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A synthetic model of collagen structure taken from bovine macrophage scavenger receptor

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A putative collagen structure from macrophage scavenger receptors binds to a wide range of ligands. In order to elucidate the ligand's binding mode, this collagen structure was constructed using short peptides. This was accomplished by the reaction of a tri-bromoacetylated branched peptide with a purified unprotected 25-residue peptide, which contained Cys, 4 repeats of the triplet, Gly-Pro-Hyp, and 12 residues from the bovine macrophage scavenger receptor (residues 332 to 343). The three identical 25-residue peptides are linked at the N-terminus. CD and NMR spectra of the N-terminus cross-linked tripeptide show that it forms a collagen structure below 10°C and an extended structure at high temperature with a midpoint of 20°C.

Peptide mimetic; Triple helix; Collagen structure; Macrophage scavenger receptor

1. INTRODUCTION

The bovine macrophage scavenger receptor is known to play a key part in atherogenesis. It has two types of structures (Type I and II) and they have been found to consist of 453 and 349 amino acids, respectively, as deduced from their nucleotide sequences [1,2]. They exist in a trimerized form and the trimerization is required for their function. They possess one membrane-spanning region (26 amino acids) and their N-terminal 50 amino acids lie in the cytoplasm with their C-terminus outside. The external region of both receptors is divided into four regions; a spacer domain (32 amino acids), an α-helical coiled-coil domain (163 amino acids), a collagen-like domain (72 amino acids), and a C-terminal type-specific domain (110 for Type I and 6 amino acids for Type II). The uptake of acetyl-LDL and oxidized LDL by the scavenger receptors is initiated by their binding to the C-terminal part of the collagen-like domain. Four Lys residues at position 327, 334, 337 and 340 in the receptor have been revealed to be responsible for the substrate binding, with Lys-337 as the most crucial [3]. The receptor binds not only acetyl or oxidized-LDL but also a variety of ligands such as maleylated BSA, polyvinyl sulfate and polyinosine (poly(I)) [4]. On the other hand, LDL, BSA and poly(C) do not bind. In order to elucidate the binding mode of the receptor and its substrates, it is most suitable to prepare the collagen structure using a short peptide. Collagen structures have been previously synthesized by using short peptides, three of which were linked at the C-terminus. These peptides have been chemically synthesized from the C-terminus to the N-terminus by either stepwise or block condensation [5,6]. However, this approach may lead to certain difficulties in the isolation step, since synthetic peptides contain mixtures of truncated peptides. We report an alternative method for preparing an N-terminus cross-linked tripeptide. The collagen-like structure of the peptide was analyzed by CD and NMR spectroscopy.

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Abbreviations: LDL, low density lipoprotein; BSA, bovine serum albumin; Fmoc, 9-fluorenylmethoxycarbonyl; Hyp, hydroxyproline; tBu, *t*-butyl; tBoc, *t*-butoxycarbonyl; Trt, trityl; BrZ, 2-bromobenzyloxycarbonyl; mBHA resin, 4-methyl benzhydrylamine resin; HPLC, high pressure liquid chromatography; NMP, *N*-methylpyrrolidone; TFA, trifluoroacetic acid; TFMSA, trifluoromethanesulfonic acid; DCC, dicyclohexylcarbodiimide; HOBt, hydroxybenzotriazole.

2. MATERIALS AND METHODS

2.1. *Synthesis of a 25-residue peptide, C(GPHyp)$_4$GQKGQKGEKGS-GNH$_2$, 1*

The peptide was synthesized by standard protocols using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry on a 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin in an Applied Biosystems peptide synthesizer, Model 430A. The side chain protecting groups used are tBu for Ser and Hyp, tBoc for Lys, OtBu for Glu, and Trt for Cys. After cleavage from the resin by treatment with TFA containing 5% ethanedithiol and anisole (1:3, v/v) for 1.5 h, the crude peptide was precipitated by addition of cold ether. The precipitate was dissolved

