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Gly-His-Lys 펩타이드가 결합된 키토산과 그의 세포증식 효과에 관한 연구

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Gly-His-Lys Conjugated Chitosan and its Cell Proliferation Effects

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요 약: 새로운 Gly-His-Lys (약자로 GHK)가 결합된 키토산을 N^{α}-Fmoc 아미노산과 BOP 커플링 시약을 사용한 고상법에 의해 제조하였다. 이를 위해 키토산 마이크로 비드를 W/O 에멀젼 상분리법으로 평균 입자크기 70 마이크로미터로 얻었다. GHK 펩타이드는 순차적으로 고상법에 의해 키토산 마이크로비드 위에 커플링 하였다. 아미노산 분석을 실시한 결과 Gly, His, Lys의 비율이 1.02 : 1.13 : 0.96의 비율로 나타나 이론치와 거의 일치함을 확인할 수 있었다. GHK 펩타이드가 결합된 마이크로비드의 세포증식 효과는 MTT 분석으로 측정하였다. 측정결과 GHK 펩타이드가 결합된 키토산 마이크로비드는 대조군인 펩타이드가 결합되지 않은 키토산 마이크로비드 자체에 비해 높은 세포증식 효과를 보였다.

Abstract: Novel GHK-conjugated chitosan was prepared by the solid-phase method using N^{α}-Fmoc amino acids/BOP coupling reagent. For this purpose, the chitosan microbeads which had a mean diameter of 70 μm were prepared by the W/O emulsion-phase separation method. The GHK was successfully coupled to the chitosan microbeads by stepwise solid-phase method. The result of amino acid analysis was in good agreement with the theoretical values; $Gly_{1.02}$ $His_{1.13}$ $Lys_{0.96}$. The cell proliferation effect of the GHK-bound chitosan microbeads was measured by MTT assay. We concluded that GHK-bound chitosan microbeads gave higher cell proliferation effect than chitosan microbeads.

Keywords: *GHK-conjugated chitosan, chitosan microbeads, cell proliferation effect, solid-phase method*

1. Introduction

Chitosan is a promising biopolymer in some industrial applications and has been used as polymeric drug delivery system[1]. Since chitosan is non-toxic and biocompatible, it is one of the best polymer matrixes for pharmaceutical application[2-6]. Recently, peptides have become an increasingly important class of molecules in biochemistry, medicinal chemistry and pharmaceutical industries. Many physiologically relevant peptides function as hormones, neurotransmitters, and growth factors. Glycyl-L-histidyl-L-lysine (GHK) is one of the peptides with mitogenic activity and is probably a major growth factor in human plasma[7]. It is known to have an important role in wound healing, tissue repair, cell recognition, etc.[8-9]. The early stages of tissue repair are sustained by fibroblast proliferation, collagen deposition, angiogenesis, and subsequent reepithelialization. Chitosan itself is also known to have the wound healing properties[4]. As a result, the GHK-chitosan conjugate is expected to exhibit more enhanced cell proliferation effect. Until now, there has been few report about the synthesis of peptide-bound chitosan[10], even no report about their physiological properties. We now report about the synthesis of GHK-bound chitosan microbeads and some preliminary results of *in vitro* assay with the peptide-bound chitosan microbeads.

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2. Experimental Part

2.1. Materials and Instruments

Chitosan was of reagent grade from Tokyo Kasei Kogyo Co., Ltd. The chitosan was found to have $M_\eta = 8.2\times10^5$[11], 78% of deacetylation[12], and specific rotation $[\alpha]_D^{22}=-4.8°$(c 1.0, 1% acetic acid). The highly deacetylated chitosan was prepared according to the method proposed by Mima *et al.*[13]. Sorbitan momooleate (Span 80, HLB 4.3) was obtained from ICI. BOP, benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate was purchased from Richeliue Biotechnologies. N^ε-Boc-N^α-Fmoc-L-Lysine, N^α-Fmoc-N^{im}-Trityl-L-Histidine, Fmoc-ε-aminocaproic acid, and Fmoc-Glycine were purchased from Bachem Inc. Methylene chloride (Oriental Chem. Co., Extra pure) was purified by distilling fractionally from phosphrous pentoxide. DMF (Oriental Chem. Co., Extra pure) was distilled from ninhydrin, and stored over 4 Å molecular sieve. Triethylamine (Aldrich) was freshly distilled before use. Other reagents were all reagent grade and purified according to the standard method[14]. UV/VIS spectra were obtained with a Cecil spectrophotometer CE-5500. FT-IR spectra were recorded by FTS-40 system of Bio-Rad Laboratories.

