Polymer Bulletin 26, 363-370 (1991)

Polymer Bulletin

© Springer-Verlag 1991

Synthesis and evaluation of oligopeptide RGDS exhibiting cell-attachment activity

Yoshiaki Hirano[1], Toshio Hayashi[2], Kunio Goto[1], and Akio Nakajima[1,*]

[1]Department of Applied Chemistry, Osaka Institute of Technology, Asahi-ku, Osaka 535, Japan
[2]Research Center for Biomedical Engineering, Kyoto University, Kyoto 606, Japan

Summary

Tetrapeptide Arg-Gly-Asp-Ser (RGDS), an amino acid sequence existing in the cell-attachment domain of fibronectin, was synthesized using improved solid-phase procedure. Cell-attachment activity of the RGDS toward L-929 fibroblast cells originating in mouse epithelia was examined by measuring (a) the number of cells attached onto RGDS-immobilized polyvinyl alcohol(PVA) film, and (b) the % inhibition of cell-attachment onto polystyrene substrate from suspension of the cells in the presence of RGDS molecules. It was found that (a) a number of cells attached to the RGDS-immobilized PVA films, and (b) RGDS molecules remarkably attached to the cells, and, as a result, RGDS inhibited the cells to adhere onto the substrate.

Introduction

Non-collagenous glycoproteins existing in extracellular matrix have not attracted attention until recently, because of the lack of structural elucidation. Along with the progress of molecular biology and gene engineering in recent years, the primary structures of cell-adhesive proteins(1), such as fibronectin(2), vitronectin(3), and laminin(4), have been examined, and biological functionalities of the proteins now became capable to be pursued in terms of their molecular structures.

In particular, fibronectin, first discovered in blood plasma by Morrison(5) (1948) has extensively been investigated(6-13) in these 10 years. Fibronectin is known as a cell-adhesive glycoprotein, and causes adhesion, spreading, and multiplication of most cells. Pierschbacher and co-workers (9-13) have promoted detailed researches on the functionalities of domain IV, which contains the cell-binding site, of fibronectin molecule, and suggested that amino acid sequence Arg-Gly-Asp-Ser (RGDS) locating at near the C-terminal of the domain IV may play an important role as the cell recognition determinant. Recently, Humphries(14) reported that a pentapeptide Gly-Arg-Gly-Asp-Ser (GRGDS) inhibits cancer metastasis to lung.

The aim of this paper is to synthesize the tetrapeptide RGDS using improved solid-phase procedure, and to elucidate the cell-attachment activity of the synthetic peptide by means of two methods: (a) cell-attachment to RGDS-immobilized polyvinyl alcohol (PVA) film, and (b) inhibition of cell-attachment to substrate (culture dish) from the suspension of cells in the presence of RGDS molecules. The cell tested is

*To whom offprint requests should be sent

L-929 fibroblast cell originating in mouse epithelia.

Experimental

Materials

t-Butoxycarbonyl-glycine (Boc-Gly), t-butoxycarbonyl-L-aspartic acid-β-benzyl ester (Boc-Asp(OBzl)), and t-butoxycarbonyl-O-benzyl-L-serine (Boc-Ser(Bzl)) were purchased from Peptide Institute, Inc., and t-butoxycarbonyl-L-arginine hydrochloride (Boc-Arg-OH·HCl·H_2O) was purchased from Kokusan Chemical Works, Ltd. Dicyclohexylcarbodiimide (DCC) used as a dehydration agent was purchased from Peptide Institute, Inc., and N,N'-diisopropylcarbodiimide (DIC) was purchased from Aldrich. N,N'-diisopropylethylamine (DIEA) used as a neutralization agent, triethylamine (TEA), and hydroxybenzotriazole (HOBt) were purchased from Aldrich, Katayama Chemicals, and Kokusan Chemical Works, respectively. Amino methylated-polystyrene resin and 4-(bromomethyl) phenylacetic acid phenylester ($BrCH_2$-⌬-CH_2COOCH_2-CO-⌬), both used for Pam resin preparation, were purchased from Peptide Institute, Inc., and Applied Biosystems, respectively. Trifluoromethanesulfonic acid (TFMSA) (Kanto Chemicals) and trifluoroacetic acid (TFA) (Peptide Institute, Inc.) were used as deblocking agents. Ninhydrin (Kishida Chemicals) was used as a coloring agent.

