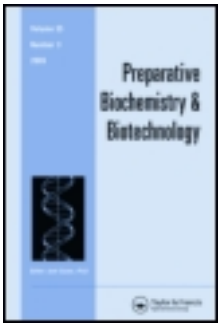

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Purification and Characterization of an Immunomodulatory Peptide from Bovine Placenta Water-Soluble Extract

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Abstract: An immunomodulatory peptide was isolated from bovine placenta water-soluble extract and purified by consecutive chromatography on DEAE Sepharose CL-6B, Sephadex G-25, and Sephasil C18 column using lymphocyte proliferation assay to identify the active fractions. The immunomodulatory peptide showed a dose-dependent stimulating effect on lymphocyte proliferation. The isoelectric point of the immunodulatory peptide was determined to be 3.82 by capillary isoelectric focusing electrophoresis. The molecular mass of the immunomodulatory peptide was determined to be 2133.52 Da by mass spectrometry. The first 10 amino acid sequence of the immunomodulatory peptide was Tyr-X-Phe-Leu-Gly-Leu-Pro-Gly-X-Thr. This immunomodulatory peptide showed no significant homology with other immunomodulatory peptides. Additionally, it was thermostable, retaining about 60% of immune activity after incubating at 80°C for 30 min.

Keywords: Bovine placenta, Water-soluble extract, Immunomodulatory peptide, Lymphocyte proliferation, Purification, Characterization

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INTRODUCTION

Dairy products have been the prevalent food of our daily life. The huge demand of dairy forces dairy corporations to expand the amount of dairy cattle. As the outgrowth of parturition, the placenta is usually discarded; this may cause environmental contamination. It is known that the placenta extraction mix has been used as precious Chinese medicines and the placenta contains and produces a wide variety of biologically active compounds which are, for the most part, proteins and peptides.[1,2] However, as the outgrowth of parturition, the placenta is usually discarded; this may cause environmental contamination. Therefore, recovering the placenta and converting it to highly valued economic products is desirable.

Immunomodulatory peptides are promising clinical therapeutic agents, and various sources of immunomodulatory peptides have been investigated. Many of them have been isolated, purified, and characterized. Migliore-Samour et al.[3] indicated the casein hydrolysates with immune activity including fragments of α_{s1}-casein (residues 194–199: Thr-Thr-Met-Pro-Leu-Trp) and β-casein (residues 63–68: Pro-Gly-Pro-Ile-Pro-Asn and 191–193: Leu-Leu-Tyr). The effects of muramyl dipeptide (MDP) synthetic analogue LK415 on the immune response of chickens immunized with a live vaccine against infectious bursal disease (IBD) were studied.[4] Georgieva et al.,[5] Marthy and Ragland,[6] and Price et al.[7] reported the nature of extracts with immune activity from different animal origins.

Additionally, the bioactivity of immunopeptides has been characterized by various *in vitro* and *in vivo* test systems. The *C*-terminal sequence 193– 209 of β-casein induced a significant proliferative response in rat lymphocytes.[8] Kayser and Meisel[9] indicated that the immunoreactivity of human peripheral blood lymphocytes was stimulated by the peptides Tyr-Gly and Tyr-Gly-Gly, corresponding to fragments of bovine α-lactorphan and κ-casein, respectively. Additionally, other cells of the immune system, and even tumor cells, had also been used to identify the immune activity.[3,10] Hadden[11] even reported that preliminary data from trials with dipeptide (Tyr-Gly) and tripeptide (Tyr-Gly-Gly), in pre-AIDS patients, are encouraging. They significantly enhanced immune and anti-infectious ability of the pre-AIDS patients and decreased their mortality.

The objective of this study was to purify the immunomodulatory peptide from a bovine placenta, using mice spleen lymphocyte proliferation assay, and then the isoelectric point, molecular weight, and partial amino acid sequence of the peptide were determined. Thermostability of the immunomodulatory peptide was investigated.

EXPERIMENTAL

Materials

Bovine placenta was obtained from a dairy company in Wuxi, China. The bovine placenta was washed several times with 0.154 M NaCl

solution until it was free of blood and was then stored at $-20^{\circ}C$ for further study.

