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Design of a Novel Plasminogen Activator Based on the Structure of Hirudin

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Abstract Using a phage library, seven peptide sequences with high affinity to human microplasminogen were obtained. Caseinolytic assay indicated that only the synthesized peptide P07 had slight fibrinolytic activity. To enhance its plasminogen activation ability, peptide P07 was fused into loop 32–35 of hirudin. *In vitro* assay demonstrated that this hirudin-like fusion protein can activate human plasminogen and retain the function of thrombin inhibition. Fusing the sequence "SPDASRL" into hirudin generated a plasminogen activation activity 100 times higher than peptide P07 in chromogenic and radial caseinolytic assay. This significant functional improvement might originate from a more specific active structure due to the hirudin scaffold.

Key words plasminogen activator; hirudin; fusion protein; thrombolytic agent; anticoagulant

Activation of plasminogen (Plgn; EC 3.4.21.7) is a key event in the fibrinolytic system that results in the dissolution of blood clots in a fibrin-dependent manner, and also promotes cell migration and tissue remodelling. The zymogen activation of Plgn results in the serine protease plasmin (Plm), which consists of five kringle domains at the amino-terminus and a serine protease domain in the carboxyl-terminus [1]. Human microplasminogen (μPlgn) is a single polypeptide of 261 residues from the carboxyl-terminal portion of native Plgn [2] and still retains the fibrinolytic function of the latter. However, like Plgn, μPlgn is the inactive precursor of the fibrinolytic enzyme microplasmin, and it can be activated by tissue-specific plasminogen activator (t-PA), vampire bat t-PA, urokinase, and two bacterial protein co-factors, streptokinase (SK) and staphylokinase (SAK) [3–5].

SK and SAK are not enzymes themselves, but form 1:1 stoichiometric complexes with Plgn and Plm, which acquire a remarkable specificity and efficiency to activate Plgn. After complex formation the specificity switches, whereas plasmin shows a preference for "extended" substrates like fibrin(ogen), the SK-Plgn and SAK-Plm complexes have activities against "narrower" substrates, such as the activation loop of Plgn [5,6]. This binding-activation mechanism of co-factors SK and SAK provides clues for new plasminogen activator (PA) design.

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Using a phage displayed random peptide library, we screened out a specific peptide with high affinity to μPlgn. However, amidolytic assay showed no PA activity for the synthesized peptide, whereas the M13 strain having the same epitope does have this ability. To restrict the conformation of this peptide, we constructed a fusion protein by inserting this sequence into loop 32–35 of hirudin. In chromogenic assay, the fusion protein (designated 8067) demonstrated PA activity, which was further proved by radial caseinolytic assay.

Materials and Methods

Materials

Bacterial strains of *Escherichia coli* DH5α and ER2738, wild-type M13 phage, yeast strain of *Pichia pastoris* and pPIC9K were stored in our laboratory. All the restriction endonucleases, T4 DNA ligase and *Pfu* polymerase were purchased from New England Biolabs (Ipswich, USA). IPTG, X-gal, tetracycline and primers were obtained from Sangon Biotech (Shanghai, China). Recombinant SAK [7], chromogenic substrate S-2390 and Plgn were prepared in our laboratory. Sephacryl S-100 and Lys-Sepharose were obtained from Pharmacia Biotech (Uppsala, Sweden). Peptides were synthesized by GL Biochem (Shanghai,

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China). All peptides were purified with HPLC to more than 90% purity. Disulfide bonds after oxidation, together with the free amino and acid group at the N- and C-termini, were verified with mass spectrometry. For example, peptide P07 was prepared with a purity of 92.2%. The sequence of P07 is GGSACSPDASRLCGGSAE, with several flanking residues at both the N- and C-termini to increase its stability and solubility. These flanking residues were derived from the Ph.D-C7C peptide library (New England Biolabs). Human Plgn was isolated from fresh frozen plasma using Lys-Sepharose affinity chromatography following the established procedures [8,9]. All other reagents were of analytic purity.