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in aqueous acetic acid and purified by preparative reversed-phase HPLC on an Aquapore Prep 10 (C-8, 300 Å pore size, Applied Biosystems) with 0.1% TFA in a water/acetonitrile gradient. The amino acid sequence was confirmed by automated Edman degradation on an Applied Biosystems Model 477A. The purified peptide was hydrolyzed in a saturated 6 N HCl atmosphere at 150°C for 1 h using a Waters PICO-TAG Work Station, and amino acid analysis was performed on a Beckman Mode6300 amino acid analyzer. Amino acid analysis (expected value in parentheses), Cys 0.7 (1), Gly 9 (9), Pro 3.9 (4), Hyp 4.1 (4), Gln/u 3.3 (3), Lys 3.2 (3), Ser 1.1 (1). FAB mass spectra, m/z = 2331.2, $(M+H)^+$. Calcd. for $C_{97}H_{155}N_{31}O_{34}S_1$ = 2331.1.

2.2. *Synthesis of a tri-N-bromoacetylated branched peptide, 2*

Using 4-methyl benzhydrylamine (MBHA) resin, Tyr, Gly, Lys, and Lys were condensed stepwise in the peptide synthesizer using Fmoc chemistry. The protecting groups of the side chain were BrZ for Tyr and tBoc for Lys. After the tetrapeptide was synthesized, the Fmoc group was removed by 20% piperidine in NMP for 20 min and the tBoc group was removed by 50% TFA in CH_2Cl_2 for 20 min followed by neutralization with 5% diisopropylethylamine in NMP. Fmoc-β-Ala and bromoacetic acid were then condensed stepwise to the peptide resin in the peptide synthesizer. Completion of each of the reactions was monitored by the ninhydrin test [7]. The peptide resin was treated with a mixture of TFA, *m*-cresol and TFMSA (9:1:1, v/v) for 1 h at room temperature. Subsequently, water was added to the mixture with cooling and the mixture was washed with *n*-pentane twice followed by ether twice. The aqueous solution was applied to reversed phase HPLC (Vydac C-4, 300 Å, 5 μm, 4.6 × 150 mm) and the fractions containing the desired branched peptide were pooled and then lyophilized. FAB mass spectra m/z = 1067.0, $(M+H)^+$. Calcd. for $C_{38}H_{57}N_{10}O_{11}Br_3$ = 1067.2

2.3. *Synthesis of an N-terminus cross linked tripeptide, 3*

The 25-residue peptide, **1**, (5 μmol) and the branched peptide, **2**, (1.4 μmol) were mixed in 0.1 M $NaHCO_3$ and 3 M GdnHCl solution (2 ml). After 6 h at room temperature, pH of the mixture was adjusted to approximately 2 by addition of TFA and the mixture was applied to reversed-phase HPLC. The branched peptide was eluted by a linear gradient of CH_3CN (10–25%) containing 0.1% TFA at a flow rate of 1 ml/min. The fractions containing the desired product were pooled and lyophilized. Amino acid analysis (expected value in parentheses), *S*-carboxymethyl cystein 3.2 (3), Gly 28 (28), Pro 11.9 (12), Hyp 12.1 (12), Gln/u 9.5 (9), Lys 11.5 (11), Ser.3.1 (3), Tyr 1.5 (1), β-Ala 3.2 (3). Ion-spray mass spectrum was measured on a triple quadruple mass spectrometer (SCIEX AP III, Canada) equipped with a standard atmospheric pressure ionization source (Fig. 3).

2.4. *Circular dichroism (CD) measurement*

CD spectra were recorded at 4, 37 and 85°C on a J-600 spectropolarimeter (Japan Spectroscope) with a 1-mm path-length cuvette. The peptide was dissolved in 5 mM sodium phosphate at pH 7, at a concentration of 10 μM. A thermal transition curve was obtained by monitoring $[\theta]_{223}$ as a function of temperature with a 2-mm path-length cuvette. The peptide concentration was 100 μM, and the temperature was increased at a rate of 1°C/min.

