibrinogen.

172 Two milliliter culture of yeast INVSc1 transformants was centrifuged at 10,600 g after 7 d
173 induction, and the supernatant was ethanol precipitated as above. The precipitates were dissolved in
174 180 μ l sterile water. Fifty four microliters of above were mixed with 6 μ l 200 mM NaAc buffer (pH
175 5.5), either incubated at 60 °C or boiled for partial or complete hydrolysis [14-18]. Three microliter 7.5
176 mU/ μ l α -Mannosidase was then added. After incubating for 20 min at room temperature (about 25 °C),
177 0.5 μ l 0.5 U/ μ l thrombin was added and mixed well, followed by addition 50 μ l of 5 mg/ml fibrinogen.

178 Serine solution (7.6 mg/ml) and mannose solution (7.4 mg/ml) were mixed with 20 μ l 200 mM
179 10X NaAc buffer (pH 5.5), and 3 μ l 7.5 mU/ μ l α -Mannosidase was then added followed by incubation
180 at 30 °C for 8 h. 0.5 μ l 0.5 U/ μ l thrombin was subsequently added and mixed well, followed by
181 addition 50 μ l of 5 mg/ml fibrinogen.

182 Statistic significances among means of various treatments were determined using Tukey test under
183 grouped factors in the one-way analysis of variance (ANOVA) of SPSS 17.0. All figures were
184 prepared using Adobe Photoshop CS4 and Microsoft Excel.

185

186

187 Results and Discussion

188 The C-terminal dodecapeptide of Hirudin has been partially randomized, attached downstream to the
189 Kex2 cleavage site of *S. cerevisiae* α -factor secretion signal peptide, and expressed in yeast as
190 chimerical peptides for secretion into the culture media. Two other cassettes have also been processed
191 likewise. The supernatant was subsequently tested for anti-thrombin activities (Fig. 2). DNA
192 sequencing indicated that the single putative positive clone L11 from oligo 3 experiment had TCCTGA
193 immediately downstream to the Kex2 cleavage site, encoding a single serine residue followed by a stop
194 codon (Fig. 3). A re-transformation of the positive clone plated on selection plate is shown (Fig. 4).
195 Subsequent experiment indicated a linear relationship between anti-thrombin activities and the volume
196 of the supernatant (Fig. 5). Ninhydrin assay showed a high content of amino acid in the supernatant of
197 the putative transformant, whereas the control transformant with the insert free vector was virtually
198 blank despite the presence of trace amount of amino acid supplements in the media (Fig. 6). Paper
199 Chromatography of the supernatant of the positive clone exhibited a R_f of 0.012, whereas the serine
200 control showed a R_f of 0.083 (Fig. 7). The supernatant of *P. pastoris* transformant showed high amino
201 acid content in Ninhydrin test (Fig. 8). After ethanol precipitation, resuspension of the positive No. 4
202 transformant equivalent to 125 μ l fermentation supernatant, displayed insoluble fibrin precipitates 28
203 minutes after the anti-thrombin assay started, whereas the control showed precipitates 3 minutes after
204 the clock began. After extraction with organic solvents, the positive clone showed inhibition time of 3
205 and half hours, whereas the control transformant and negative control (Tris-Cl (pH 7.0) buffer) treated
206 likewise began to show precipitates 15 minutes after the clock started. Electrospray Mass Spectrometry
207 showed distinct peak at 430.5 Daltons when the expression products of the *P. pastoris* transformant

were examined (Fig. 9), suggesting that the molecule may be dimannosylated serine, as yeast possesses O-glycosylation at serine residue.

Supernatant of *S. cerevisiae* cultures had a pH of about 3.0 after induction, and low pH was moderately inhibitory to thrombin. Therefore, 50 mM Tris-HCl (pH 7.5) was used to prepare thrombin in the initial screening to partly neutralize the pH, as well as the use of 0.25 unit of thrombin in each assay to cancel off some inhibitory effects. Subsequent assays with *S. cerevisiae* cultures were all performed on samples of pH 7.0 adjusted with 1 M Tris-HCl (pH 7.5).