2.2. Preparation of Chitosan Microbeads

Chitosan microbeads were prepared by a modified method of Sakai *et al.*[15]. 6.34 g of chitosan (degree of deacetylation=94%, $M_\eta = 6.8\times10^5$) was dissolved in 1% aqueous acetic acid to give 634 g of chitosan solution. The resulting solution was mixed with 460 g of toluene, 3 g of sorbitan monooleate and 1 g of n-hexanol. Thus formed W/O emulsion was stirred at 400 rpm for 1 h to stabilize the emulsion. Thereafter, the emulsion was added into 2.4 L of 12% NaOH solution with stirring at 400 rpm. After stirring for 3 h, the phase separation occurred at the interface of the W/O emulsion. Then, 2 L of ethanol was poured into the above system to harden the spherical gels. Thus formed beads were collected *via* filtration, followed by washing with water to neutrality. After successive washing with methanol and acetone, the beads were isolated by suction on a G-4 glass filter, and finally dried over P_2O_5. Particle size distribution of the microbeads was measured by using nests of U.S. standard sieves.

2.3. Determination of Surface Amine Content

The surface amine content of the chitosan microbeads was determined by a modified method of the original picric acid titration[16]. The beads were allowed to swell in methylene chloride (1×5 min), neutralized with 5% TEA in methylene chloride (2×3 min), washed with methylene chloride (3×2 min), treated with 0.1 M picric acid in methylene chloride (2×15 min). The picrate was eluted with 5% TEA in methylene chloride (5 ×5 min), finally washed with 95% ethanol (5×5 min). After dilution with 95% ethanol, the amount of picrate was measured spectrophotometrically at 358 nm. The surface amine content was determined to be 0.53 mmol/g.

2.4. Coupling of GHK on Chitosan Microbeads

2.4.1. Preparation of Fmoc-ε-aminocaproic Acid (ACA)-chitosan

The coupling of Fmoc-ε-ACA to the chitosan microbeads was performed on a shaking apparatus[17]. The chitosan microbeads (370 mg, surface amine content: 0.53 mmol/g) were washed with methylene chloride and DMF and then allowed to react with Fmoc-ε-ACA (228 mg, 0.648 mmol), BOP (287 mg, 0.65 mmol), TEA (68.7 μL, 0.57 mmol) in DMF (15 mL). The mixture was shaken for 3 h, and the coupling was repeated two times for 1 h each. The beads were washed with DMF (2×), methylene chloride (2×), iso-PrOH (1×), acetone (1×), and anhydrous ethyl ether (1×). The resulting beads were dried under vacuum. Kaiser's ninhydrin test[18]: negative. The substitution level was determined to be 0.49 mmol/g by fulvene-piperidine adduct analysis [19], IR(KBr): 3064 (aromatic C-H), 1660 (amide I), 1553 (amide II).

2.4.2. Preparation of GHK-bound Chitosan Microbeads

Fmoc-Lys(Boc) was coupled to Fmoc-ε-ACA-chitosan as follows: (1) wash Fmoc-ε-ACA-chitosan beads (280 mg), DMF; (2) deblocking, 20% piperidine in DMF (3 + 17 min); (3) wash, DMF (4×); (4) swell with 10 mL of DMF; (5) Fmoc-Lys(Boc) (388 mg, 0.83 mmol) was added into the reaction vessel and shaken for 3~5 min; (6) BOP (366 mg, 0.83 mmol) and TEA (19.2 μL) was added; (7) shake for 3 h; (8) wash, DMF (3×); (9) wash, methylene chloride (3×); (10) repeat (3)~(9), two times; (11) wash, MeOH (1×); (12) wash, acetone

(1×); (13) wash, anhydrous ethyl ether (1×); (14) dry in vacuum. Kaiser's ninhydrin test[19]: negative. IR (KBr): 3064 (aromatic C-H), 1660 (amide I), 1553 (amide II)

Fmoc-His (Trt) and Fmoc-Gly were sucessively coupled to Fmoc-Lys(Boc)-ε-ACA-chitosan with the same procedure. The resulting Fmoc-Gly-His(Trt)-Lys-ε-ACA-chitosan was deprotected as follows: (1) wash with DMF; (2) deblocking, 20% piperidine in DMF (3+17 min); (3) wash, DMF (4×); (4) swell with 15 mL of DMF; (5) Reagent K (TFA : phenol : thioanisole : H_2O : thioethanol = 82.5 : 5 : 2.5 : 2.5, v/v) was added and shaken for 1 hr; (6) wash, DMF (3×); (7) wash, methylene chloride (1×); (8) wash, acetone (1×); (9) wash, anhydrous ethyl ether (1×); (10) dry in vacuum. Pauly test[20] : positive. IR (KBr): 1660 (amide I), 1553 (amide II)

