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Full Length Research Paper

Preparation and characterization of antigenic properties of gramicidin A- keyhole limpet hemocyanin and gramicidin A- ovalbumin conjugates

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A rapid, simple and low cost procedure for preparing hapten-protein conjugates was developed using gramicidin A (GA) and two other water-soluble proteins, keyhole limpet hemocyanin (KLH) and ovalbumin (OVA). GA was a kind of antimicrobial peptides. Two lysines and a cysteine were linked to amino- terminus and carboxyl-terminus of the peptide chain, respectively, in order to form sulfhydryl groups and improve its water solubility. And the molecule weight and purity of the modified peptide chain were checked by electron spray ionization mass spectrometry (ESI-MS) and high performance liquid chromatography (HPLC). Then the activated KLH and OVA were conjugated to GA by using 4-(N-maleimidomethyl) cyclohexane-1-carboxylic acid 3-sulfo-N-hydroxysuccinimide ester sodium salt (sulfo-SMCC) to form disulfide linkage. In order to obtain artificial antigen of GA, GA was linked to KLH and OVA by sulfo-SMCC coupling at room temperature. The conjugates of KLH-Peptide and OVA-Peptide were identified by infrared and ultraviolet spectrophotometry SDS-PAGE and DTNB method, which could prove the activity of the sulfhydryl-groups. In this way, we have obtained a novel artificial immunogen and coating antigen that could be used to raise polyclonal antibody through immunized rabbits.

Key words: Protein conjugates, Gramicidin A, characterization, artificial immunogen, coating antigen.

INTRODUCTION

Tyrothricine is consisted mainly of tryocidine and gramicidin, and gramicidin A (GA) is accounted for above 70% in the mixture. The GA has 15 amino acids including 6 L-amino acids and its sequence is L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp. In medicine, the GA presents a broad antimicrobial activity and can be used to treat some diseases caused by susceptible gram-positive pathogens, particularly *Streptococci*, *Pneumococci* and *Staphylococci*. Baker et al. (1985) studied the antimicrobial function of GA in the respiratory system and got the same results. And that it had hemolytic cytotoxicity, nephrotoxicity, hepatotoxicity resulting from its low specificity (Paul and Christof, 2007). It was also reported that GA exerts a kind of inhibition in RNA synthesis procedure (Townsley et al., 2001). It can be seen that the study of application and determination of GA has a significant role in clinical medicine. It has been confirmed that many types of commercial pharmaceuticals contained gramicidin, like Dequalinium Chloride, Tyrothricin Lozenges®, Collagenase Ointment®, Compound Triamcinolone Acetonide Cream® and so on, which are produced in China. The Chinese government and the European Union expressly prohibited gramicidin as an additive used in food and cosmetics industries because of its toxicity. It had a complicated pre-processing using instrument analytical technique, such as HPLC; and poor accuracy and precision, using conventional chemical methods, such as modern electrochemical techniques, linear sweep voltammetry, cyclic voltammetry, square wave voltammetry and chronocoulometry, combining with UV spectroscopy. This is because Gramicidin, as a peptide, is a biomacromolecule. These analytical methods are laborious and it takes time to achieve a confirmed identification, which could not be adapted for rapid, specific and sensitive

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detection; leading to disadvantages for food and cosmetics administration and disease prevention. Therefore, exploring and applying an accurate, efficient, sensitive and reliable analysis method of GA is important for the safety of food, cosmetics and pharmaceutical products.

Immunoassay is one of the most sensitive and rapid methods. The reaction is based on the interaction between antigen and antibody (Liu et al., 2007). An antibody response is elicited in an animal, following injection of a foreign material (that is, antigen). But, substances of molecular weight less than 8000 Dalton (haptens) are not immunogenic by themselves and need to be conjugated to highly immunogenic macromolecules (e.g., proteins or synthetic polyamino acids) in order to stimulate a potent immune (Erlanger, 1973). The small molecule was conjugated to a carrier protein by various chemical reactions to form antigen for the purpose of getting high affinity polyclonal antibody. The carrier protein contains bovine serum albumin (BSA), ovalbumin (OVA), casein and hemocyanin (KLH), human serum albumin (HSA) and a synthetic homopolymer like Poly-DL-lysine (James, 1996). KLH and OVA can be activated by sulfo-SMCC and used as carrier protein (Allyn et al., 1997). In this paper, we reported that GA was attached to KLH and OVA, and the synthesis of artificial antigen, KLH-Peptide and OVA-Peptide was gained and identified.