Isolation of the Immunomodulatory Peptide

All subsequent steps were carried out at $4^{\circ}C$. Frozen placenta (100 g) was cut into small pieces and suspended in 20 mM phosphate buffer at a ratio of 1:2 (wt/v) and then homogenized. The homogenate was centrifuged at 12,000 g for 20 min. The supernatant was subjected to ultrafiltration through a 10 kDa cut-off membrane and divided into the large and small (less than 10 kDa) molecular fractions. The fraction containing the immunomodulatory peptide was loaded onto a DEAE Sepharose CL-6B column and eluted with 20 mM phosphate buffer and then a linear gradient was applied from 0.5 M-1 M NaCl in 20 mM phosphate buffer at a flow rate of 1.0 mL/min. The fractions were collected and assayed for immune activity by a lymphocyte proliferation assay. Fractions with immune activity were then separated on a Sephadex G-25 gel filtration column which was previously equilibrated in 20 mM phosphate buffer and eluted with 20 mM phosphate buffer at a flow rate of 12 mL/h. Fractions were assayed for immune activity. Subsequently, the fractions with immune activity were fractionated on a Sephasil C_{18} column using an isocratic elution of 5% acetonitrile and then a linear gradient from 5%−40% (v/v) acetonitrile at a flow rate of 1 mL/min. Fractions were assayed for immune activity.

Cell Preparation

Spleen lymphocytes were obtained from Kunming mice following gentle teasing of the spleen with forceps in RPMI 1640. The cell suspensions were filtered through stainless steel mesh (105 μm), washed 2 times (400 g, 10 min) and suspended at a concentration of 1×10^6/mL RPMI 1640. The cells were grown in 5%(v/v) CO_2 at $37^{\circ}C$. The overall viability of 95% of the cells was proved by typan blue exclusion.

Lymphocyte Proliferation Assay

For the lymphocyte proliferation assay, cells were plated at 1×10^5/well in 96-well plates. Spleen lymphocytes' response to immunomodulatory peptide was measured using the methyl-thiazoyl tetrazolium (MTT) assay. The immunomodulatory peptide was incubated with the cells for 68 h, after which time, 20 μL MTT was added per well. Cells were incubated with the MTT for 4 h in a CO_2 incubator. The formed formazan crystals were dissolved by the addition of 100 μL dimethyl sulphoxide (DMSO) per well. The degree of MTT variation in each sample was subsequently assessed by

measuring absorbance at 570 nm. Background absorbance values, as assessed from cell-free wells, were subtracted from the absorbance values of each test sample. The lymphocyte proliferation assay was carried out in triplicate.

Biophysical Characterization

Capillary Isoelectric Focusing Electrophoresis (CIEFE)

The CIEFE was carried out on a P/ACE 5,000 capillary electrophoresis system (Beckman, USA) by using neutrally coated capillaries with a total length of 27 cm (20 cm from the window to inlet) × 50 μm i.d. (Beckman, USA). Ribonuclease A (pI = 9.45), carbonic anhydrase (pI = 5.90), and CCK peptide fragment (pI = 2.75) were used as pI determination markers. The anode electrolyte was CIEFE gel solution containing 91 mM H_3PO_4 and 2% (v/v) of Pharmalyte (pH 2.75–10). Twenty millimolar NaOH solution was employed as the cathode electrolyte and 10 mM H_3PO_4 was used for rinsing. The sample and the pI markers were added into the CIEFE gel solutions containing Pharmalyte before the determination. Electrophoretic separation was performed at a constant voltage of 13.5 kV and was monitored at 280 nm. The capillary was rinsed with pure water for 1 min before filling with sample solution. The focused strips were then rinsed through the detection window by high pressure at 13.5 kV.

Mass Spectrometry

Matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra were acquired with a Bruker Reflex-TOF-MS instrument (Bruker, Germany) with a delayed extraction of 200 ns. The mass spectrometer was equipped with a SCOUT multiprobe inlet and N_2 laser (337 nm, 3 ns pulse width with a pulse energy of 200 μJ). The acceleration voltage was 19 kV and the reflectron voltage was 15.1 kV. The matrix compound was gentisic acid DHB (2,5-dihydroxy benzoic acid) dissolved in acetone/Milli-Q-water 9:1 (v/v) at a concentration of 2% (w/v). The sample was diluted in acetone/Milli-Q-water 9:1 (v/v) at a concentration of 0.1–0.5% (w/v) and then mixed 1:1 (v/v) with the matrix solution for the analysis. The measurements were done in the linear mode. For positive ion spectra, 200 laser shots were applied. The following molecular weight standards (Sigma-Aldrich Chemie Gmbh Steinheim, Germany) were used to calculate accurate molar masses: 756.9 (Bradykinin fragm. 1–7); 1619.9 (Bombesin), and 2465.7 (Adrenocorticotropen ACTH 18–39 clip human).