2.5. *Nuclear magnetic resonance (NMR) measurement*

Proton NMR spectra were recorded on a Bruker AM600 spectrometer. The peptide was dissolved in 5 mM sodium phosphate buffer at a concentration of 5 mg/ml (about 0.6 mM) at pH 7.2. Chemical shifts are expressed in ppm to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS).

3. RESULTS AND DISCUSSION

The collagen structure exhibits scavenger receptor ligand binding. Due to its broad binding specificity, it is valuable to construct the essential collagen structure to elucidate its manner of binding to substrates. When evaluating the predicted structure of the scavenger receptor, it is possible to predict that an α-helical coiled-coil domain is likely to fold first and the comformation propagates to the collagen domain [1,2]. Type II scavenger receptor, in particular, in the C-terminal type specific domain, has only 6 amino acids (Ser-Met-Gln-Arg-Pro-Gly) which are not likely to form any secondary structures. The C-terminus domain is also not likely to be a nucleation center in forming the tertiary structure of the scavenger receptor. The six amino acid residues are not involved in the substrate binding [3]. To date, a number of chemical syntheses of collagen have been reported. The collagen is usually synthesized from the C- to N-terminus of the tri-branched peptide. However, although a peptide covalently cross-linked at the N-terminus has also been reported, this peptide contains only Pro, Ala and Gly, which have no functional groups in the side chains [8].

In order to generate the collagen structure of the scavenger receptor more precisely, the N-terminus, rather than the C-terminus, of the peptide should be covalently cross linked to form the collagen structure. Recent advances allow peptides with chain lengths of approximately 30 to be synthesized with high purity by conventional automated peptide synthesizers. Our strategy, therefore, is based on an initial purification of the unprotected peptide, followed by cross-linking at the N-terminus to induce trimerization. Selective reaction of an *N*-bromoacetyl derivative and sulfhydryl group of Cys is reported to produce the peptide-protein conju-

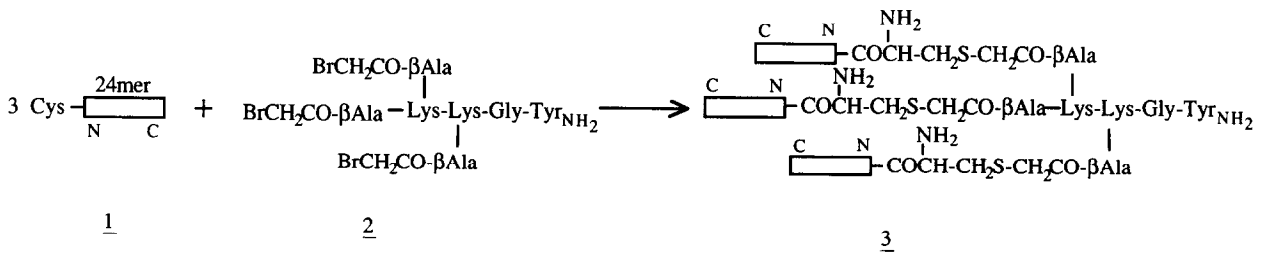

Fig. 1. Synthetic protocol of the N-terminus crosslinked tripeptide as a collagen model. The open box indicates the 24-residue peptide, $(GPHyp)_4GQKGQKGEKGSG_{NH_2}$.

gate or cyclic peptide [9]. Here, three identical peptide chains with Cys at the N-terminus are coupled to a tri-*N*-bromoacetylated branched peptide, as shown in Fig. 1.