α -Mannosidase treatments increased activities of the product from the L11 positive clone, but not from control (Fig. 10; $p < 0.001$), possibly through trimming of long glycosidic chain [19-20]. Moreover, after hydrolysis of glycosidic chains by boiling in acidic condition, further trimming with the enzyme removed anti-thrombin activity ($p = 0.001$). Synthesis of mannosyl serine via the reverse reaction of the α -Mannosidase showed modest anti-thrombin activities ($p = 0.099$). Synthesis reactions performed with serine or mannose alone in the presence of α -Mannosidase only yielded coagulation free time of 5 to 25 seconds (data not shown).

Future Perspective

Current peptide synthesis technology cannot produce dimannosylated serine, and expression in *P. pastoris* did not show significant enhancement in the yield of this product over *S. cerevisiae*. However, it remains an attractive candidate for drug development given its potent activities. The randomization of peptides is a valuable strategy for generating large number of variants, yet their expression has not been straight forward. If a protein fragment is to be randomized, its terminals should be devoid of stressful peptides to confer less strain to the expression host. This is particularly true for highly virulent viral proteins and peptides [7, 21]. Residues generally enriched in terminals of natural proteins are best to be present in the ends of designed chimerical proteins and peptides. Since thrombosis is most common in winter and at seasonal transitions, it may suggest a link between blood clotting and energy metabolism.

Conclusion

In summary, the cost effective randomization approach increased the repertoire of peptides which could be screened as drug leads. The production of dimannosylated serine in yeast may suggest that the molecule passed a preliminary safety test in eukaryotes. The glycosylated serine may be a good candidate for future pursuit of a novel antithrombotic drug. The anti-thrombin assay can be automated and adopted for large-scale screening.

Funding

This work was supported by the Guangdong Science and Technology Program (2016B020204001, 2008B020100001), Science and Technology Planning Project of Guangzhou City (201510010158), Guangzhou Science and Technology Program (201804010328), Guangdong Natural Science Foundation (S2011010004264), Open Fund of MOE Key Laboratory of Aquatic Product Safety, Foreign Expert Program at Sun Yat-sen University, Open Fund of Laboratory (20160215) and Key Project Budget at Sun Yat-sen University, and The National Natural Science Foundation of China (30370799, J1310025) to Q.L.