MATERIALS AND METHODS

Materials

Keyhole limpet hemocyanin (KLH), ovalbumin (OVA), 4-(N-Maleimidomethyl) cyclohexane-1-carboxylic acid 3-sulfo-N-hydroxysuccinimide ester sodium salt (Sulfo-SMCC), 5,5-dithiobis (2-nitrobenzoic) acid (DTNB), cysteine were purchased from Sigma, USA, and acrylamide, Tris, SDS, TEMED, protein-marker were purchased from Takara, Japan. Amino acids and GA were purchased from Sangon, Canada. All other chemical reagents were purchased from Aladdin, China.

Phosphate-buffered saline (PBS: 10 mM KH_2PO_4/K_2HPO_4, 150 mM NaCl, pH 7.2) was used for dissolving KLH and OVA and diluting test samples.

Activation of GA

The GA was activated through two lysines and a cysteine residue added to the amino-terminus and carboxyl-terminus of the peptide chain, respectively, in order to form sulfhydryl groups at C-terminus and improve its water solubility. The activated peptide sequence is L-Lys-L-Lys-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-L-Cys. The synthesis of activated GA used solid-phase peptide synthesis (SPPS) with N-Fmoc protection from C-terminus to N-terminus. It was synthesized on a peptide synthesizer at the 25 μmol scale using a 10 fold excess of Fmoc-amino acids (200 mM) relative to the resin. Rink amide PEGA resin (0.33 mmol/g), Fmoc-amino acids, 5-chloro-1-[bis (dimethylamino)methylene]-1H-benzotriazolium 3-oxide hexafluorophosphate (HCTU), 400 mmol N-methylmorpholine (NMM) in dimethylformamide (DMF) and 20% piperidine were ready for experiments. Deprotection was performed using 20% piperidine/DMF. Coupling was performed using 1:1:2 amino acid/activator/NMM in DMF. Side protecting groups for amino acids were trityl (Trt) for L-Val, Gly, L-Ala, D-Leu, D-Val, L-Val and L-Cys, *t*-butyloxycarbonyl (*t*Boc) for L-Trp and L-Lys. Cleavage was carried out using volume ratio of TFA/water/anisole/ EDTA to 95:2:2:1 for 2 h. Crude peptides were precipitated using ice-cold ether, redissolved in distilled water and lyophilized to white powder for next experiments (Christina et al., 2008). Peptides were then analyzed on a Varian HPLC system (Prostar 240, USA) with 5 □m and 250 × 4.6 mm C-18 column over 25 min. The mobile phase was a gradient solution which started with solution A (0.1% trifluoroacetic in 100% acetonitrile) and solution B (0.1% trifluoroacetic in distilled water) in the ratio of 48:52(v/v); and the solution A was increased up to 78% in 25 min at 1 ml/min. UV detection was at 220 nm. The peptide masses were confirmed by LC-ESI-MS and compared with GA.

Preparation of GA-protein conjugates

Sulfo-SMCC modified KLH and OVA (sulfo-SMCC-KLH, sulfo-SMCC-OVA)

Sulfo-SMCC 5 mg was dissolved in 5 ml deionized water with final concentrations of 1 mg/ml, 20 mg KLH and 20 mg OVA in 10 ml of 10 mM PBS with pH 7.2. The amount of 5 ml KLH and OVA mixed with 1 ml sulfo-SMCC, respectively, and stirred mildly on oscillator at room temperature for 1 h is outlined in Figure 1. After the reaction, the product was desalted by passing through a column of Sephadex G-25 using 10 mM pH 7.2 PBS as mobile phase. The first liquid whose peak showed in computer was collected and the other liquid containing sulfo-SMCC was discarded. The two kinds of liquid collected were activated KLH and OVA (sulfo-SMCC-KLH, sulfo-SMCC-OVA). They were lyophilized and stored at -20°C for next experiments.

Preparation of GA-KLH conjugate and GA-OVA conjugate

A total of 2.6 mg activated gramicidin A and 2.6 mg sulfo-SMCC-KLH was dissolved in 1 ml of 10 mM PBS (pH 7.2), respectively. The former solution was added dropwise to the sulfo-SMCC-KLH solution with constant stirring mildly. The reaction was allowed to proceed for 2 h at room temperature as outlined in Figure 1. Analysis of GA-KLH with DTNB method shows no change in the number of amino groups compared with normal KLH (Qian et al., 2009). The reaction solution was dialyzed for 2 days with several changes of 10 mM PBS (pH 7.2) to remove unreacted peptide and other small chemical molecules for analysis and preparation of polyclonal antibody (Zhu et al., 2007). GA-OVA was also prepared in a similar way. The conjugates of KLH-Peptide and OVA-Peptide were identified by infrared and ultraviolet spectrophotometry, SDS-PAGE and DTNB method which could prove the activity of the sulfhydryl-groups. The GA-KLH and GA-OVA were artificial immunogen and solid coated antigen respectively in the experiments.