Cloning, expression and purification of μPlgn and fusion protein

The structural gene of μPlgn was retrieved from the genomic DNA of *Homo sapiens* by polymerase chain reaction amplification and cloned into yeast expression vector pPIC9K [10]. The full-length gene of hirudin-like fusion protein (8067) was synthesized (Sangon Biotech) then cloned into pPIC9K (**Fig. 1**). Both μPlgn and hirudin-derived fusion protein were expressed in *P. pastoris*. The supernatant of yeast fermentation was precipitated using 40% ammonium sulfate solution and loaded onto a Sephacryl S-100 column using a low velocity of flow (0.5 ml/min, 0.02 M phosphate buffer, pH 7.8) to avoid pigment contamination, then purified by ion exchange chromatography (QFF) (**Fig. 2**). Mass spectrometry verified the molecular weight of 8067 as 7286 Da. Purified proteins were lyophilized and stored at –20 ºC.

Panning using epitope mapping phage display

The 7 mer random peptide library (Ph.D-C7C peptide library kit) was purchased from New England Biolabs. Using the purified μPlgn as the target protein, the panning process was carried out according to the manual (**Table 1**).

Radial caseinolytic assay

Functional activity of fusion protein was also estimated by radial caseinolytic assay. Petri dishes containing 1.2% agarose, 1% skim milk and 10 μg/ml Plgn were prepared [11]. On this solidified agarose plate, wells of equal diameter were bored and an equal quantity of protein was added and kept at 37 ºC for 6–7 h. The diameter of the halo around the well was measured to check the fibrinolytic activity.

Amidolytic assay

Amidolytic activity assay was carried out with small

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1   ATG GTT GTT TAC ACT GAC TGT ACT GAA TCT GGT CAA AAC TTG TGC
1   Met Val Val Tyr Thr Asp Cys Thr Glu Ser Gly Gln Asn Leu Cys
46  TTG TGC GAA GGT TCC AAC GTT TGC GGT CAA GGT AAC AAA TGC ATC
16  Leu Cys Glu Gly Ser Asn Val Cys Gly Gln Gly Asn Lys Cys Ile
91  TTA GGT TCT CCA GAT GCT TCT AGA TTG AAA AAC CAA TGT GTT ACT
31  Leu Gly Ser Pro Asp Ala Ser Arg Leu Lys Asn Gln Cys Val Thr
136 GGT GAA GGT ACC CCA AAA CCA CAA TCC CAC AAC GAT GGT GAC TTC
46  Gly Glu Gly Thr Pro Lys Pro Gln Ser His Asn Asp Gly Asp Phe
181 GAA GAA ATT CCA GAA GAT TAC TTG CAA TAA
61  Glu Glu Ile Pro Glu Asp Tyr Leu Gln End
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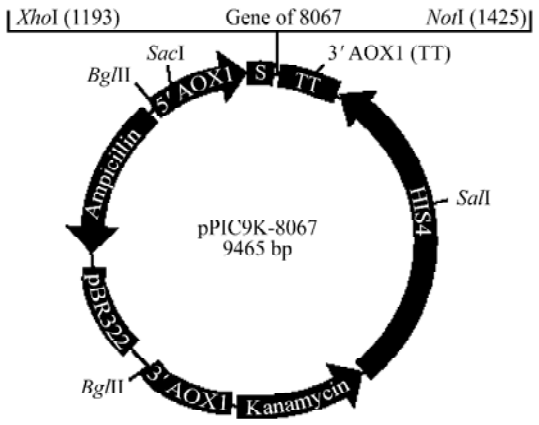

Fig. 1 Cloning strategy of hirudin-like fusion protein 8067
The gene of 8067 was synthesized and inserted at multiple cloning sites of vector pPIC9K (digested with restriction endonucleases *Xho*I and *Not*I). The expression plasmid was named pPIC9K-8067. The shadowed sequence represents the inserted P07 motif.

