Bound Biotin-neutravidin Inducing Steric Hindrance Used for Controlling Bioactivity of Bradykinin Linked with Biotin

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Abstract Biotin-linked bradykinin was synthesized by solid phase peptide synthesis for development of a functionalized peptide and study on structure-relevant bioactivity in biological system. In PC12 cell system, bioactivity of the synthetic peptide was evaluated and found to be controllable in the presence of neutravidin and free biotin. The controlling mechanism had been discussed and could be ascribed to steric hindrance induced by binding of neutravidin to the linked biotin. Moreover, influence of competitive binding between the free biotin and the linked biotin to the neutravidin had also investigated into and could be employed for switching the bioactivity on and off.

Key words bradykinin, steric hindrance, bioactivity, fluorescence, biotin, neutravidin

Controlling bioactivity of a biomolecule has been drawing a great interest in scientific community for many years[1~5], and many approaches, so far, have been proposed to the bioactivity controlling. Of those, one method, forming a steric hindrance to the interaction among biomolecules by changing local and/or whole structure of one biomolecule, is widely used in the controlling. For example, photoisomerization of a photochromic group modified chemically onto a biomolecule had been attempted to control the bioactivity by many researchers[3~5]. In addition, the binding of antibody to antigen incorporated into a biomolecule was also considered to be able to form the steric hindrance[6] to the interaction and, consequently, could be utilized in the controlling. In this research, a biotin-linked bradykinin was manually synthesized with solid phase peptide synthesis (SPPS) and, in particular, controlling its bioactivity had been carried out by addition of neutravidin to solution of the synthetic peptide. The binding of neutravidin to biotin was expected to exert a big influence on the bioactivity of the biotin-linked bradykinin owing to the steric hindrance as mentioned above. Bradykinin, Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7-Phe^8-Arg^9-OH, which is formed from a large precursor polypeptide (kininogen) by the action of the enzyme, kallikrein (kininogenase)[7], and has been implicated in the pathogenesis[7,8] of inflammation, pain, asthma, and other disease. Thus, controlling bioactivity of bradykinin is of great interest in study on the structure-relevant bioactivity in biological system.

1 Material and methods

1.1 Synthesis of biotin-modified bradykinin

In SPPS, a support, Fmoc-NH-SAL Resin (0.05 g, 0.025 mmol NH_2 group) (WataNabe chemical industries, LTD), was used in peptide syntheses. The normal procedure for peptide synthesis is as follows. Deprotection of Fmoc group was carried out with 20% piperiding in N, N-dimethylformamide (DMF) for 10 minutes; Ninhydrin test was used to indicate that the protecting group was removed from the protected amino group, which was now ready for coupling of the next Fmoc-amino acid. The Fmoc-amino acid was coupled to the amino group for one hour in DMF solution containing coupling reagents: 1-hydroxybenztriazole (HOBt), N, N-diisopropylethylamine (DIEA) and benzotriazol N-oxytrisdimethylaminophosphonium hexaflurorophosphate (BOP). Ninhydrin test guaranteed completeness of the coupling. Repetition of the above steps could result in peptide with sequence as designed; Finally, cleavage of the peptide was exe-cuted with trifluoroacetic acid (TFA) containing scavengers, m-cresol and thioanisol (m-cresol: thioanisol: TFA was 2: 12: 86). The structure of the biotin-linked bradykinin is schematically represented in Figure 1(a). The mass spectrum, as shown in Figure 1(b), indicates the success in the synthesis of the peptide (calculated mass [M + H^+] = 1 286.65

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and experimental mass [M + H$^+$] = 1286.28). The synthetic peptide was purified to single peak in HPLC chart with ODS column (data not shown here).