Spectrophotometric measurements

Ultraviolet spectrophotometric measurement

For analytical purpose, we prepared 1 mg/ml KLH, OVA and GA in 10 mM PBS (pH 7.2) and GA-KLH and GA-OVA were diluted to 2 folds. They were assessed through a quartz cuvette with a dual beam spectrophotometer and recorded between 190 and 400 nm using PBS buffer as control (Pazur and Li, 2004; Venkatesh and Yeldur, 2007). We could compare with the absorption spectra of the five samples to ensure the successful coupling between the peptide and carrier proteins.

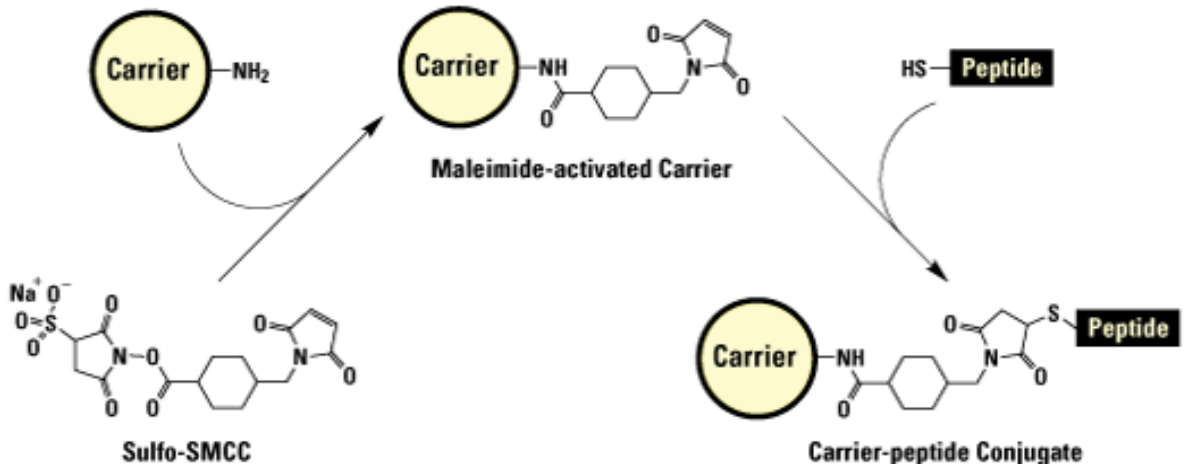

Figure 1. Scheme for the preparation of sulfo-SMCC modified carrier proteins like KLH and OVA and activated GA conjugated to carrier proteins.

Infrared spectrophotometric measurement

Hydrosulfuryl was an important functional group in proteins or peptides. We could use Fourier-Transform infrared spectrum of hydrosulfuryl whose wave number was about 2500 cm^{-1} and observed the diversity of the five substances above in order to confirm the correctness. As the same as the ultraviolet spectrophotometric measurement, we viewed the changes of spectra from 4500 ~ 500 cm^{-1} using the same samples.

Electrophoresis

OVA and GA-OVA were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Protein gels were detected by Coomassie Blue staining. The band densities were determined, using a GS-800 Calibrated Densitometer (Bio-rad, USA). In the SDS-PAGE experiments, two type of gels were used: upper and lower gels. Upper gel- 5% gel was prepared as follows: 0.33 ml 30% acrylamide solution, 0.25 ml 1.0 M pH 6.8 Tris-HCl, 0.02 ml 10% SDS, 0.02 ml 10% ammonium persulfate solutions and 0.002 ml TEMED were mixed and adjusted to 2 ml with distilled water. Lower gel-12% gel was prepared as follows: 2.0 ml 30% acrylamide solution, 1.3 ml of 1.5 M pH 8.8 Tris-HCl, 0.05 ml 10% SDS, 0.05 ml 10% ammonium persulfate solutions and 0.002 ml TEMED were mixed and adjusted to 5 ml with distilled water. First, the lower gel was prepared between the glass plates and then 1 ml ethyl alcohol was used to clear up the bubbles occurring in the upper part of the gel. After the gel was dried, the upper part was got rid of. After drying, upper gel was applied, followed by the injection of the samples. The samples were heated at 100°C for 5 min.