Procedure of RGDS synthesis

Scheme of synthesis is shown in Figure 1. First, t-butoxycarbonyl-O-benzyl-serine-4-(oxymethyl)phenylacetoaminomethyl-resin (Boc-Ser(Bzl)-$COOCH_2$-Pam-Resin) was prepared by the procedure proposed by Michell(15). To which, serine, aspartic acid, glycine, and arginine units, each carrying blocking residues, were successively bonded to respective precursor.

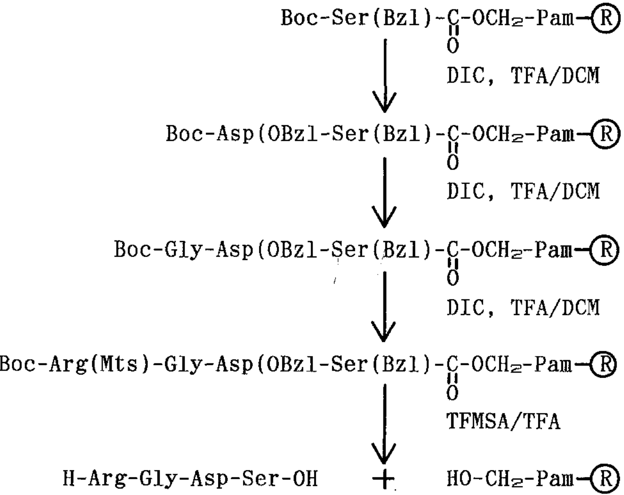


Figure 1 Scheme of synthesis of H-Arg-Gly-Asp-Ser-OH by solid-phase procedure.

Regarding the Arg unit, by taking into consideration the deblocking reaction, t-butoxycarbonyl-N^G-mesitylene-2-sulfonyl-L-arginine (Boc-Arg(Mts)-OH) was synthesized after Yajima(16) by the following procedures: Under reduced temperature, cooled down with ice and NaCl, mesitylene-2-sulfonyl chloride (67.9g, 0.3mol) dissolved in acetone (300ml) was added dropwise to a stirred solution of Boc-Arg-OH·HCl·H_2O (50.0g, 0.15mol) dissolved in a mixture of 4N NaOH (175ml, 0.7mol) and acetone (750ml), for 40 min. After stirring for 30 min in the ice-bath followed by stirring at room temperature, the solvent was evaporated, and the residue was dissolved in water. The aqueous phase was washed with ethyl acetate, and then acidified with citric acid. The resulting precipitate was extracted with ethyl acetate, and the extract was washed with ice-chilled 0.2N HCl and NaCl-saturated water, dried over Na_2SO_4 and then evaporated. An oily residue obtained was dissolved in methanol, and cyclohexylamine (CHA: 17.3ml, 0.15mol) was added. The resulting solid was recrystallized from acetonitrile. Boc-Arg(Mts)-OH·CHA salt thus obtained was dissolved in methanol, and then 0.1N HCl was added. The solvent was evaporated, and the residue was extracted with ethyl acetate. The organic phase was washed with 10% citric acid and NaCl-saturated water, dried over Na_2SO_4, and evaporated. In this manner, Boc-Arg(Mts)-OH was obtained.

Boc-Ser(Bzl)-$COOCH_2$-Pam-Resin was synthesized by reacting amino-methylated-polystyrene resin (NH_2-CH_2-⌬-Ⓡ) with Boc-O-benzyl-seryl-4-(oxymethyl)phenylacetic acid (Boc-Ser(Bzl)-$COOCH_2$-⌬-CH_2COOH), which was synthesized from Boc-Ser(Bzl)-COO^- and $BrCH_2$-⌬-CH_2COOCH_2-CO-⌬ .