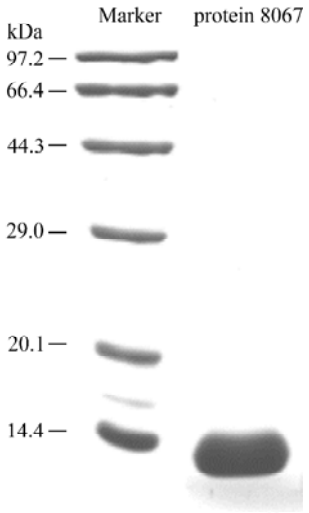

Fig. 2 Isolation of the recombinant hirudin-like fusion protein 8067 from yeast supernatant
After gel filtration and ion exchange chromatography, the target protein preparation was obtained with a purity above 90%.

Table 1 Recovery of panning steps

	Recovery of cycle I	Recovery of cycle II	Enrichment
7 mer polymer peptide library	$1.0\times10^{3}/1.0\times10^{12}=1.0\times10^{-9}$	$3.0\times10^{4}/1.0\times10^{12}=3.0\times10^{-8}$	$3.0\times10^{-8}/1.0\times10^{-9}=30$

1.0×10^{12} pfu of freshly prepared phage were used as input. Recovery was calculated as pfu input vs. pfu output. The enrichment was directly measured by comparing the number of phage recovered in second round library panning to that of first round.

modifications. Considering the relative low PA ability of the screened peptide, we used S2390 (D-Val-Phe-Lys-*p*-nitroanilide) as the chromogenic substrate instead of S2251 (D-Val-Leu-Lys-*p*-nitroanilide) [12]. S2390 has a lower K_m value than the standard S2251, and it also has a much higher V_{max}/K_m value than most other substrates of this type [13]. Plgn (1.25 μM) was incubated with sample moieties (25 μM) at 37 ºC in activation buffer (10 mM phosphate buffer, pH 7.4, 0.01% Tween 80). S2390 was added to a final concentration of 1 mM and initial velocity determinations for the enzymatic activities were performed by monitoring the hydrolysis of the tripeptidyl-*p*-nitroanilide at 405 nm. The absorbance change (ΔA_{405}) was monitored for up to 12 h using a spectrophotometer (Microplate Reader Benchmark; Bio-Rad, Hercules, USA).

Determination of specific activity of hirudin-like fusion protein by thrombin titration

Human α-thrombin was obtained from Huashan Hospital (Shanghai, China). The recombinant hirudin variant 1 (rHV1) was prepared in our laboratory and standardized with a specific activity of 10,000 ATU/mg [14]. The specific activity of hirudin-like fusion protein 8067 was determined using a modified thrombin titration method [15, 16]. Briefly, 200 μl of 0.5% fibrinogen solution was added to a polystyrene cuvette (path length=1 cm). Assay working buffer was composed of 50 mM sodium chloride, 50 mM Tris-HCl, 0.1% BSA and 0.1% PEG-6000, and was adjusted to pH 7.4. Fibrinogen was incubated at 37 ºC with 50 μl of sample for 5 min. Then 5 μl of thrombin (20 NIH units/ml) was added, mixed and incubated at 37 ºC for 1 min with 8067 or rHV1. The addition of thrombin increased turbidity because of the fibrin clot formation, and the absorbance was monitored by the microplate reader at 405 nm.

Results

Panning of phage library

After two cycles of panning, the random library was enriched with strains representing highly specific epitopes for μPlgn (**Fig. 3**). Thirty blue phage plaques were picked out for sequencing, from which seven different sequences were identified, as shown in **Fig. 3**. We named the peptides P02, P07, P08, P16, P17, P21 and P22, with the suffix number designating the serial number of the clone by which the sequence was identified. Among the 30 picked blue plaques, three had the same sequence as P07; six clones had different sequences as P02, P08, P16, P17, P21 and P22; five clones had sequences partly unreadable; and the remaining 16 plaques were identified as wild-type M13 phage (vanishingly small levels of contaminating environmental wild-type phage has a growth advantage over the library phage). These seven peptides were subsequently synthesized for function assay.