References

- [1] Froemel D, Fitzsimons SJ, Frank J et al (2013) A review of thrombosis and antithrombotic therapy in microvascular surgery. *Eur Surg Res* 50(1):32-43
- [2] Linkins LA, Dans AL, Moores LK et al (2012) Treatment and prevention of heparin-induced thrombocytopenia: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. *CHEST J* 141(2_suppl):e495S-e530S
- [3] Chapin JC and Hajjar KA (2014) Fibrinolysis and the control of blood coagulation. *Blood Rev* DOI: 10.1016/j.blre.2014.09.003
- [4] Bompiani KM, Lohrmann JL, Pitoc GA et al (2014) Probing the Coagulation Pathway with Aptamers Identifies Combinations that Synergistically Inhibit Blood Clot Formation. *Chem Biol* 21(8):935-44
- [5] Kelly AB, Maraganore J M, Bourdon P, Hansont SR, Harkert AL (1992) Antithrombotic effects of synthetic peptides targeting various functional domains of thrombin. *Proc Natl Acad Sci* 89(13):6040-6044
- [6] Sambrook J, Fritsch E F and Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
- [7] Yu W, Yang X, Gao Y et al (2012) Batch isolation of novel sequences targeting regions of rapid viral variations. *Indian J Anim Sci* 82 (7):665–670
- [8] He R, Cui L, Cao Y et al (2013) A Combinatorial Yeast Overlay Method for the Isolation of Antibacterial Oligopeptides. *Proc Natl Acad Sci, India, Sect B Biol Sci* DOI 10.1007/s40011-013-0257-8
- [9] Wang S, Luo Y, Yi X et al (2007) A highly efficient and highly reliable protocol for transformation of *Escherichia coli* by electroporation. *J Rapid Meth Aut Mic* 15(3):253-258
- [10] Xu Z, Zhang Y, Wang Z et al (2010) A highly efficient protocol for transformation of *Saccharomyces cerevisiae* and *Pichia pastoris* by electroporation. *J Sun Yat-sen Univ* 49(3):98-101
- [11] He R, Luo X, Wang Q et al (2005) Rapid and efficient generation of PCR templates from *Escherichia coli*, *Saccharomyces cerevisiae* and *Oryza sativa* using a microwave and by boiling. *J Rapid Meth Aut Mic* 13(1):19-28
- [12] Burton RB, Zaffaroni A, Keutmann EH (1951) Paper chromatography of steroids II. Corticosteroids and related compounds. *J Biol Chem* 188(2):763-771
- [13] Schröder S, Matthes F, Hyden P et al (2010) Site-specific analysis of N-linked oligosaccharides of recombinant lysosomal arylsulfatase A produced in different cell lines. *Glycobiol* 20(2):248-59
- [14] Kasaai MR, Arul J and Charlet G (2013) Fragmentation of Chitosan by Acids. *Sci World J* 2013:508540
- [15] Marzo M, Gervasini A and Carniti P (2012) Hydrolysis of disaccharides over solid acid catalysts under green conditions. *Carbohydr Res* 347(1):23-31
- [16] Tiboni M, Grzybowski A, Baldo GR et al (2014) Thermopressurized diluted phosphoric acid pretreatment of ligno(hemi)cellulose to make free sugars and nutraceutical oligosaccharides. *J Ind Microbiol Biotechnol* 41(6):957-64
- [17] Deng W, Liu M, Zhang Q, Tan X, Wang Y (2010) Acid-catalysed direct transformation of cellulose

296 into methyl glucosides in methanol at moderate temperatures. Chem Commun (Camb)
 297 46(15):2668-2670

298 [18] Hu S, Smith TJ, Lou W, Zong M (2014) Efficient Hydrolysis of Cellulose over a Novel
 299 Sucralose-Derived Solid Acid with Cellulose-Binding and Catalytic Sites. J Agric Food Chem
 300 62(8):1905-11

301 [19] Lommel M, Bagnat M and Strahl S (2004) Aberrant Processing of the WSC Family and Mid2p
 302 Cell Surface Sensors Results in Cell Death of *Saccharomyces cerevisiae* O-Mannosylation Mutant
 303 s Mol Cell Biol 24(1):46-57

304 [20] Gemmill, T. R., and R. B. Trimble. 1999. Overview of N- and O-linked oligosaccharide structures
 305 found in various yeast species. Biochim Biophys Acta 1426:227–237

306 [21] Fu M, Zhang X, Lai X et al (2013) Generation of sequence variants via accelerated molecular
 307 evolution methods. Recent Pat DNA Gene Seq 7(2):144-156
 308

309

310 **Table 1** List of primers and oligos

311

oligo or primer	sequence
1	GCGAATTCAAAAATGAGATTTCTTCAA
2	CGTCTAGATCA(N) ₃ ACT(N)₉ATC(N)₉ATG <i>TCTTTCTCGAGAGATAC</i>
3 [#]	CGTCTAGATCACA AAATATTCTTCTGG(N)₃TTCTTCAAAATC(N)₆TCTTT <i>TCTCGAGAGATAC</i>
4	CGTCTAGATCA(N) ₉ ACATTCTCTTTTCTCGAGAGATAC
5	CCTCTATACTTTAACGTCAAGGAGAAAAAACCCCGGATCG□
6	GGCGTGAATGTAAGCGTGACATAACTAATTACATGATGCG□
7	CTGAAGCTGTCATCGGTTAC
(sequencing) *	
8	GCCATTTTCCAACAGCAC
(sequencing) *	