We first synthesized a 25-residue peptide, **1**. It contains 12 amino acids (Gly-Gln-Lys-Gly-Gln-Lys-Gly-Glu-Lys-Gly-Ser-Gly) corresponding to the C-terminus (332 to 343) of the predicted collagen structure of the bovine scavenger receptor and four repeats of a tripeptide, Gly-Pro-Hyp, and Cys, at the N-terminus. Tripeptide, Gly-Pro-Hyp, has a strong tendency to form a collagen structure and therefore can stabilize the collagen structure [10]. Cys is used to covalently cross-link at the N-terminus. Though the 12 amino acid residues may be insufficient for biological assay since one Lys at 327 is omitted, our initial approach is to synthesize the collagen structure to evaluate the utility of synthetic method. The peptide, **1**, was synthesized using an automated peptide synthesizer and purified by reversed phase HPLC. The structure was confirmed by amino acid analysis, peptide sequence analysis and FAB-mass analysis.

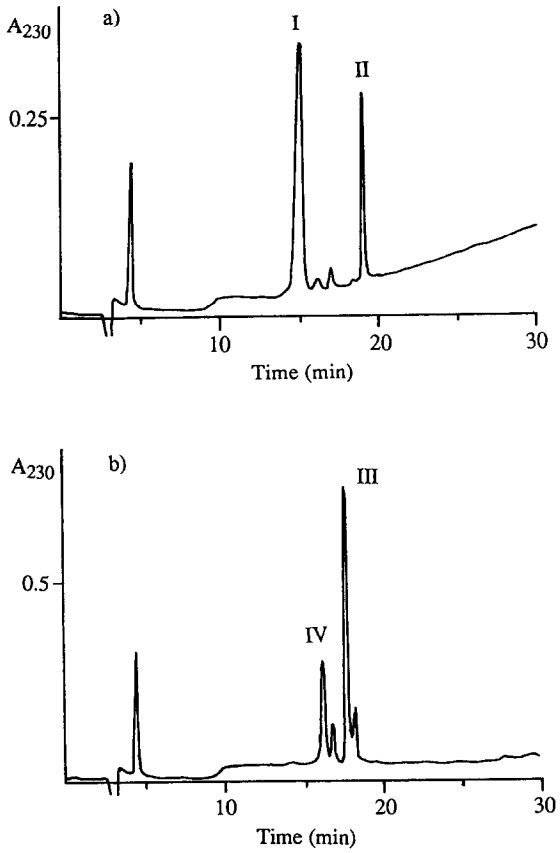

Fig. 2. HPLC analysis of the reaction mixture of **1** and **2** at (a) $t = 0$ and (b) $t = 6$ h. I, the 25 residue peptide, **1**; II, tri-*N*-bromoacetylated branched peptide, **2**; III, cross linked tripeptide, **3**; IV, dimerized **1**. Chromatography was carried out on a TSKgel ODS-120T (4.6 mm × 22 cm, TOSOH) at a flow rate of 1 ml/min with a linear gradient of CH_3CN (0–50%) for 30 min in 0.1% TFA.

Synthesis of the tri-*N*-bromoacetylated branched peptide was carried out as follows. On MBHA resin, Tyr was attached for quantitation. A BrZ group was used for Tyr side chain protection since this group is stable under the TFA treatment conditions required for side chain deprotection of Lys, but removed by treatment with TFMSA. After condensation of Fmoc-Gly as a spacer, two Fmoc-Lys(tBoc) were condensed. The Fmoc and tBoc groups were removed by piperidine and TFA, respectively. The N^{α}-amino and two N^{ε}-amino groups of Lys in Lys-Lys-Gly-Tyr(BrZ)-MBHA resin were condensed with Fmoc-β-Ala and bromoacetic acid activated by DCC and HOBt. The peptide resin was treated with a mixture of TFA, *m*-cresol and TFMSA for 1 h. The peptide did not precipitate in ether. The mixture was washed with *n*-pentane and ether and then **2** was isolated by reversed phase HPLC. The structure of **2** was confirmed by FAB-mass analysis.