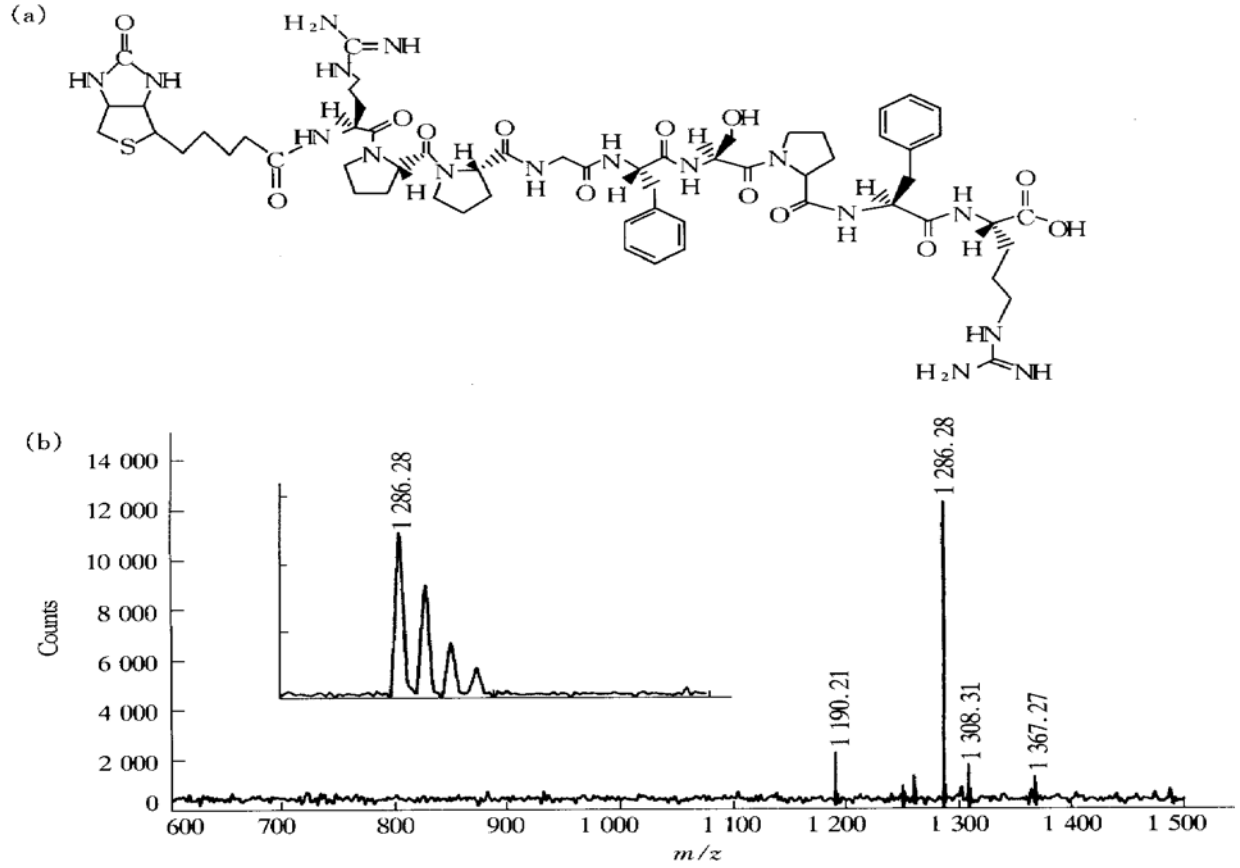

Fig. 1 Schematic diagram of structure (a), and mass spectrum (b) for the biotin-linked bradykinin: Biotin-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH

Inset shows a magnified spectrum.

1.2 Evaluation on bioactivity of biotin-bradykinin

The bioactivity of biotin-linked bradykinin was evaluated by monitoring Ca^{2+}-induced fluorescence variation under confocal microscopy (Bio-Rad). The fluorescence intensity increase was caused by binding of Ca^{2+}-indicator (Fluo-3) with released Ca^{2+} ions which were resulted from the response of PC-12 to bradykinin. Therefore, the fluorescence intensity variation could be used for evaluating the bioactivity of bradykinin in PC-12 system.

Prior to the monitoring, PC-12 cells of 1 ml with concentration of 10^5 cell/ml were put into a glass dish (Wako) and cultured in RPMI Medium 1640 (Life Technologies, Inc.) for 2~3 h at 37℃, 5% CO_2. Then Fluo-3 (Molecular Probes) dissolved in dimethylsulfoxide (DMSO) was added into the dish, resulting in final Fluo-3 concentration of 1 μmol/L. For the purpose of loading Fluo-3 into PC-12 cells, the cells in the medium containing Fluo-3 were again incubated for 15 minutes at 37℃, 5% CO_2. Next, removal of the medium by aspiration and twice washes of those PC-12 cells adhered onto bottom of the dish with 2-[[4-2-hydroxyethyl]-1-piperazinyl] ethanesulfonic acid (HEPES) buffer saline (HBS, pH 7.4), was followed by filling the dish with fresh HBS of 1 ml and putting it on the stage of confocal microscopy for the evaluation on the bioactivity of the peptides. Last, solution of synthetic bradykinin in HBS was injected into the dish to stimulate PC-12 cells. As responsive to bradykinin addition, fluorescence variation of Fluo-3 excited by argon-ion laser source at wavelength of 488 nm was recorded in time course mode under confocal microscopy.