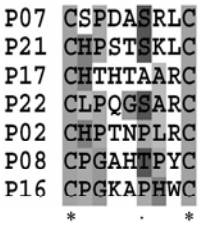

Fig. 3 Sequence alignment of seven peptides screened from a 7 mer random peptide library

A disulfide bond formed by the lateral cysteines (carried by the library itself) was used to fix the conformation of the random sequence. "*" indicates positions which have a single, fully conserved residue; "." indicates positions of the relative weaker conserved residue; Sequences were colored on the basis of alignment consensus (if the consensus is above 50%), and the residues in each column were colored according to the consensus character assigned to that column (e.g., column 6 had a consensus of 80%) [17].

Radial caseinolytic assay

The seven synthesized peptides were assayed. However, at a final concentration of 5 mM, only P07 showed a small lytic halo (**Fig. 4**). The hirudin-like fusion protein 8067 showed little fibrinolytic activity at 10–20 μM, whereas 50 μM of 8067 led to a clearly visible lytic circle (**Fig. 5**). The pattern of caseinolytic assay indicated concentration-

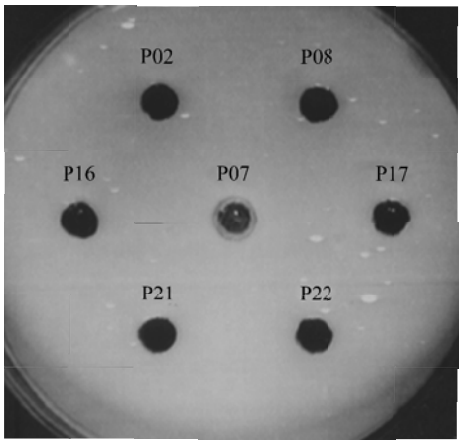

Fig. 4 Radial caseinolytic assay of seven synthesized peptides screened out from a 7 mer random peptide library

Of the 5 mM of peptides tested, only P07 (central hole) showed a small lytic circle.

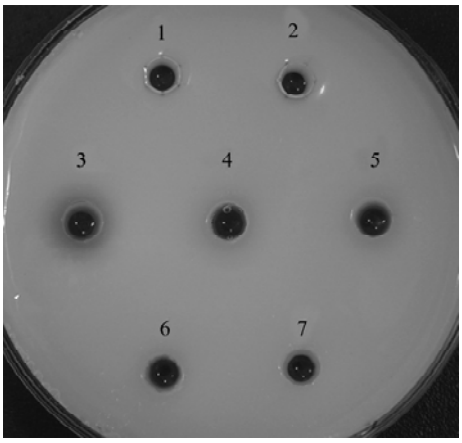

Fig. 5 Radial caseinolytic assay of fusion protein 8067 at different concentrations

1, phosphate-buffered saline (control); 2, hirudin variant 1 (rHV1; 50 μM); 3, 8067 (50 μM); 4, 8067 (20 μM); 5, 8067 (10 μM); 6, 8067 (5 μM); 7, 8067 (1 μM). Sample 3 (8067 at 50 μM) produced a visible lytic halo within 1 h. This fibrin plate assay indicated the dose-dependent fibrinolytic ability of 8067.

dependent activity of protein 8067 in the fibrinolytic process.

Amidolytic activity assay

To examine the Plgn activation properties of hirudin-like fusion proteins, the pattern of Plgn activation by protein 8067 was compared with that of SAK, a specific and robust activator for μPlgn. Protein 8067 activated Plgn into Plm very slowly, to approximately 80% of Plgn activation in approximately 12 h. Activation of Plgn in the presence of 8067 occurred progressively with a prolonged lag phase followed by an exponential increase in Plgn activation. The synthesized peptide P07 showed only marginal (approximately 8%) PA activity. In contrast, the catalytic amount of SAK (5 nM) induced rapid activation of Plgn to Plm, resulting in more than 95% of Plgn activation within 6 min. Hirudin demonstrated no specific PA ability compared with the control (PBS buffer) (**Fig. 6**). PBS control and rHV1 showed an almost identical noise pattern. These results suggested that the ability of 8067 to activate Plgn is much lower than SAK but significantly higher than P07.

