

Synthesis of RGDFPASS-containing Cystine Peptides and Isolation of RGD-recognizing Receptor in Sand Dollar Embryo by the Peptide-Affinity Chromatography

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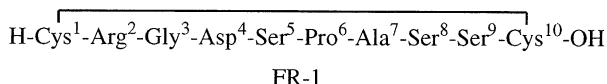
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RGDFPASS-containing cystine decapeptides were synthesized by the solution method. These peptides strongly inhibited platelet aggregation. N-Acetyl and C-methoxy peptide ([Phe⁵]-FR-1-AM) having amphiphilic structure exhibited especially high activity as a platelet aggregation inhibitor. A 240 kDa RGD-recognizing receptor was obtained from a sand dollar embryo by RGDFPASS-containing peptide-affinity chromatography.

The Arg-Gly-Asp (RGD)-containing peptides inhibit tumor progression,¹ platelet aggregation,² and sand dollar embryo genesis.³ Tripeptide RGD is essential for recognizing cell surface receptor.⁴ We attempted to specify the secondary structure of RGDFPASS sequence of fibronectin (FN), a cell adhesion protein, and synthesized RGDFPASS-containing cystine peptides, FR-1 and FR-1 analogs.^{5a-c}



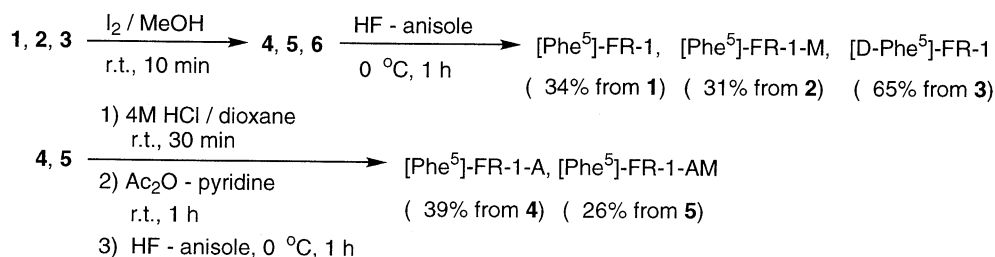
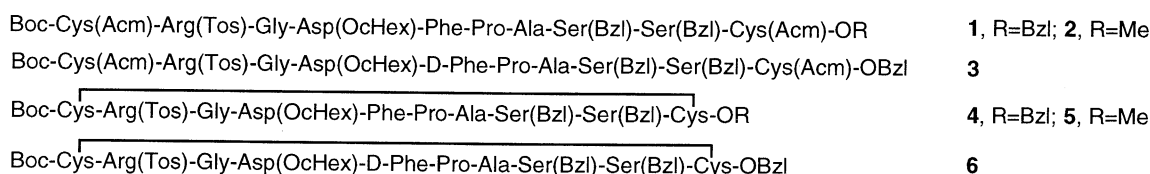
From our previous studies,^{5c} we expect that the inhibitory activity of [Phe⁵]-FR-1 for platelet aggregation would be high, while that of [D-Phe⁵]-FR-1 would be low. In order to investigate the relationship between the biological activity and the hydrophobicity of [Phe⁵]-FR-1, we placed Ac and/or MeO at the N- and/or C-termini in the [Phe⁵]-FR-1. We describe the synthesis and biological activity of [Phe⁵]-FR-1 and its derivatives. We also discuss the relationship between amphiphilic structure and biological activity of RGDFPASS-containing cystine peptide. In addition, we successfully isolated RGD-recognizing receptor in the sand dollar embryo.