312 Italicised: coding sequences of *S. cerevisiae* α -factor secretion signal peptide

313 Bold letters: randomized coding sequences for hirudin peptides and other peptides

314 [#] oligo encoding the randomized Hirudin peptide

315 * sequencing primers located on the coding sequence of *S. cerevisiae* α -factor secretion signal
316 peptide

317

318

319

320

321 **Fig. 1** Chimeric DNA fragments encoding *S. cerevisiae* α -factor secretion signal and oligopeptides
 322 were generated from PCR amplifications using oligos 2 (lane 2), 3 (lane 3) and 4 (lane 4) respectively.
 323 Lane 5 was a negative control and DL2000 (Takara, Dalian, China) was used as molecular marker

324

Fig. 2 Anti-thrombin assay: (a) Assay with 30 μ l supernatant from insert free vector control clone (left) and putative positive clone (right) without pH adjustment; (b) left to right: control with 50 mM Tris-HCl, 5 μ g/ml serine, 50 μ g/ml Serine; putative positive clone; all with adjusted pH of 7.0

Fig. 3 Sequencing chromatogram of the single putative positive clone L11: insert is shown in arrow

333

334 **Fig. 4** A re-transformation of the positive clone L11 into yeast strain INVSc1 was plated on Ura⁻
335 selection plate

336

337

338 **Fig. 5** Anti-thrombin activities versus volume of supernatant of the positive transformant (square) and
 339 control (triangle) after adjusting pH to 7.0

340

341

342

343 **Fig. 6** Ninhydrin Amino Acid Indicator assay. left to right: 30 μ l supernatant of control with insert
 344 free vector , 30 μ l supernatant from putative positive clone; 5 μ g/ml serine

345

346

347

348

349 **Fig. 7** Paper chromatography of the supernatant from induced expression. Left: serine standard; right:
350 the supernatant of the positive clone

351

352

353

354
355 **Fig. 8** Ninhydrin Amino Acid Indicator assay. Left: supernatant of *Pichia* transformant; right:
356 supernatant of control transformant with insert free vector

357

358

359

360

Fig. 9 Electrospray Mass Spectrometry of samples purified with organic solvents. a: distinct peak at 430.5 in the expression products of the *P. pastoris* transformant; b: expression products of the control transformant with insert free vector

367

368 **Fig. 10** α -Mannosidase treatments. a: it increased activities of the products from the L11 positive clone;
369 b: group 1: L11 products were mixed with NaAc buffer and incubated at 60 °C for 2 min without
370 subsequent addition of α -Mannosidase; group 2: L11 products treated likewise were incubated at 60 °C
371 for 6 min without subsequent addition of α -Mannosidase; group 3: L11 products treated likewise were
372 incubated at 60 °C for 25 min without subsequent addition of α -Mannosidase; group 4: L11 products
373 treated likewise were boiled for 10 min without subsequent addition of α -Mannosidase; group 5: L11
374 products treated likewise were incubated at 60 °C for 25 min with subsequent addition of
375 α -Mannosidase; group 6: L11 products treated likewise were boiled for 10 min with subsequent
376 addition of α -Mannosidase; c: Synthesis of mannosyl serine via the reverse reaction of the
377 α -Mannosidase; group 1: 90 μ l serine solution plus 90 μ l mannose solution; group 2: 45 μ l serine
378 solution plus 45 μ l mannose solution; group 3: 18 μ l serine solution plus 18 μ l mannose solution; group
379 4: 9 μ l serine solution plus 9 μ l mannose solution; group 5: reactions without serine and mannose. Each
380 group was repeated in triplicate and one standard deviation is shown
381