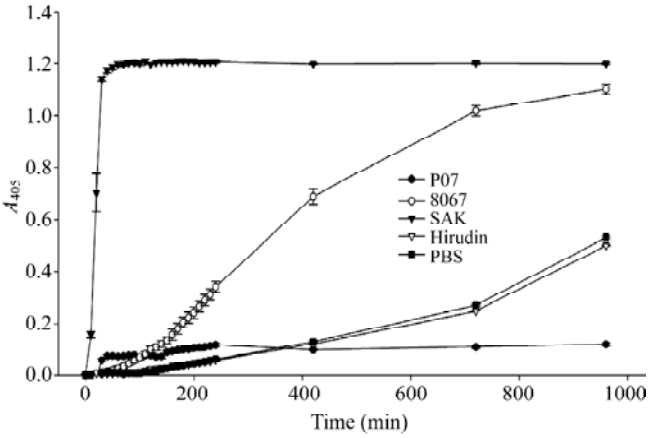

Fig. 6 Measurement of plasminogen (Plgn) activation ability of the hirudin-like fusion protein 8067

Plgn activation ability of 8067 was measured by chromogenic assay. Human Plgn (1.25 μM) was incubated with native 8067 (25 μM), P07 (5 mM), hirudin variant 1 (rHV1; 25 μM), native staphylokinase (SAK; 5 nM) and phosphate-buffered saline (PBS control; 0.05 M) in the presence of 1 mM chromogenic substrate S2390 at 37 ºC in 0.01 M phosphate buffer, pH 7.4, containing 0.01% Tween 80. The rate of plasmin generation was monitored at 405 nm at different time intervals.

Inhibition of thrombin by the hirudin-like fusion protein 8067

To provide evidence that the fusion protein 8067 binds to thrombin and is able to inhibit the activity of thrombin, we carried out the titration assay of anti-thrombin activity (**Fig. 7**). Protein 8067 and rHV1 at concentrations of 500 ng/ml and 100 ng/ml, respectively, were sufficient to completely inhibit the clotting activity of thrombin. Our results showed that the inhibition of thrombin catalytic activity by 8067 (2000 ATU/mg) was impaired markedly compared to that of rHV1 (10,000 ATU/mg).

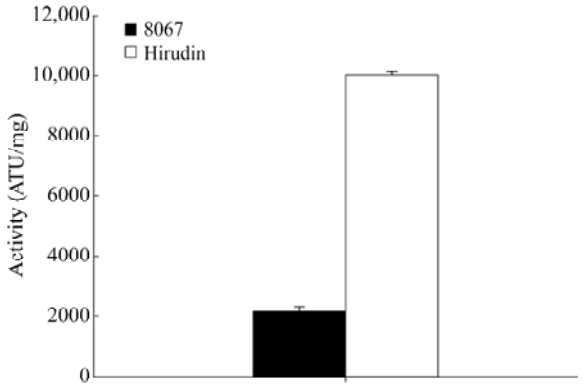

Fig. 7 Inhibition of thrombin by the hirudin-like fusion protein 8067

Standard sample of hirudin variant 1 (rHV1) had a specific activity of thrombin inhibition of 10,000 ATU/mg; the anti-thrombin ability of 8067 was reduced to 20% of rHV1. However, in light of hirudin's potency, several ten nano-molars (about 70 nM) of 8067 is sufficient to completely inhibit the clotting activity of thrombin. The thrombin titration assay indicated 8067 is still an effective anticoagulant.

Discussion

The fibrinolysis mechanism is mainly responsible for the maintenance of the potency of blood vessels during the elimination of intravascular clots. Plasminogen activators play a key role in fibrinolysis. Current clinically approved thrombolytic agents have significant drawbacks, including re-occlusion and bleeding complications. Recently, attempts have been made to obtain hybrid proteins on the basis of known PA such as t-PA, SAK and thrombin inhibitors like hirudin [18–24]. They consist of hybrid fusion proteins from active compounds, or fragments which are necessary for hemostasis. However, simple incorporation of functional domains into one molecule might raise problems such as difficulties in gene engineering and expression, incorrect protein folding and sophisticated interaction between different original fragments.