Decapeptides **1**, **2**, and **3** were synthesized by the solution method in the same manner as with FR-1.^{5a} The FABMS data of **1**, **2**, and **3** were as follows, 1790 (M+H)⁺, 1714 (M+H)⁺, and

1790 (M+H)⁺, respectively. The formation of an intramolecular disulfide bond was achieved by I₂ oxidation in high dilution (1×10⁻³ M) for 10 min at room temperature (Scheme 1). The cyclization yields of **4**, **5**, and **6** were 75, 55, and 87%, respectively. Cyclic peptides **4**, **5**, and **6** were confirmed by FABMS: 1646 (M+H)⁺, 1592 (M+Na)⁺, and 1646 (M+H)⁺, respectively. Protected peptides **4**, **5**, and **6** were treated with liq. HF (1 h, 0 °C) to obtain [Phe⁵]-FR-1, [Phe⁵]-FR-1-M, and [D-Phe⁵]-FR-1, respectively (Scheme 1). On the other hand, cyclic peptides **4** and **5** were treated with 4M HCl/dioxane (30 min, room temperature) for deprotection of the Boc group. These free N-terminal peptides were treated with Ac₂O and pyridine (1 h, room temperature) and were treated with liq. HF (1 h, 0 °C) for deprotection (Scheme 1). The final products⁶ were purified by HPLC. Peptide **6** was prepared in a remarkably high yield (87%). The high yield reflects that the side chains of D-Phe⁵ and Pro⁶ do not interact with each other and the turn structure of [D-Phe⁵]-FR-1 is rigid.

RGD-containing peptides bind to fibrinogen receptor on the platelet surface and inhibit the platelet aggregation.⁷ Using human platelet rich plasma, the IC₅₀ of FR-1, [Phe⁵]-FR-1, [Phe⁵]-FR-1-A (A: N-terminal acetyl), [Phe⁵]-FR-1-M (M: C-terminal methyl ester), [Phe⁵]-FR-1-AM, and [D-Phe⁵]-FR-1 were found to be 7.6, 2.5, 0.74, 1.8, 0.23, and 515 μM, respectively. The binding potency of [Phe⁵]-FR-1 to the fibrinogen receptor was stronger than those of FR-1 and [D-Phe⁵]-FR-1. These results show that the side chains' repulsion between AA⁵ and Pro⁶ (i+1 and i+2 positions in the turn) is important for binding to the receptor. Hydrophobic [Phe⁵]-FR-1-AM exhibited the highest activity as a platelet aggregation inhibitor in our cyclic peptides.

Previously, we found that dansyl-labeled FR-1 specifically



Scheme 1.

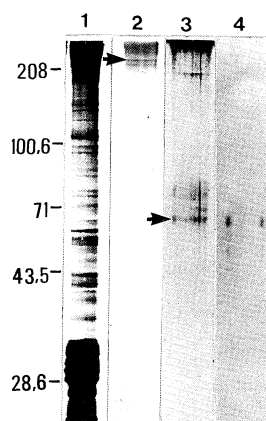
bound to the basal surface of the ectoderm and to the cytoplasm of primary mesenchyme cell in embryo of the sand dollar, *Clypeaster japonicus*.⁸ This indicates that RGD-recognizing receptor (FR-1 receptor) is present in the sand dollar embryo.

We attempted to isolate this receptor in the sand dollar embryo. An affinity matrix was prepared by coupling between activated CH-Sepharose 4B (Pharmacia Inc.) and [Phe⁵]-FR-1 by the method described by Izzo and Gantt.⁹ The solution of the sand dollar embryo lysate was loaded to the peptide-affinity column for isolation of FR-1 receptor. Specific elution of FR-1 receptor was performed by RGDS peptide solution.

Despite the presence of numerous protein bands separated from mesenchyme blastula lysate (Figure 1 lane 1), FR-1-affinity column has successfully separated a single band at 240 kDa region in 10% SDS-PAGE gel under non-reducing condition (Figure 1 lane 2) and 57 kDa region under reducing condition (treated with 2-mercaptoethanol) (Figure 1 lane 3). This suggests that FR-1 receptor is a tetrameric protein. Lane 4 in figure 1 shows that FR-1 receptor preserved its binding potency after affinity purification. On the other hand, FR-1 receptor was not obtained efficiently by the use of affinity chromatography with [D-Phe⁵]-FR-1. This result indicates that the side chains' repulsion between AA⁵ and AA⁶ in cyclic decapeptide is also important for binding to FR-1 receptor.