Amino Acid Sequencing of the Immunomodulatory Peptide

The sample was dissolved in 5 μL of an acetonitrile/water/formic acid (50:49.9:0.1) solution. Three μL of the solution was spotted onto a

precycled Biobrene Plus TM-coated micro-TFA filter. Edman degradation was performed using an automated protein sequencer (Applied Biosystems, USA). The peptide was treated with phenylisothiocyanate, which combined with the *N*-terminal amino acid residue in the peptide chain. The consequence of this reaction was that the first peptide bound in the chain became labile and could be easily broken by treatment with mild acid. The removed amino acid, which was modified, could be chromatographically identified. The modified amino acid was loaded onto a column (Spheri-5 PTH, 5 μm, 220 × 2.1 mm) with a binary gradient elution being performed using a microgradient system, and the modified amino acids were detected by a programmable absorbance detector (Applied Biosystems, USA). All *N*-terminal sequencing reagents required for the Edman degradation and all the solvents required for gradient elution were obtained from Applied Biosystems, USA. The amino acid was identified by comparison of the elution time of the unknown with the elution times of the standard mixture components. These steps were carried out repeatedly to determine other amino acids.

Thermal Resistance

Thermal resistance of the immunomodulatory peptide was assessed by incubation at six different temperatures (−20°C, 4°C, 20°C, 40°C, 60°C, and 80°C) for 30 min. The samples were prepared in distilled water at a final concentration of 200 μg/mL. The immune activities of the different samples were measured by a lymphocyte proliferation assay.

Far-ultra-violet circular dichroism (far-UV CD) spectra were obtained with a spectropolarimeter (Jasco, Japan) equipped with a 1 mm path-length cell. The scanning range was from 190 to 250 nm. The thermostability of the immunomodulatory peptide treated at different temperatures was measured. The samples were incubated at a special temperature (20°C to 80°C) for 30 min, then cooled down to room temperature before measurement. The samples were prepared in distilled water at a final concentration of 200 μg/mL.

Statistical Analysis

Results were analyzed using a Statistical Analysis system (SAS) software.

RESULTS AND DISCUSSION

Isolation of the Immunomodulatory Peptide

The protein extract of bovine placenta was obtained by suspending it in 20 mM phosphate buffer and then homogenizing and centrifuging. Its

immune activity was determined by a lymphocyte proliferation assay. The placenta protein extract showed a significant stimulating effect on lymphocyte proliferation.

To isolate the immunomodulatory peptide, the placenta protein extract was ultrafiltrated through a 10 kDa cut-off membrane. The low molecular weight fraction (less than 10 kDa) showed a significant stimulating effect on lymphocyte proliferation, determined by lymphocyte proliferation assay. The filtrate from the ultrafiltration membrane was then loaded onto a DEAE Sepharose CL-6B column and resolved into 4 major fractions (Fig. 1A). The immune activities of the four fractions were determined by lymphocyte proliferation assay and the P-A fraction exerted the significant stimulating effect on lymphocyte proliferation. Therefore, the P-A fraction was then used for further isolation by Sephadex G-25 column chromatography. The eluate was divided into two fractions from the column (Fig. 1B) and the immune activity was assayed as above. Lymphocyte proliferation assay showed that the P-M fraction had a significant stimulating effect on lymphocyte proliferation. The P-M fraction was then subjected to Sephasil C_{18} column chromatography (Fig. 1C). Among the fractions, P-R showed a significant stimulating effect on lymphocyte proliferation. The isolated peptide was re-analyzed by capillary electrophoresis, thereby confirming its purity (Fig. 2). The isolated peptide, by these consecutive steps, retained its significant stimulating effect on lymphocyte proliferation and showed dose-dependency (Fig. 3).

Biophysical Characterization

The immunomodulatory peptide was identified by capillary isoelectric focusing electrophoresis, mass spectrometry, and peptide sequencing.

To obtain the accurate pI of an unknown sample, ribonuclease A (pI = 9.45), carbonic anhydrase (pI = 5.90), and CCK peptide fragment (pI = 2.75) were added to the sample as the running markers. The CIEF profile of the pI markers and sample (Fig. 4) showed that the retention time of each protein is proportional to its pI ($y = 0.6015x + 14.6410$), indicating that the isoelectric point of the immunomodulatory peptide was 3.82.