In this work we take advantage of peptide mimics to simplify the structure of chimera molecules. The goal is to obtain effective thrombolytic and antithrombotic protein and simplify the way the agent is given, to restore vessel potency.

To obtain active peptide mimics of PA, we used a random peptide library that was conformationally restricted by a disulfide bond (formed by two cysteines at both termini of the random sequence region). We had previously screened a linear peptide library, which led to more unified sequences, but had less potential to improve its PA activity. Cyclic peptide has a more steady structure and possibly favors its interaction with target molecules. As the PA activity of the primary peptide P07 was quite poor, we selected hirudin as a scaffold for further molecular design.

Hirudin is a classic anticoagulant and very robust. Both fragments derived from its C-terminal tail and N-terminal core have inhibition abilities to thrombin [25,26]. Loop 32–35 of rHV1 is relatively distant from the active region, as mentioned above, making it an ideal site for engineering. In addition, the neighboring β-strands are tightly strained, enabling this loop a typical β-turn motif [27], which is similar to the architecture of the cyclic peptide P07. With these factors in mind, we introduced the sequence "SPDASRL" into loop 32–35 of rHV1 by replacing mutation. The disulfide bond of P07 was substituted by the native structure of rHV1 for motif configuration (**Fig. 8**).

Fusion of "SPDASRL" into rHV1 generated a PA activity 100 times higher than P07 in chromogenic and radial caseinolytic assays. This significant functional improvement might originate from the more specific active structure due to the rHV1 scaffold. Although the fusion protein has a high affinity motif for Plgn displayed at the molecular surface, protein 8067 is believed to be a non-enzymatic PA, as our research originated from the binding-activation theory. A mechanism is proposed for this enhancement that involves the rendezvous and local concentration of Plgn. By complex formation with trace Plm, the Plm-8067 complex activates Plgn and initiates the enzyme-catalyzed reactions. This hypothesis is based on the Plgn activation

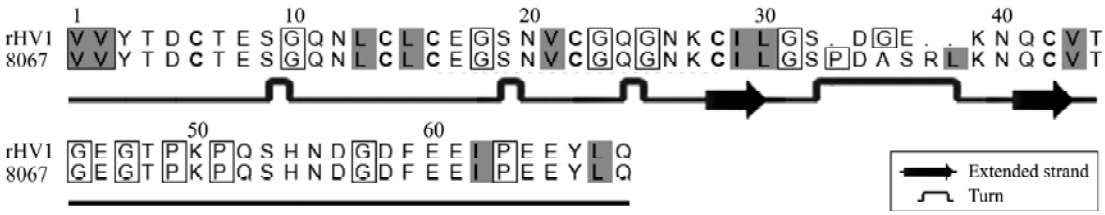

Fig. 8 Sequence alignment of hirudin variant 1 (rHV1) and hirudin-like fusion protein 8067

The P07 motif was fused into loop 32–35 of rHV1.

mechanism of SAK [28,29], which acts as a co-factor in the fibrinolytic process. More evidence is needed to confirm the mechanism of Plgn activation by 8067.

Anti-thrombin activity of 8067 was reduced remarkably compared with rHV1, suggesting insertion of the sequence "SPDASRL" in loop 32–35 does impair the interaction between hirudin and thrombin. The specific activity of 8067 was approximately 20% of rHV1. In spite of this drawback, 8067 remains an effective anticoagulant due to its potent parent form.

In conclusion, a functional epitope screen targeting μPlgn provided clues for developing a peptide agonist of Plgn. Our finding revealed a new PA or co-activator, although the mechanism needs further research. By grafting the peptide in hirudin, we developed a new agent with evident thrombolytic and anticoagulant properties. Ongoing studies on the fusion protein will provide further details about its functional mechanism and therapeutic potential.

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