The results of the inhibitory activity suggest that the more hydrophobicity the cyclic peptide had, the higher its affinity was to the fibrinogen receptor on the platelet surface. [Phe⁵]-FR-1-AM had hydrophilic groups (the side chains of Ser, Asp, and Arg) and hydrophobic groups (the side chains of Pro and Phe, and C-terminal Me and N-terminal Ac). We predicted that this amphiphilic structure is important for binding to fibrinogen receptor on the cell surface.

Figure 1. Affinity purification of FR-1 receptor. Silver stained 10% SDS-PAGE gel analysis of whole embryo lysate (lane 1), affinity-purified FR-1 receptor under non-reducing condition (lane 2 arrow), and reducing condition (lane 3). FR-1 receptor blotted to nitrocellulose filter bound to horseradish peroxidase-conjugated FR-1. This lane was visualized by 0.5 mg/ml DAB (lane 4).



The present receptor's relative molecular mass indicates that FR-1 receptor is not integrin β subunit, a known 90-110 kDa RGDS peptide receptor.¹⁰ However, further thorough studies ought to be done to specify molecular properties of FR-1 receptor.

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References and Notes

- Abbreviations: Acn, acetamidomethyl; Boc, *t*-butoxy-carbonyl; Bzl, benzyl; cHex, cyclohexyl; DAB, 3, 3'-diaminobenzidine; Tos, *p*-toluenesulfonyl.
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 - [Phe⁵]-FR-1: FABMS *m/z* 1040 (M+H)⁺; Amino acid analysis(theoretical): Asp 1.00(1), Ser 1.78(2), Gly 0.98(1), Ala 1.00(1), (Cys)₂ 0.91(1), Phe 1.00(1), Arg 1.02(1), Pro 0.98(1); Found: C, 39.68; H, 4.42; N, 11.94%. Calcd for C₄₁H₆₁N₁₃O₁₅S₂·3TFA·3H₂O: C, 39.28; H, 4.87; N, 12.67%. [Phe⁵]-FR-1-A: FABMS *m/z* 1082 (M+H)⁺; Found: C, 41.03; H, 4.41; N, 11.94%. Calcd for C₄₃H₆₃N₁₃O₁₅S₂·3TFA·1.5H₂O: C, 40.52; H, 4.41; N, 12.54%. [Phe⁵]-FR-1-M: FABMS *m/z* 1054 (M+H)⁺; Amino acid analysis (theoretical): Asp 0.98(1), Ser 1.71(2), Gly 0.96(1), Ala 1.00(1), (Cys)₂ 0.70 (1), Phe 0.96(1), Arg 0.87(1), Pro 0.93(1); Found: C, 39.92; H, 4.56; N, 12.00%. Calcd for C₄₂H₆₃N₁₃O₁₅S₂·3TFA·3.5H₂O: C, 39.51; H, 5.04; N, 12.00%. [Phe⁵]-FR-1-AM: FABMS *m/z* 1096 (M+H)⁺; Found: C, 40.76; H, 5.49; N, 12.87%. Calcd for C₄₄H₆₅N₁₃O₁₅S₂·2TFA·5H₂O: C, 30.57; H, 4.83; N, 12.49%. [D-Phe⁵]-FR-1: FABMS *m/z* 1040 (M+H)⁺; Amino acid analysis (theoretical): Asp 1.01(1), Ser 1.85 (2), Gly 1.00(1), Ala 1.00(1), (Cys)₂ 0.68(1), Phe 1.01(1), Arg 0.97(1), Pro 0.99 (1); Found: C, 38.54; H, 4.96; N, 12.60%. Calcd for C₄₁H₆₁N₁₃O₁₅S₂·3TFA·4.5H₂O: C, 38.58; H, 5.03; N, 12.44%.
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