As a popular and versatile analysis method, MALDI-TOF mass spectrometry provides a tool for analyzing the different samples. Here, the mass spectrum of the immunomodulatory peptide by MALDI-TOF mass spectrometry is shown in Fig. 5. The molecular weight of the peptide was found to be 2133.52 Da.

The purified immunomodulatory peptide was subjected to the analysis of N-terminal amino acid sequence. The first 10 N-terminal amino acid sequence was Tyr-X-Phe-Leu-Gly-Leu-Pro-Gly-X-Thr (X corresponds to unidentified residues).

Although the sequences of many of immunomodulatory peptides have been known, the partial sequence of the immunomodulatory peptide obtained

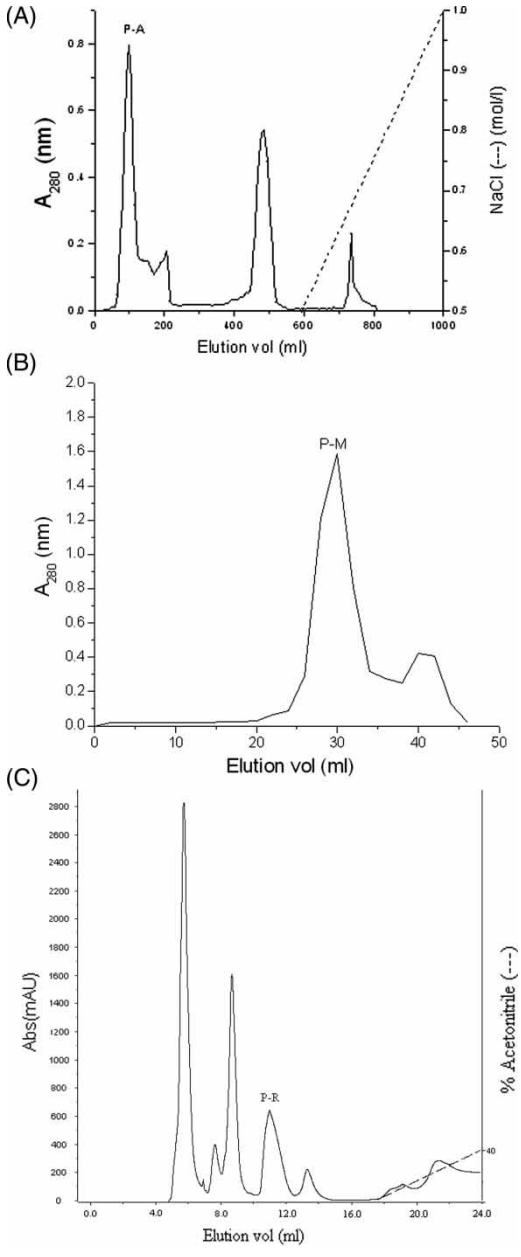

Figure 1. A) DEAE Sepharose CL-6B column chromatography profile of the placenta protein filtrate from the 10 kDa cut-off membrane; B) Sephadex G-25 column chromatography profile of the P-A fraction from DEAE Sepharose CL-6B column chromatography; C) Sephasil C_{18} column chromatography profile of the P-M fraction from Sephadex G-25 column chromatography.

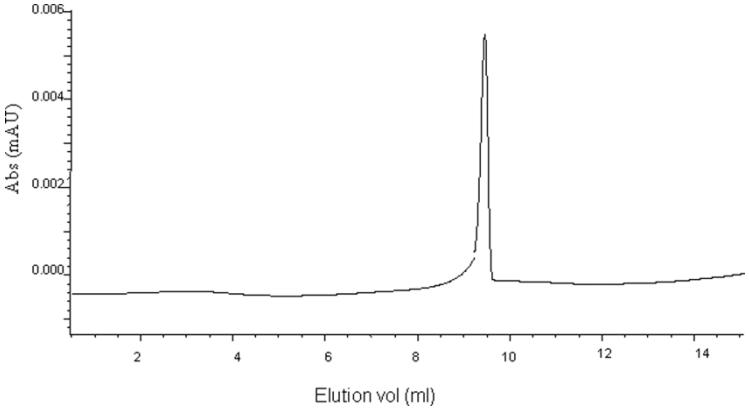

Figure 2. Purity identification profile of the immunomodulatory peptide by capillary electrophoresis.