2.5. Amino Acid Analysis

10 mg of GHK-bound chitosan sample was introduced into 6 inch test tube and 1 mL of conc-HCl and propionic acid (1:1) mixture was added. The sample was freezed in a dry ice and acetone bath. The tube was connected to a well-trapped vacuum pump and evacuated. After refreezing the sample and sealed off the test tube under vacuum, the sample was hydrolyzed at 105℃ for 24 hr. After hydrolysis, the sample solution was evaporated and sent to the Life Science Laboratory at the Korea Basic Science Center (KBSC) for the amino acid analysis.

2.6. Evaluation of Cell Proliferation

2.6.1. Cell Culture

Normal human fibroblasts, isolated from the dermis of freshly obtained neonatal foreskins, were subcultured in a tissue culture flask (Falcone 3813, Becton Dickinson & Company) at subconfluent cell density in Dulbecco's Modified Medium (DMEM) supplemented 10% fetal bovine serum (FBS), 2 mM L-glutamate, 1 mM sodium pyruvate, 100 μg streptomycin sulfate/mL and 0.25 μg/mL amphotericin B (DMEM complete). Cultures were maintained at 37℃ in a 5% CO_2 (90% humidity), and were used for experiments between passage 4 and 12.

2.6.2. Cell Proliferation Assay

100 μL of 5% FBS was placed into 96 well multiplate (Falcon 3072, Becton Dickinson & Company), treated with test samples diluted in complete DMEM containing 2% FBS, and cultured for 7 days. Cell proliferation was measured by MTT assay[21,22]. The optical density at 570 nm was determined spectrophotometrically using Microplate EL 310 Autoreader (Bio-Tek Instruments). The test was also performed for the untreated controls.