2 Results and discussion

The biotin-linked bradykinin in HBS with final concentration of 3 nmol/L was injected into the dish containing PC12 cells in HBS, and the fluorescence variation was recorded, as shown in Figure 2(b). The result indicated that the biotin-linked bradykinin could preserve the bioactivity as native bradykinin (sigma), as shown in Figure 2(a).

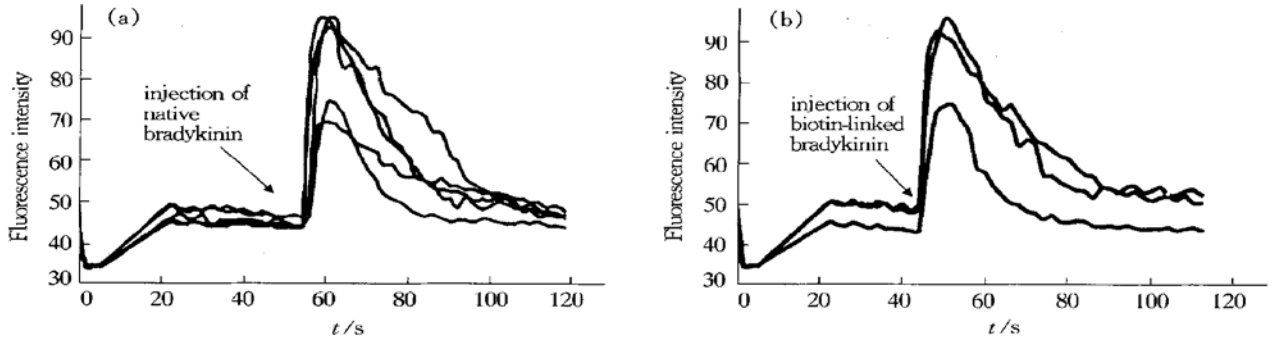

Fig. 2 Fluo-3 fluorescence variations recorded with confocal microscopy in time course mode

(a) native bradykinin (3 nmol/L); (b) biotin-linked bradykinin (3 nmol/L).

A mixed solution of the biotin-linked bradykinin with final concentration of 3 nmol/L and the neutravidin with final concentration of 15 nmol/L was prepared and injected into the fresh dish. From Figure 3 (a), no responsive peak could be seen, indicating the inhibition on the bioactivity of the biotin-linked bradykinin. An explanation to the result was given as that the binding of the linked biotin to the neutravidin hindered the interaction of bradykinin with the receptors on surface of PC12 cells and thereby inhibited the response of PC12 cells to the addition of the biotin-linked bradykinin.

In addition, another mixed solution of the biotin-linked bradykinin, the neutravidin and free biotin with final concentrations of 3 nmol/L, 15 nmol/L and 80 nmol/L respectively was prepared and injected into a fresh dish containing PC12 cells in HBS. In this case, a competitive binding between the free biotin and the linked biotin to the neutravidin had happened, and binding sites on the neutravidin were dominantly occupied by the free biotin because of its excessive amount in the solution. The result was shown in Figure 3(b).

Furthermore, executed was an investigation into the influence of the competitive binding on the bioactivity, as shown in Figure 3(c). Firstly, a mixed solution of the biotin-linked bradykinin and the neutravidin with final concentrations of 50 nmol/L and 250 nmol/L respectively was added into the dish. 15 minutes later, the free biotin with final concentration