revealed that it was not identical with these peptides.[12] Additionally, the presence of lipids and carbohydrates had been detected in some other kinds of peptides,[13,14] and it was well known that such elements interfere with peptide sequencing procedures; it was our belief that the repetitive failure of the

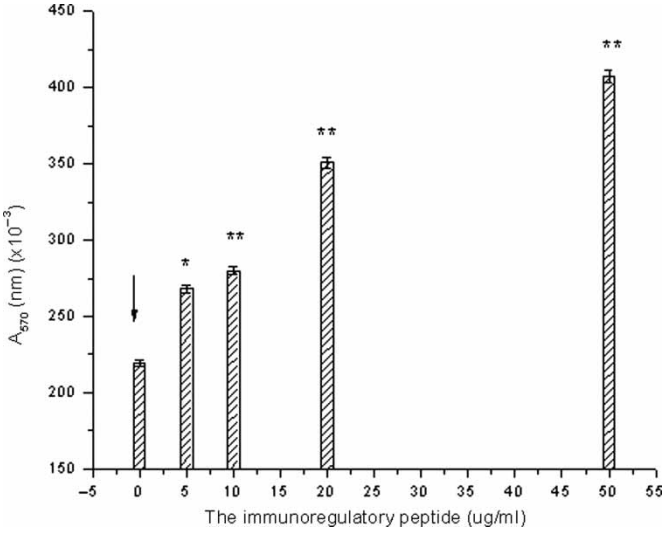

Figure 3. The stimulating effect of the immunoregualtory peptide on lymphocyte proliferation. Arrow shows the control cultures of lymphocyte running in the absence of the immunomodulatory peptide. Others show the cultures of lymphocyte running in the presence of the immunomodulatory peptide. The immunomodulatory peptide shows a dose-dependent stimulatory effect on the lymphocyte proliferation ($^{*}P < 0.05$, $^{**}P < 0.01$).

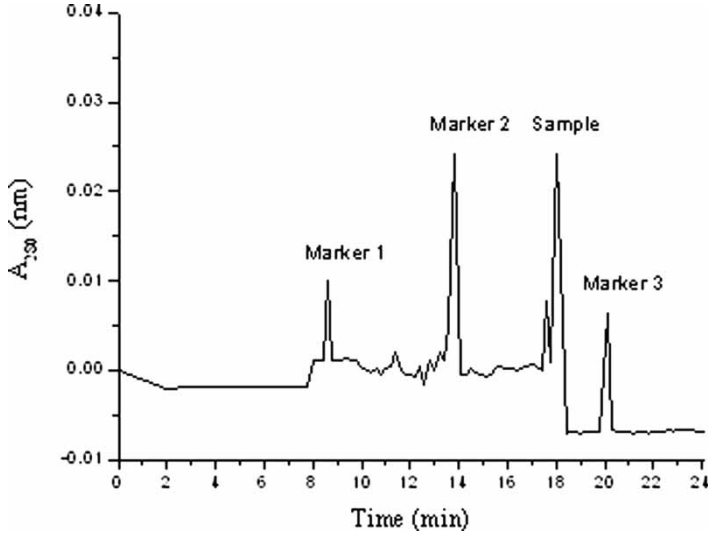

Figure 4. The capillary isoelectric focusing electrophoresis profile of the immunomodulatory peptide.

identification of several residues of the immunomodulatory peptide sequence was a consequence of the presence of one or several of these elements. According to these findings, the molecular mass determined by mass spectrometry may be related to the peptide moiety and these elements together.

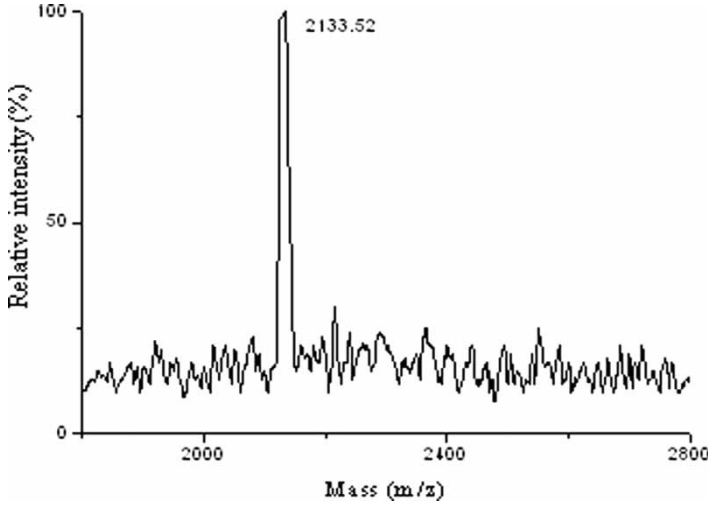

Figure 5. MALDI-TOF mass spectrum of the purified immunomodulatory peptide from bovine placenta.