DTNB method for detection of hydrosulfuryl content and calculation of the couple ratio

DTNB was an ellman reagent (Sashidhar et al., 1994) and used for detecting the hydrosulfuryl content of bio-samples with colorimetric determination, showing a maximum peak at 412 nm.

Fabrication of standard curve for cysteine

15 mM cysteine was weighed into 10 ml of 10 mM pH 7.2 PBS buffer leading to the final concentration of 1.5 mM as mother solution. Mother solution was dilated to seven different concentrations as 1.5 ~ 0.25 mM, with 10 mM pH 7.2 PBS buffer. The test samples were prepared as follows: 20 µl of each diluted solution of seven gradients to seven different tubes, 400 µl PBS, 40 µl 1 mg/ml DTNB, were mixed and reacted at 27°C for 15 min. Then 150 µl test sample was added to polystyrene 96-well microtitration plates for detection at 412 nm using microplate-reader. The absorption values of sevens gradients were analyzed and the standard curve of which the X axis standard for molar concentration and the Y axis standard for absorption at 412 nm was drawn. The regression equation was got through the standard curve for calculating the content of hydrosulfuryl in GA, KLH, OVA, GA-KLH and GA-OVA.

Determination of the content of hydrosulfuryl and calculation of the couple ratio between peptide and carrier proteins

As a similar method was used for the determination of cysteine, the test samples were determined. The couple ratio of each conjugate was determined using the following equation:

$$\text{the couple ratio} = \frac{a - b}{a} \times 100\%$$

Where a is the content of hydrosulfuryl in GA; b is the content of hydrosulfuryl in GA-carrier protein.

RESULTS

Activation of GA

The GA and activated GA were identified by LC-ESI-MS spectroscopy. The spectra of activated GA were easy to read; the molecular weights and their actual states of existence in PBS are characterized in Figure 2 (b). The molecular weights of the activated GA were 2170.75 from the mass spectra comparing with the natural GA's 1811.26 in Figure 2 (a). The 359.49 of molecular difference