After the reaction of the 25-residue peptide, **1**, and the tri-*N*-bromoacetylate branched peptide, **2**, for 6 h in 3 M GdnHCl at pH 7.5, the desired product, **3**, identified as the main product by monitoring of the reaction by means of reversed phase HPLC (Fig. 2). The product was isolated at a 70% yield. The structure of **3** was confirmed by amino acid analysis. Ion spray mass spectrometry indicated that the average mass was 7822.2 ± 0.4 (calculated MW, 7821.7 Da for $C_{329}H_{519}N_{103}O_{113}S_3$) (Fig. 3).

Formation of the collagen structure was analyzed by CD spectra (Fig. 4). CD spectra of the peptide at 4°C and pH7 showed a positive band around 223 nm and a minimum band around 198 nm, indicating that the peptide formed a collagen structure. At 37°C for biological assay, the minimum at 198 nm in the CD spectrum shifted to 202 nm with a decreased magnitude, showing the melting of the triple helix. The thermal transition curve was obtained by monitoring $[\theta]_{223}$ as a function of temperature. It exhibited a sharp thermal transition with a midpoint of 20°C.

NMR spectra in D_2O of the collagen structure of the peptide with the amino acid sequence $(Pro\text{-}Hyp\text{-}Gly)_{10}$ showed amido protons of Gly at around 8 ppm [11]. The N-terminus cross linked tripeptide, **3**, synthesized here, also had amido protons around 8ppm (Fig. 5). This peak remains even after 2 days at 4°C, indicating the peptide has a relatively stable structure. The amido proton signal decreases as the temperature increases finally disappearing at 20°C due to a rapid H–D exchange. This result is consistent with that obtained from CD spectra. It also suggests that the peptide forms a collagen structure.

In conclusion, this N-terminus cross linked tripeptide could be easily synthesized and purified by condensation with an unprotected purified peptide which has Cys at the N-terminus and a tri-*N*-bromoacetylated branched peptide. The synthesized peptide forms a collagen structure below 10°C, but unfolds at 25°C. For