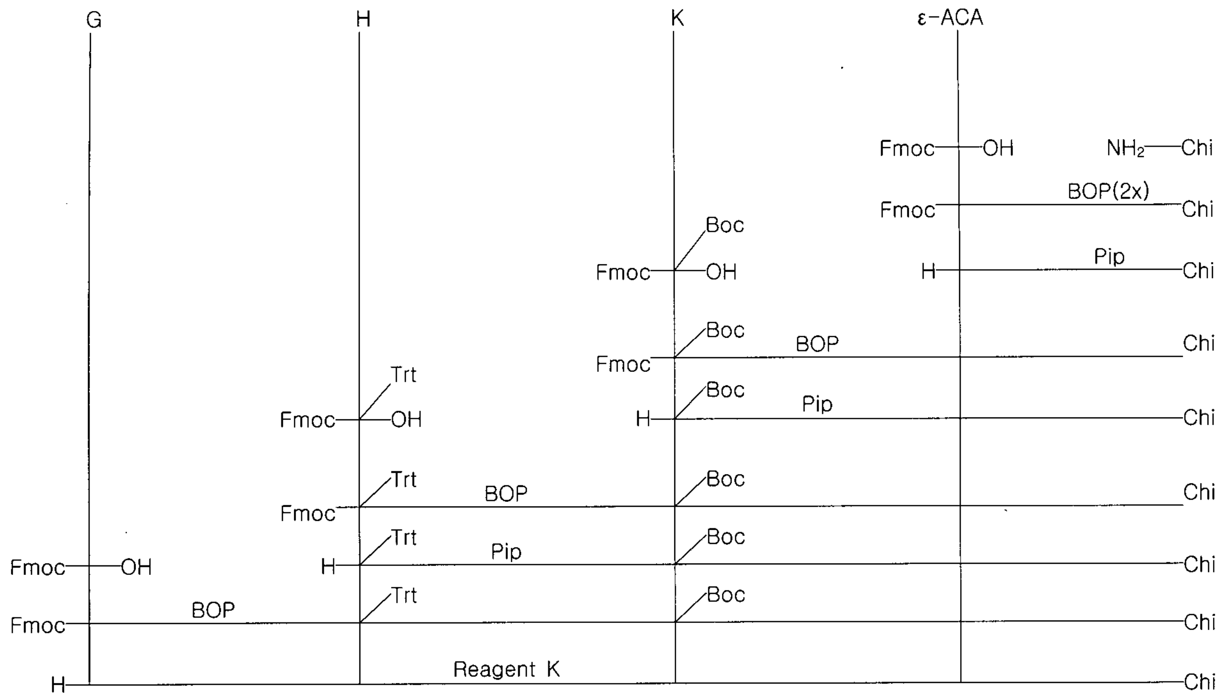
3. Results and Dscussion

3.1. Preparation and Characterization of Chitosan Microbeads

We have successfully prepared a new class of polymer support-chitosan microbeads for solid phase synthesis by using W/O emulsion-phase separation method. To make a stable W/O emulsion, we used sorbitan monooleate, W/O emulsifier and n-hexanol as a cosurfactant. We found that sorbitan monooleate (0.3 wt%) and n-hexanol (0.1 wt%) gave the most suitable microbeads (mean diameter of 70 μm) for solid-phase synthesis. Thus obtained chitosan microbeads revealed a hydrophilic character. Therefore, to determine the surface amine content of the beads properly, a modified Gisin's method should be applied [16]. The adsorption time should be elongated to complete the adsorption of picric acid to the beads. We found that 15 min was enough for complete adsorption equilibrium. The picric acid titration revealed that the surface amino content of the beads was 0.53 mmol/g, which means that the chemically available amino group of the beads was about 10% of the total amine content. We guess that most of the amine groups are buried at the inner crystalline region during the preparation process of the chitosan microbeads.

3.2. Solid-phase Synthesis of GHK-bound Chitosan Microbeads

The synthetic route to produce the GHK-chitosan conjugates was summarized in Scheme 1. All the N^{α}-Fmoc amino acids were successively coupled to the chitosan microbeads after following the general procedure of stepwise solid-phase peptide synthesis[23]. After completion of the coupling reactions, the amino acid analysis showed $Gly_{1.02}$ $His_{1.13}$ $Lys_{0.96}$.



Scheme 1. Synthetic route to GHK-ε-ACA-chitosan microbead.

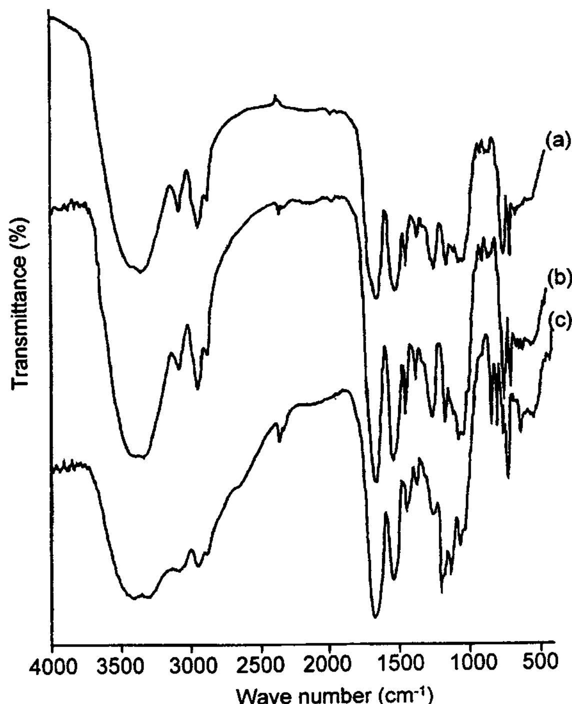


Figure 1. FT-IR spectra of various chitosan derivatives.
(a) Fmoc-Gly-His(Trt)-Lys(Boc)-ε-ACA-chitosan,
(b) Gly-His(Trt)-Lys(Boc)-ε-ACA-chitosan,
(c) Gly-His-Lys-ε-ACA-chitosan.

In the first step of synthesis, the amino groups on the chitosan microbeads were coupled with N^{α}-Fmoc-ε-aminocaproic acid by using BOP reagent[19]. The N^{α}-Fmoc protecting group was removed by treating with 20% piperidine in DMF. After appropriate washing step, the next N^{α}-Fmoc amino acids carrying orthogonal protecting groups were coupled by using BOP reagent. Each coupling step was usually brought to completion except the early stages of the couplings where repeated coupling steps were necessary, and it was judged by the Kaiser's ninhydrin test[18]. We have chosen Fmoc protective groups which can be removed by mild basic condition since the chitosan microbeads are soluble in organic acid medium. After each coupling reactions, the amount of the amino acid coupled to the beads was determined by UV/VIS spectrophotometer after deblocking of the Fmoc groups by piperidine[20]. The results showed that the degree of substitution of the amino acid on the beads was about 0.49 mmol/g, which is in good agreement with the results of picric acid analysis. FT-IR spectra of Fmoc-G-H(Trt)-K(Boc)-ε-ACA-chitosan is shown in Figure 1. The absorbances at 1660 cm^{-1} (the amide I band) and 1533 cm^{-1} (the amide II band) is strong due to the formation