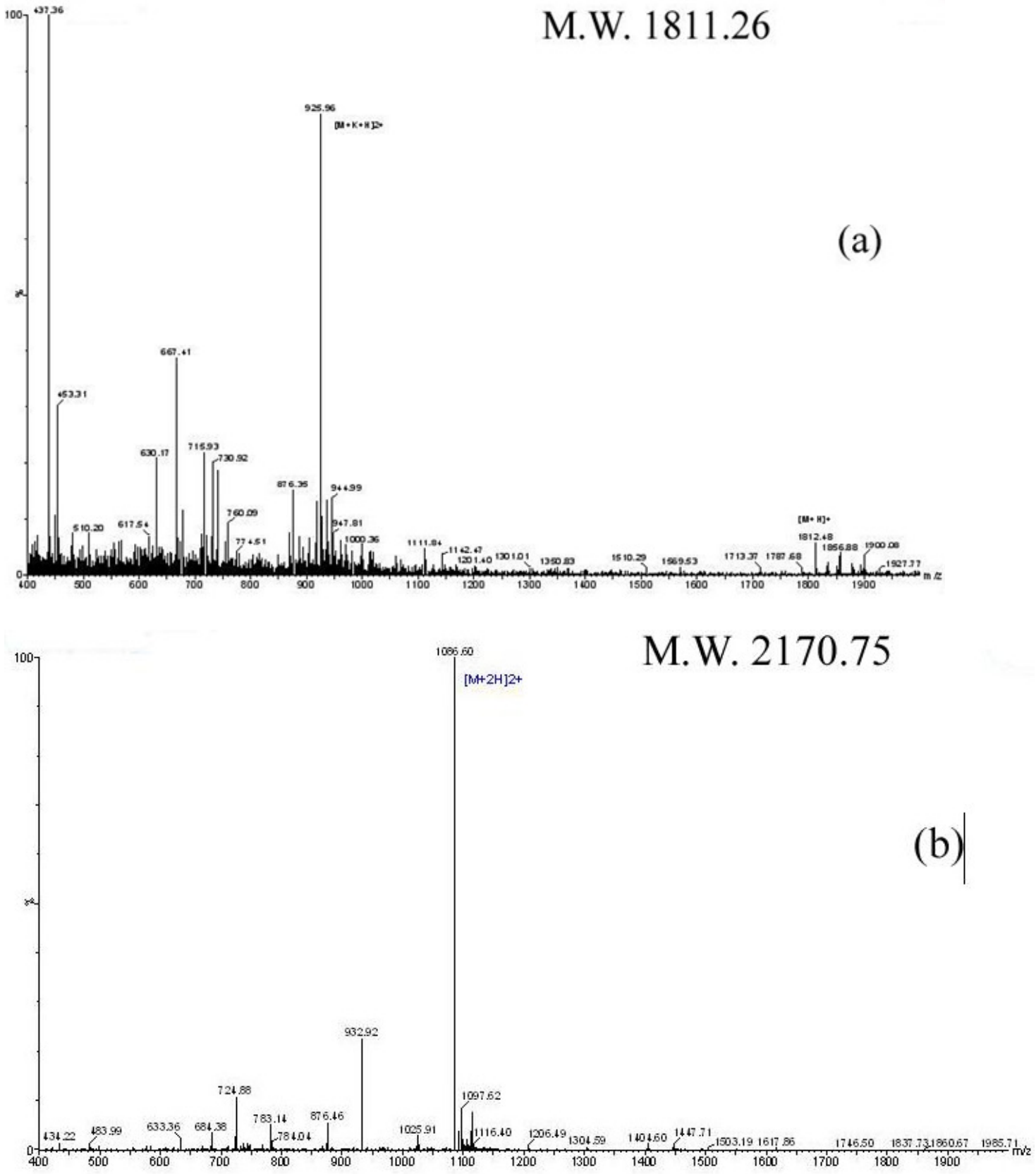

Figure 2. The ESI-Mass spectra of **(a)** natural gramicidin A , and **(b)** activated gramicidin A.