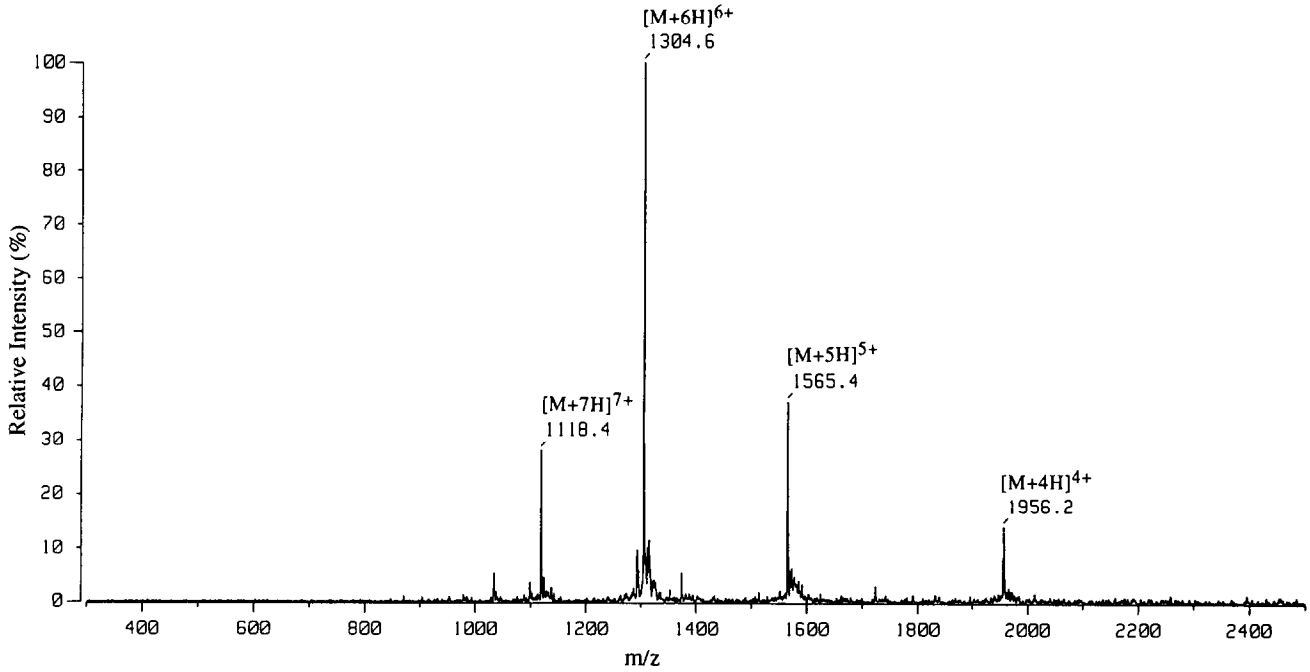

Fig. 3. The ion-spray mass spectrum of the cross-linked tripeptide, **3**. From each *m/z* value of the multiply charged ion signals ($[M+nH]^{n+}$), the molecular weight of **3** was calculated to be 7822.2 ± 0.4.

biological assay, one should make a stable collagen structure at 37°C. One way to do this is to increase the number of the tripeptide, Gly-Pro-Hyp. An other way is to elongate the amino acid sequence of the scavenger receptor so as to contain the 4 Lys residues responsible for full activity.

This synthesis can be done in the presence of GdnHCl, suggesting that a secondary structure, which sometimes leads to difficulties during the condensation reaction, is not formed during the reaction. This method is, therefore, useful for not only construction of collagen structures, but also for synthesis of 4-helical bundle proteins [12,13].

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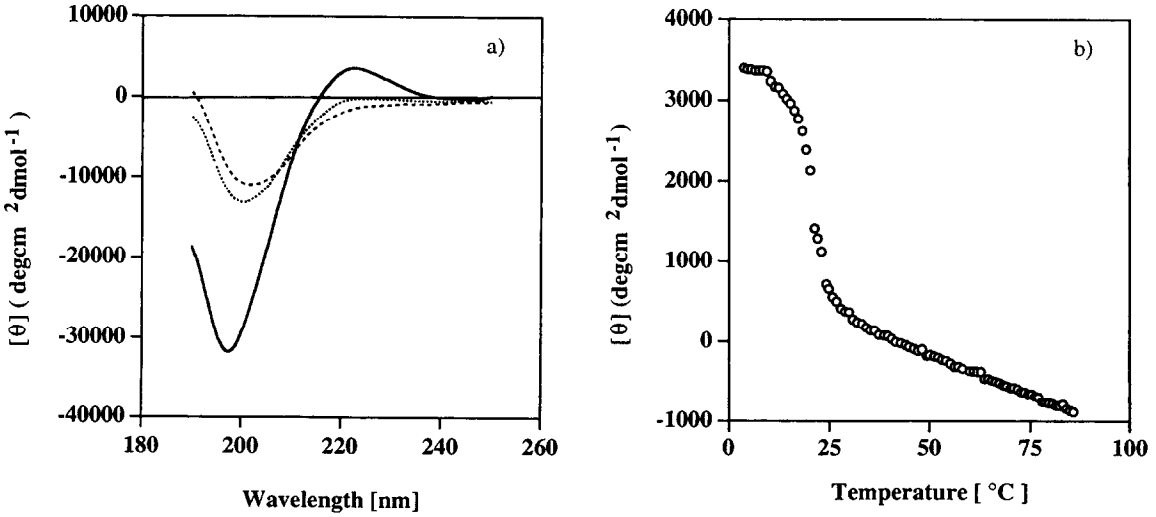

Fig. 4. (a) CD spectra of the cross linked tripeptide, **3**, at 4° (—), 37° (···) and 85°C (- - -) in pH 7. (b) Thermal transition curve of **3** in pH 7.