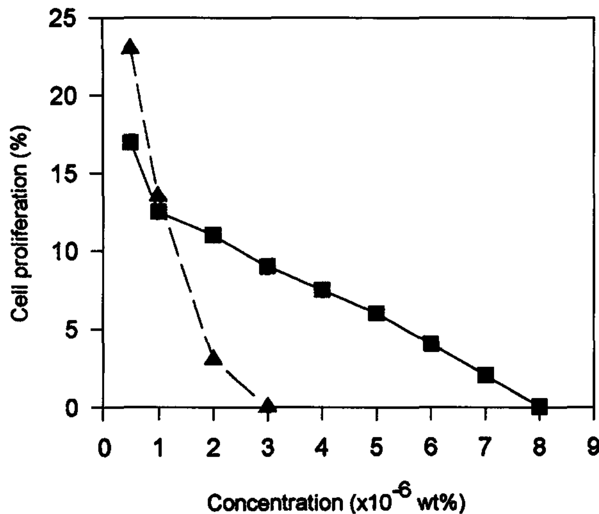


Figure 2. The results of MTT colorimetric assay.
▲: chitosan beads, ■; GHK-bound chitosan beads.

of the amide bonds. The aromatic C-H stretching band at 3064 cm^{-1} proves the existence of the trityl group at the side chain of histidine and Fmoc protecting group. After removing Fmoc protecting groups, FT-IR spectra was examined (Figure 1(b)). The protecting im-trityl group of histidine was removed by Reagent K[19,24]. The removal of the trityl group from the imidazole nucleus was also confirmed by FT-IR spectra and the appearance of red-orange color in Pauly test[20]. The absorption band due to the aromatic C-H stretching mode was nearly disappeared, as shown in Figure 1(c).

3.3. MTT Colorimetric Assay

A semiautomated MTT assay has been used to measure the cell survival and chemosensitivity[21,22]. The assay is based on the color reaction that tetrazolium ring of MTT is cleaved by the action of succinic dehydrogenese in mitochondria to give MTT formazan. So, the absorbance of MTT formazan can be correlated to the cell number. The cell proliferation (%) was calculated by the following equation:

$$\text{Cell proliferation } (\%) = \left[\frac{A_{570} \text{ (treated)}}{A_{570} \text{ (control)}} - 1 \right] \times 100$$

As shown in Figure 2, the cell proliferation with the chitosan microbeads was about 13~22% at the concentration of $0.5 \times 10^{-6} \sim 1 \times 10^{-6}$ wt%. But the effect was markedly decreased with increasing the concentration of chitosan bead because of the cytotoxicity of the beads. Above 3×10^{-6} wt%, the chitosan microbeads had little effect on cell proliferation. In contrast, at the concentration of $1 \times 10^{-6} \sim 8 \times 10^{-6}$ wt%, the GHK-bound chitosan microbeads showed higher cell proliferation than the chitosan microbeads. This result indicates that the GHK which is bound on the surface of the chitosan microbeads could suppress the cytotoxicity and improve the cell proliferation of fibroblast. We expect that the GHK-chitosan conjugate can exhibit the improved wound healing effects than chitosan itself.

4. Conclusion

Novel GHK-conjugated chitosan was prepared by the solid-phase method using N^{α}-Fmoc amino acids and BOP coupling reaction. For this purpose, the beads which had a mean diameter of 70 μm and 0.53 mmol/g surface amine content were prepared by the W/O emulsion-phase separation method. A tripeptide, GHK was coupled to the chitosan microbeads with a spacer of ε-amino-caproic acid by stepwise solid-phase method. The result of amino acid analysis of the GHK-bound chitosan microbeads was in good agreement with the theoretically values; $Gly_{1.02}$ $His_{1.13}$ $Lys_{0.96}$. The cell proliferation effect of the GHK-bound chitosan microbeads was measured by MTT assay. The results revealed that the GHK-bound chitosan microbeads showed higher cell proliferation of fibroblast than the uncoupled chitosan microbeads.

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