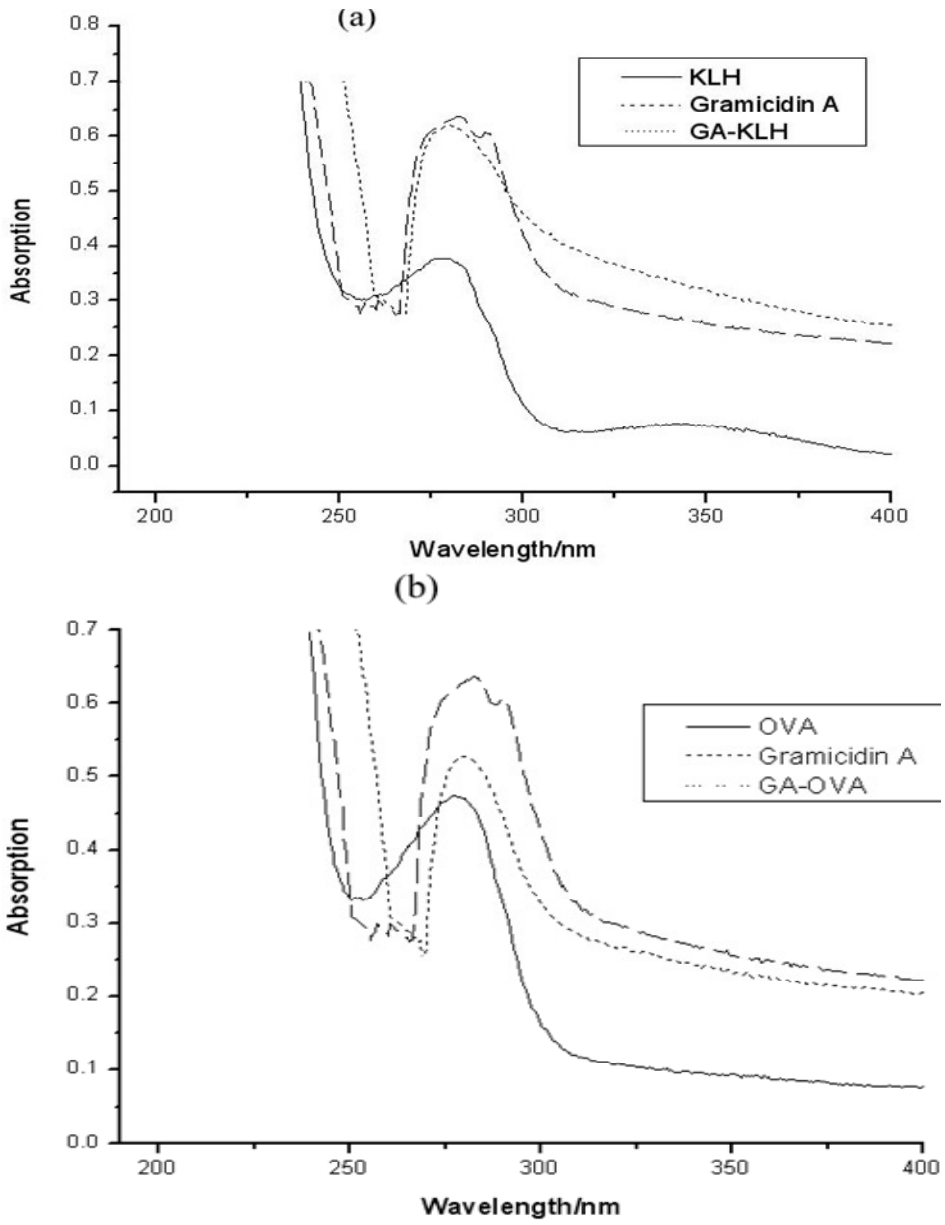

Figure 3. Comparison of UV spectra of **(a)** gramicidin A, KLH and GA-KLH, and **(b)** gramicidin A, OVA and GA-OVA.

formed from two lysines and a cysteine residue losing three water molecules was just right.

Spectrophotometric measurement

The UV spectra of KLH, GA and GA-KLH were recorded between 190 and 400 nm as shown in Figure 3(a). GA (1 mg/ml) dissolved in PBS exhibited two peaks of similar intensity at 282.5 and 289.5 nm, respectively, whereas KLH (1 mg/ml) presented a major peak at 279.5 nm. Moreover, a high absorbance contribution of the GA-KLH conjugates at 280 nm. These was spectral characteristics of KLH relatively and the peaks of gramicidin disappeared. The UV spectra of OVA, GA and GA-OVA are also displayed in Figure 3 (b). Likewise, the major peak of OVA was at 277.5 nm and the highest absorbance of GA-OVA was at 280 nm. All of these prima facie evidence showed that GA had been conjugated to OVA and KLH.

Infrared spectrophotometric (IR) spectra were used for detection of structure of samples. The aim of this experiment was mainly observing the spectral variation of hydrosulfuryl in GA, KLH, OVA, GA-KLH and GA-OVA at 2600 ~ 2400 cm^{-1}. The IR spectra of GA, KLH and GA-KLH were shown in Figure 4 (a). It was demonstrated the