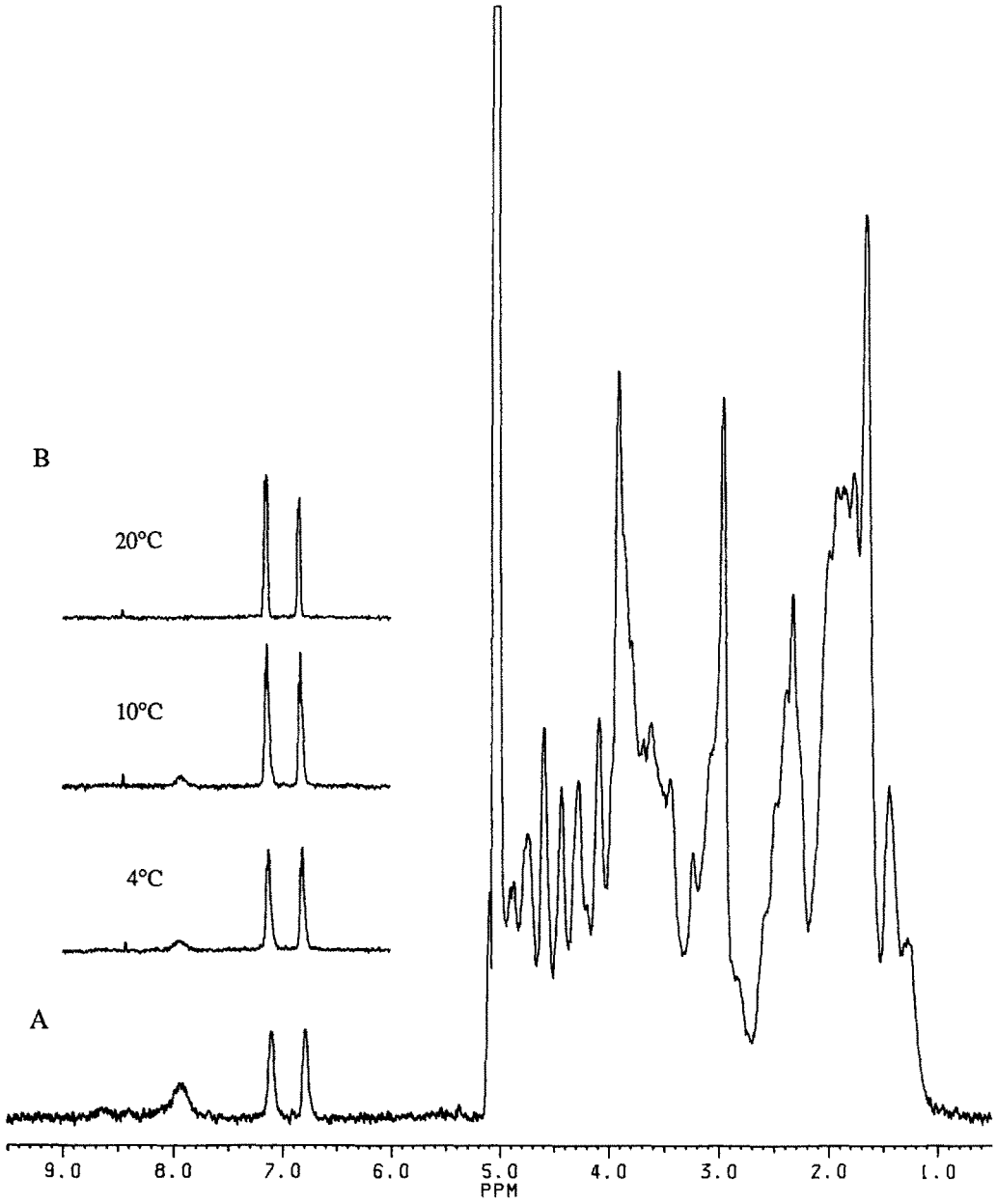

Fig. 5. 600MHz NMR spectra of the peptide, **3**. The lyophilized peptide, **3**, was dissolved in D_2O, pH 7.2 at 4°C. (A) After 5 min, the NMR spectrum was measured at 4°C. (B) After 2 days at 4°C, the NMR spectra were measured at 4°, 10° and 20°C.

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