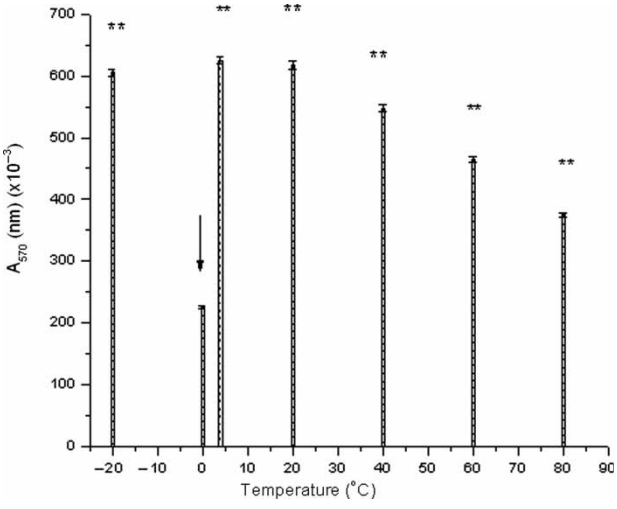

Figure 6. Effect of temperature on the immunomodulatory peptide stability. Arrow shows the control cultures of lymphocyte running in the absence of the immunomodulatory peptide. Others show the cultures of lymphocyte running in the presence of the immunomodulatory peptide. ($^{*}P < 0.05$, $^{**}P < 0.01$).

Thermal stability of the immunomodulatory peptide was investigated by lymphocyte proliferation assay (Fig. 6). Figure 6 showed that no significant loss of activity was detected and the immunomodulatory peptide which was treated at 80°C for 30 min still had more than 60% relative residual activity, compared with the immune activity retained at 20°C.

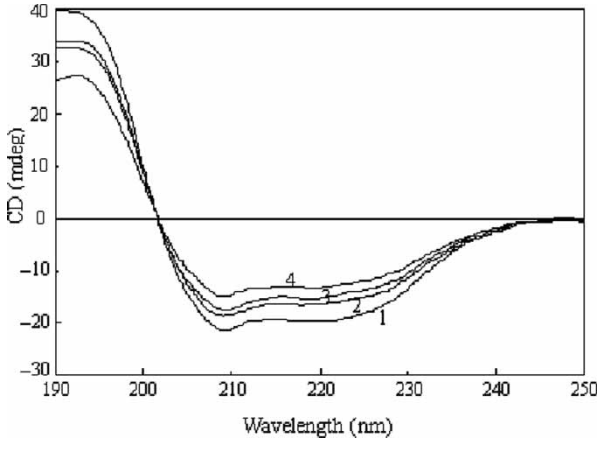

Figure 7. CD spectra of the immunomodulatory peptide at different temperature. The temperatures for lines 1–4 are 20, 40, 60, and 80°C, respectively.

Table 1. Percentages (±S.D.) of different secondary structures in the immunomodulatory peptide as a function of the different temperature treatment

Temperature (°C)	Helix	Sheet	Turn	Random
20	45.6 ± 2.1	21.2 ± 1.3	7.5 ± 1.1	25.3 ± 1.5
40	45.9 ± 3.2	20.8 ± 1.7	7.7 ± 0.5	24.7 ± 2.1
60	46.3 ± 1.9	21.3 ± 1.1	7.3 ± 1.0	25 ± 1.0
80	46.9 ± 2.0	20.4 ± 2.2	7.1 ± 1.4	25.5 ± 1.1

Additionally, the changes in the CD spectra of the immunomodulatory peptide at different temperatures are shown in Fig. 7. It is commonly known that two negative peaks at 208 and 222 nm are characteristic of the a-helix secondary conformation of proteins and the one at 214 nm was characteristic of the β-sheet secondary conformation of proteins.[15,16] Analysis of the spectra of the immunomodulatory peptide shows that the secondary structures decreased gradually with increasing temperatures (Table 1) and the curve was in a sigmoidal shape, which showed the significant cooperativity of secondary structures during thermal denaturation. The results here further confirmed the results above.

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