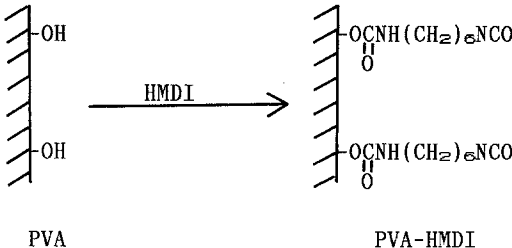
In the course of synthetic route of H-Arg-Gly-Asp-Ser-OH, shown in Figure 1, deblocking of Boc group from Boc-Ser(Bzl)-$COOCH_2$-Pam-Resin was performed by 60% TFA in CH_2Cl_2(DCM). The product was washed with DCM and DMF, neutralized with 10% DIEA in DCM, and again washed with DCM and DMF. The deblocked product, NH_2-Ser(Bzl)-$COOCH_2$-Pam-Resin, was coupled with Boc-Asp(OBzl) by using DIC with HOBt, dehydration-coupling agent. In this step, the Kaiser test was carried out by means of ninhydrin to confirm complete coupling of amino acid. The obtained Boc-Asp(OBzl)-Ser(Bzl)-$COOCH_2$-Pam-resin was washed with DCM and DMF. In a similar manner, Boc-Gly and Boc-Arg(Mts) were successively coupled to amino-peptidyl-OCH_2-Pam-Resin. At each coupling step, deblocking and purification procedures were carefully performed.

Finally, Boc-tetrapeptidyl-$COOCH_2$-Pam-Resin (1.0g) obtained was stirred in a mixture of thioanisole (1.76ml), 1,2-ethanedithiol (0.88ml), m-cresol (0.2ml), and TFA (11.9ml) at room temperature, and then deblocked and cleaved from the resin by TFMSA (1.33ml) in an ice-bath for 2 hr. Diethyl-ether was added to the reaction system, and then the precipitate and resin were filtered and washed with diethylether. The deblocked H-Arg-Gly-Asp-Ser-OH peptide was dissolved in a small amount of water and treated with CM-Sephadex C-25 ion-exchange chromatography (ammonium solution form, Pharmacia Fine Chemicals). The eluates were lypophilized and dried over P_2O_5 in vacuum. The sample obtained was tested by amino acid analysis.

Immobilization of tetrapeptide RGDS to polyvinyl alcohol film

The scheme of surface activation of polyvinyl alcohol (PVA) film is

shown below.



PVA films were purified in a methanol Soxhlet extraction system, and then placed in 10% hexamethylene diisocyanate (HMDI) solution in toluene, to which 0.07vol% di-n-butyl tin dilaurate was added. The solution was stirred for 1 hr in N_2 atmosphere. After the reaction was over, films were rinsed with toluene and acetone.

The activated films were dipped in 1mg/ml RGDS buffer solution and stirred for 3 hr at pH 3. The RGDS-immobilized PVA films were washed with pure water, phosphate buffer solution (PBS), and again with pure water by using ultrasonic cleaner for 10 min.

-OCNH$(CH_2)_6$NCO (C=O) → H_2N-RGDS → -OCNH$(CH_2)_6$NCNH-RGDS (C=O, C=O)

-OCNH$(CH_2)_6$NCO (C=O) → -OCNH$(CH_2)_6NH_2$ (C=O)

PVA-HMDI PVA-HMDI-RGDS

The surface composition of the immobilized PVA film was tested by means of C1s and N1s spectra measured with a Shimadzu 750 ESCA spectrometer using $MgK\alpha_{1,2}$ exciting radiation.

Cell-attachment activity test for RGDS

L-929 fibroblast cell was cultured in Eagle MEM containing antibiotics (Kanamycin, 60mg/l) and 10% fetal calf serum (FCS: M. A. Bioproducts, Maryland, USA). Primary-cultured mouse fibroblast was taken from skin of fetal mouse using trypsin and ethylenediamine tetraacetic acid disodium salt (EDTA). L-929 cells were trypsinized from culture dish, washed once in a medium containing 10% FCS, and once serum-free medium. The cell density was adjusted to 1.76×10^5 cells/ml for cell-attachment test in both serum medium and serum-free medium. The RGDS-immobilized PVA films were stirred in 70% ethanol for overnight, and ethanol was displaced with PBS for 1 day. The test films were placed in 24-well cell culture dish (Corning, N.Y.). 1 ml cell suspensions were added to each of the polymer films placed in the wells, and kept for predetermined periods in humidified incubator conditioned to 37 ℃, 5% CO_2 and 95% air atmosphere. After incubation, the unattached cells

were removed from the film surface by washing with PBS. Adhered cells were fixed by using glutalaldehyde, stained by Giemsa, and by glycerin. The number of cells attached to the films was counted.

For cell inhibition test, the starting cell density was adjusted to 1.76×10^5 cells/ml in serum-free medium. 1 ml of cell suspension containing 2mg of RGDS peptide was placed in a 24-wells cell culture dish, and kept for predetermined periods in a humidified incubator conditioned to 37℃, 5% CO_2 and 95% air atmosphere. The number of cells attached to the culture dish was counted as a function of incubation time.