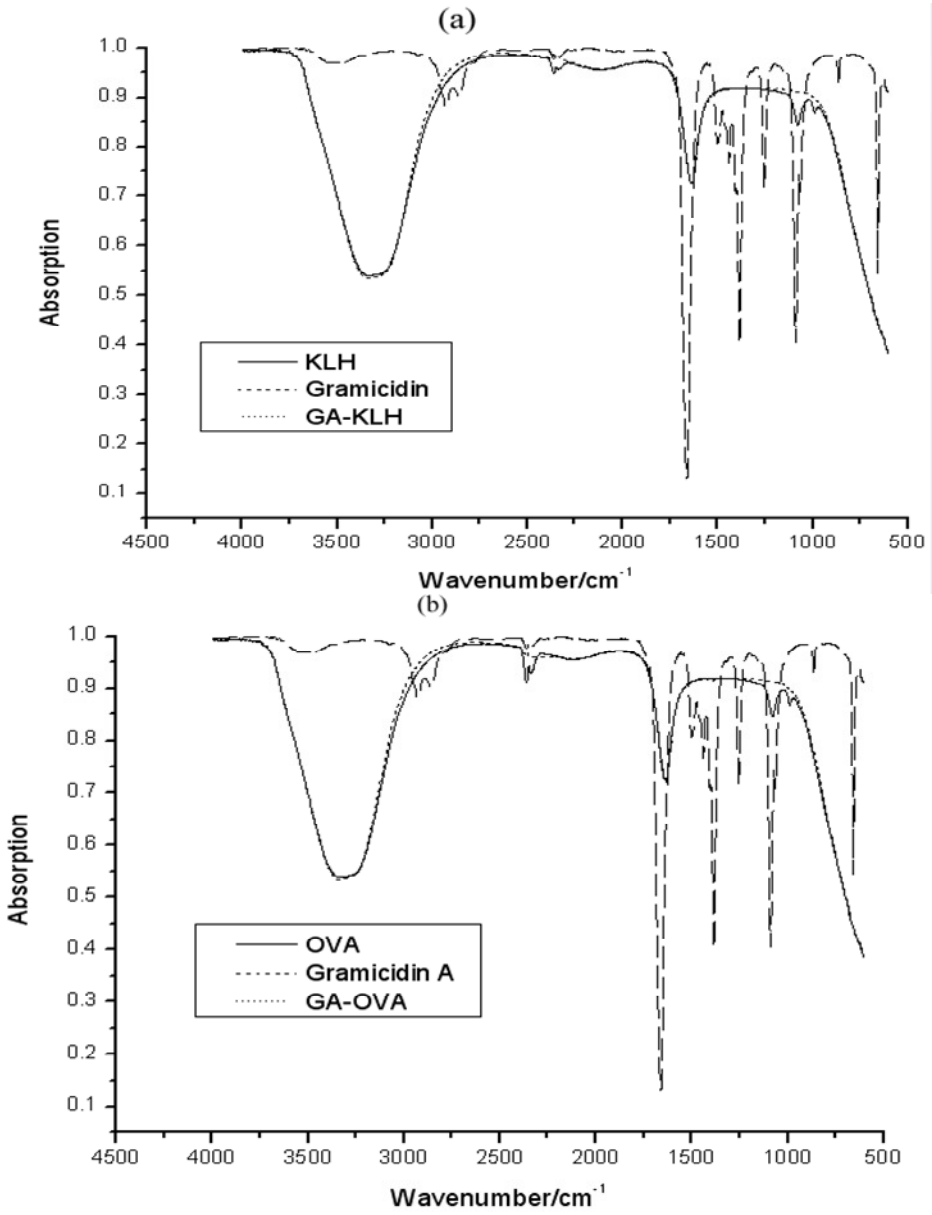

Figure 4. Comparative IR spectra of **(a)** gramicidin A, KLH and GA-KLH and **(b)** gramicidin A, OVA and GA-OVA.

existence of hydrosulfuryl in GA and KLH, whereas the GA-KLH did not have. Because the obvious peaks appeared in KLH and GA, the GA-KLH was flat. The Figure 4 (b) described analogical IR spectra of OVA, OVA-GA and the peaks of hydrosulfuryl were appearing in GA and OVA, but the GA-OVA did not have. This evidence showed the conjugate of GA to OVA or KLH was succeeded, on the other hand.

Electrophoresis assay

After Coomassie Blue staining, the SDS-PAGE results demonstrated the successful coupling between GA and OVA because of the feeble differences of displacements between OVA and GA-OVA. But the electrophoresis bands of KLH and GA-KLH yielded non-conclusive results caused by their high molecular weight as Figure 5 revealed.

Assay of hydrosulfuryl content and calculation of the couple ratio

The OD values of hydrosulfuryl in seven gradients of cysteine were measured as the method described in

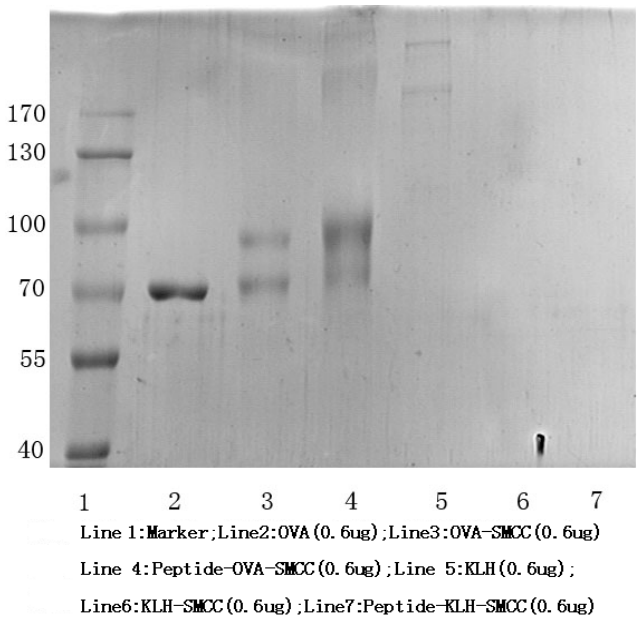

Figure 5. SDS-PAGE electrophoretogram of OVA, KLH and their conjugations.

above. A standard curve of seven gradients of cysteine was constructed. The adequacy and high accuracy of these dosages were deduced from the respective values of the correlation coefficient which was up to 0.9935 and the equation: Y = 0.1821X + 0.1005 was derived. In this equation, Y was the absorbance at 412 nm and X was hydrosulfuryl content (mM). The equation of the regression straight line was used to measure the content of hydrosulfuryl in activated GA, GA-KLH and GA-OVA.

The content of hydrosulfuryl in activated GA, GA-KLH and GA-OVA were determined by the method described above. The OD value of GA was 2.987 at 412 nm, the GA-KLH was 0.582 and the GA-OVA was 0.436. The molar concentration of hydrosulfuryl in GA deduced from the regression equation was 15.85, 2.64 mM in GA-KLH and 1.84 mM in GA-OVA. According to the formula of the calculation of the couple ratio, the couple ratio of GA-KLH was 83.3% and that of the GA-OVA was 88.4%.