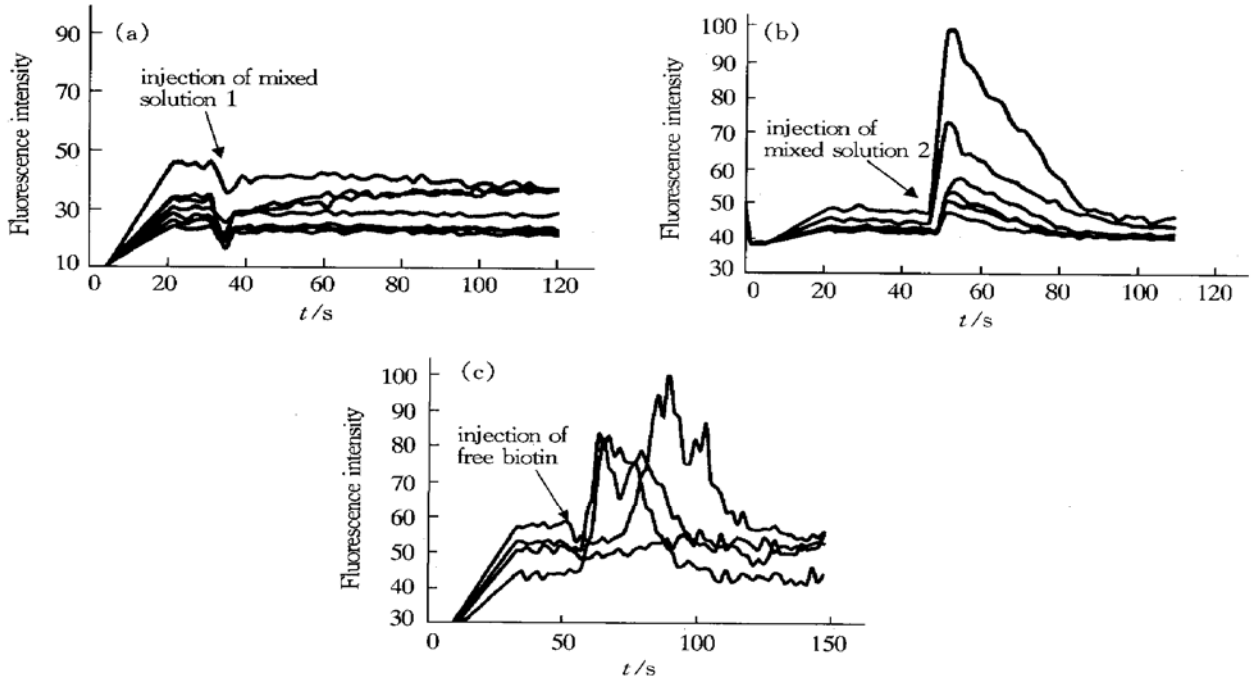

Fig. 3 Fluo-3 fluorescence variations recorded with confocal microscopy in time course mode

(a) mixed solution 1: a mixed solution of biotin-linked bradykinin (3 nmol/L) and neutravidin (15 nmol/L); (b) mixed solution 2: a mixed solution of the biotin-linked bradykinin (3 nmol/L), neutravidin (15 nmol/L) and free biotin (80 nmol/L); (c) mixed solution 3: a mixed solution of biotin-linked bradykinin (50 nmol/L) and neutravidin (250 nmol/L), 15 minutes later, addition of the free biotin (2 μmol/L). (Each curve corresponds Fluo-3 fluorescence variation in a cell. Exciting wavelength was 488 nm).

of 2 μmol/L was added into a fresh dish containing PC12 cells in HBS. The competitive binding of the free biotin to the neutravidin resulted in release of neutravidin from the linked biotin and, therefore, the freed biotin-linked bradykinin could stimulate PC12 cells, causing increase in the fluorescence.

In conclusion, successfully synthesized was the biotin-linked bradykinin without loss of its bioactivity. The steric hindrance caused by the binding of the neutravidin to the linked biotin could be exploited for controlling the bioactivity of the biotin-linked bradykinin. Furthermore, the influence of competitive binding between the free and the linked biotin to the neutravidin on the bioactivity was also examined and discussed, and could be used for switching the bioactivity on and off.

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用生物素-中性抗生物素蛋白结合导致的位阻来控制连接生物素的缓激肽生物活性

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摘要 缓激肽是一含有9个氨基酸残基的多肽，其残基序列为 Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7-Phe^8-Arg^9-OH，在激肽释放酶的作用下，从其大的前体多肽——激肽原而形成的．许多发病机理，如发炎、疼痛、哮喘等都与缓激肽有关．它能与PC12细胞表面的受体作用，引起细胞器内的钙离子释放，在共焦显微镜下，通过观察钙指示剂Fluo-3荧光增加来监测缓激肽的生物活性．在这项研究中，利用固相肽合成方法合成了连接生物素的缓激肽，Biotin-Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7-Phe^8-Arg^9-OH，通过对其生物活性的研究发现：a. 它能保持象天然的缓激肽那样的生物活性；b. 由于中性抗生物素蛋白与连接的生物素的结合引起的空间位阻阻碍它与细胞表面受体的相互作用，从而抑制了它的生物活性；c. 在有自由的生物素存在的条件下，自由生物素与连接生物素与中性抗生物素蛋白的竞争结合，能够使得与中性抗生物素蛋白结合的连接生物素的缓激肽从抗生物素蛋白上脱离，因而恢复其生物活性．因此，可利用生物素和抗生物素蛋白来控制连接生物素的缓激肽的生物活性．这对于研究生物体系中生物活性的结构相关性具有重要的意义．

关键词 缓激肽，空间位阻，生物活性，荧光，生物素，中性抗生物素蛋白

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