Results and Discussion

Synthesis of RGDS and immobilization of RGDS to PVA

Boc-Ser(Bzl)-$COOCH_2$-Pam-Resin obtained was hydrolyzed with 6N-HCl/ propionic acid for 24 hr, the product was assayed by amino acid analysis to determine the degree of introduction of Boc-Ser(Bzl) to the resin. It was found that Boc-Ser(Bzl) residues were introduced to 82% of amino residues on the NH_2-CH_2-⟨○⟩-Resin.

The results of amino acid analysis and of elemental analysis on H-Arg-Gly-Asp-Ser-OH (RGDS) synthesized by solid-phase procedure were given in Table 1.

Table 1. Results of analyses on RGDS ($C_{15}H_{27}N_7O_8$)

	Amino acid analysis			Chemical analysis	
	Count (nmol)	Ratio		Calc	Expr
Ser	11.6	1.00	C	41.6%	41.4%
Asp	12.3	1.06	N	6.2%	6.6%
Gly	11.1	0.96	H	22.6%	22.7%
Arg	12.7	1.09			

The results obtained from both analyses indicate that the RGDS peptide was really synthesized.

Figure 2 and 3, respectively, illustrate C1s and N1s ESCA spectra for PVA, PVA-HMDI, and PVA-HMDI-RGDS. The C1s spectrum of the RGDS-immobilized PVA exhibits a shoulder at 289-291 eV, which is characteristic to carbon atom of the amide bond. The theoretical % ratio of oxygen to carbon atom (O/C ratio) of the bulk PVA is 50%. However, as reported in our previous paper(17), experimental value for the PVA was 35.2%. This fact may suggest that about 70% of OH residues locates on the film surface, and remainders are buried in the film interior. O/C ratio of RGDS-immobilized PVA was reported as ca.36%. Regarding N/C ratio, reported values(17) for PVA and RGDS-immobilized PVA were 0 and 10.28%, respectively. N/C value for PVA-HMDI obtained in this work is 4.58%. These data clearly support that RGDS tetrapeptide molecules are really immobilized to the PVA film via HMDI as a spacer.

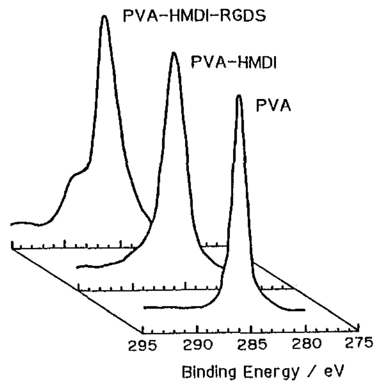


Figure 2 C1s ESCA spectra of PVA, PVA-HMDI, and PVA-HMDI-RGDS surface.

Figure 3 N1s ESCA spectra of PVA, PVA-HMDI, and PVA-HMDI-RGDS surface.

Cell-attachment activity of RGDS for L-929 cell

In Figure 4, logarithmic number of L-929 cells attached to RGDS-immobilized PVA film was plotted against the incubation time. In serum-free medium, the number of attached cells onto RGDS-immobilized PVA film was about 15 times and 10 times as many as control(PVA-HMDI), at 1 hr and 5 hr incubation, respectively. In the medium with serum, a similar behavior was observed as shown in Figure 4.

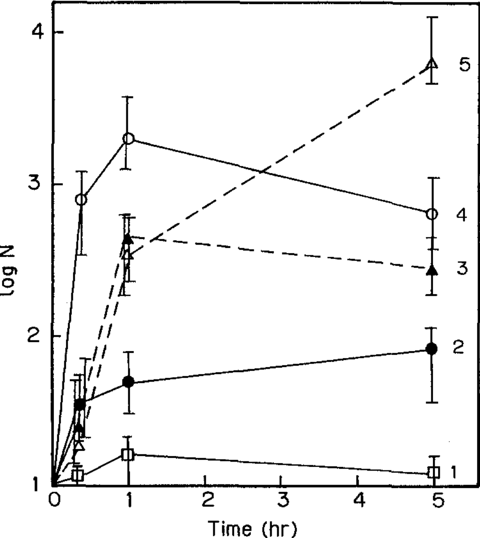


Figure 4 Logarithmic number of L-929 cells attached onto RGDS-immobilized PVA film plotted against time.
1. PVA(serum-free), 2. PVA-HMDI(serum-free),
3. PVA-HMDI(with serum), 4. PVA-HMDI-RGDS(serum-free),
5. PVA-HMDI-RGDS(with serum).