DISCUSSION

Enzyme immunoassays are widely used in medicine, food, cosmetics fields to help rapid, simple, accurate and specific, but also cost-effective, quantification of many biologically essential small molecules. However, such technology needs to be improved or designed especially for rare haptens like hormones, drugs, insecticide, marine toxins or mycotoxins (Kim et al., 2003; Brun et al., 2004; Tomasz et al., 2009). The first challenge for researchers is to develop a method to prepare effective immunogens by coupling haptens to carrier proteins or poly-L-lysine (PLL). Towards this goal an impressive number of chemical and photochemical procedures have been published but few of them are applicable to very tiny amount of material with sufficient yield.

In a previous experiment, we had used the mixed acid anhydride reaction through dicyclohexylcarbodiimide (DCC) and 1-ethyl-3(3-dimethyl-amminopropyl) carbodiimide hydrochloride (EDC) to connect the GA with carrier proteins referred to by Edward et al. (1983). But we failed because of its low couple ratio which may be caused by the instability of existing molecular structure of the peptides. In this present report, we describe a rapid and efficient method to couple the peptide through free hydrosulfuryl by which we added a cysteine residue to the carboxyl-terminus of GA. It can be detected using DTNB reagent at 412 nm. Hydrosulfuryl group plays an important role in interaction with proteins or peptides. We make use of this kind of covalent bond called disulfide bond to achieve the purpose. At the same time, HPLC and ESI-MS were utilized to demonstrate the purity of the activated GA and the variation of molecular weight before and after the reaction. The single chromatograph of the activated GA showed the purity of the synthetic substance which is up to 99% calculated by work station of HPLC. On the other hand, the ESI-MS demonstrates that two lysines and one cysteine residues have been linked to the GA through the molecular weight. Hence, coupling reaction has happened between activated peptide and activated carrier proteins.

After coupling the artificial immunogen and solid coated antigen, characterization is absolutely necessary. For characterization of the conjugates, we compared the absorption spectra of the conjugates, the peptide and the carrier proteins alone using UV spectrophotometer from 400 to 190 nm and IR spectrophotometer from 4500 to 500 cm^{-1} to indicate disulfide bond formed. Moreover, a convincing evidence, that shows that there is obvious difference of UV and IR spectra among the KLH, OVA, GA and conjugates, demonstrates the success of coupling. The fine changes of molecular structure or chemical bond cause spectral absorption changes, using UV and IR spectrophotometers. At the same time, determination of hydrosulfuryl using DTNB reagent is a method of quantitative analysis which could control the course of the reaction and calculate the couple ratio. We have an efficient reaction to get the products; meanwhile the by-products can be removed by dialysis.

Unfortunately, the electrophoresis bands of KLH and GA-KLH yielded non-conclusive results by SDS-PAGE because the molecule weight of KLH is too large to move in the 12% acrylamide gel. Therefore, we only identified the clear electrophoresis bands of OVA and its relative products. From the electrophoretogram, a tiny longitudinal displacement happens among OVA, OVA-SMCC and GA-OVA due to the molecular weight (M.W.) 436.37 of sulfo-SMCC and M.W. 2170.75 of GA and the influence for electrophoresis is small.

In conclusion, high-quality artificial immunogen and coating antigen of GA were coupled using KLH and OVA with hydrosulfuryl conjugated method. The characterization of conjugates used infrared and ultraviolet spectrophotometry, SDS-PAGE and DTNB method. The conjugates can be exploited to prepare polyclonal antibody with rabbits and a number of immunological applications such as the preparation of synthetic vaccines and detection kit through the specific induction of high titer antibodies with higher affinities.

Abbreviations:

GA, gramicidin A; **KLH,** keyhole limpet hemocyanin; **OVA,** ovalbumin; **ESI-MS,** electron spray ionization mass spectrometry; **HPLC,** high performance liquid chromatography; **Sulfo-SMCC,** 4-(N-maleimidomethyl) cyclohexane-1-carboxylic acid 3-sulfo-N-hydroxy succinimide ester sodium salt; **SDS-PAGE**, sodium dodesyl sulfate-polyacrylamide gel electrophoresis; **HSA,** human serum albumin; **DTNB,** 5,5-dithiobis (2-nitrobenzoic) acid; **PBS,** Phosphate-buffered saline; **SPPS,** solid-phase peptide synthesis; **UV,** ultra-violet; **PLL,** poly-L-lysine; **TEMED,** N,N,N',N'-tetramethylethylene- diamine; **PEGA,** Polyethylene glycol polyacrylamide; **EDTA,** ethylene diamine tetraacetic acid.

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