The % cell-attachment onto polystyrene dish from cell suspension including RGDS molecules was plotted against the incubation time in Figure 5. Obviously, cell-attachment is inhibited about 80% at the initial stage of the incubation time. This fact indicates that RGDS molecules bind to the fibronectin receptor of the L-929 cell, the receptor is being inhibited by the RGDS molecules, and thus the cell-attachment onto the cell culture dish is largely reduced. It was found that RGDS peptide interacts strongly with fibronectin receptor of the cell. Some specific site of protein distributing on the cell surface of L-929 is considered to act as a receptor toward the RGDS peptide as ligand.

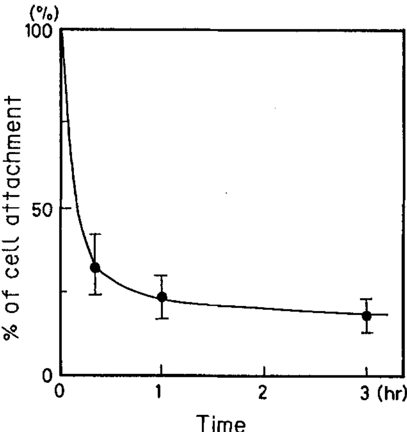


Figure 5 Inhibition effect of RGDS molecules on attachment of L-929 cell to substrate dish.

The authors wish to thank Professor H. Yajima, and Dr. S. Funakoshi, Faculty of Pharmaceutical Science, Kyoto University, for their suggestions for peptide synthesis, and Mr. H. Kobayashi, Research Center for Medical Polymer and Biomaterials, Kyoto University, for his guidance in cell-attachment experiments. Thanks are also due to Messrs. H. Yamamoto, S. Sinohara, and Y. Kando, Osaka Institute of Technology, for their assistance in the experiments. This work was supported by the Grant-in-Aid #62604018 to A. N., for Scientific Research on Priority Area, the Ministry of Education, Science, and Culture, Japan.

References

1. Yamada K M, Akiyama S K, Hasegawa T, Hasegawa E, Humphries M J, Kennedy D W, Nagata K, Urushihara H, Olden K, Chen W T (1985) Cellular Biochem 28: 79
2. Kornbliht A R, Umezawa K, Vibe-Pedersen K, Baralle F E (1985) EMBO J 4: 1755
3. Suzuki S, Oldberg A, Hayman E G, Pierschbacher M D, Ruoslahti E (1985) EMBO J 4: 2519
4. Sasaki M, Kato S, Kohno K, Martin G R, Yamada Y (1987) Proc Natl Acad

Sci USA 84: 935
5. Morrison P, Edsall J T, Miller S G (1948) J Amer Chem Soc 70: 3103
6. Suzuki S (1987) Protein Nucleic acid Enzyme 32: 1305
7. Hasegawa T, Hayashi M (1986) ibid 31: 425
8. Petersen T E, Thøgersen H C, Skorstengaard K, Vibe-Pedersen K, Sahl P, Scottrup-Jensen L, Magnusson S (1983) Proc Natl Acad Sci USA 80: 137
9. Pierschbacher M D, Ruoslahti E, (1984) Nature 309: 30
10. Pierschbacher M D, Hayman E G, Ruoslahti E (1983) Proc Natl Acad Sci USA 80: 1224
11. Yamada K M, Kennedy D W (1985) J Cellular Biochem 28: 99
12. Armant D R, Kaplan H A, Mover H, Lennarz W J (1986) Proc Natl Acad Sci USA 83: 6751
13. Pierschbacher M D, Ruoslahti E (1987) J Biol Chem 262: 17294
14. Humphries M J, Olden K, Yamada K M (1986) Science 233: 467
15. Mitchell A R, Kent S B H, Engelhard M, Merrifield R B (1987) J Org Chem 43: 2845
16. Yajima H, Takeyama M, Kanaki J, Nishimura O, Fujino M, (1978) Chem Pharm Bull 26: 3752
17. Nakajima K, Hirano Y, Iida T, Nakajima A (1990) Polymer J 22: 985

Accepted July 9, 1991 S