Front. Agric. China 2008, 2(3): 355–360
DOI 10.1007/s11703-008-0044-0

RESEARCH ARTICLE

Fengshan GAO, Lei WANG, Xinsheng LI, Yungang LI, Huifei WANG, Qinmei FANG, Huifang HAO, Chun XIA

Cloning α and β chains of SLA-DR loci and reconstruction of their complex *in vitro*

Abstract In order to investigate the prompt conformations of swine major histocompatibility complex (MHC), a swine MHC-II protein complex (SLA-II) was reconstructed *in vitro*. *DRA* and *DRB* were cloned from a crossbred commercial pig (Changbai-Dalan). Subcloned extracellular parts of *DRA* and *DRB* were linked together by a linker containing rich glycine/serine (G4S)3, and the whole length of two genes, named *DRA-linker-DRB*, was amplified by splicing overlap extension PCR (SOE PCR). *DRA-linker-DRB* was inserted into pMAL-p2X prokaryotic system and expressed. The expressed fusion protein MBP-DRA-(G4S)3-DRB was identified by Western-blot, purified and cleaved to obtain the protein of interest DRA-(G4S)3-DRB. The secondary structure of this protein was determined in circular dichroism (CD) apparatus. The results indicated that the subsequent protein MBP-DRA-(G4S)3-DRB was soluble and its molecule weight was 83.4 ku in consistent with the Western-blot. Cleaved by Factor Xa, the protein of interest was separated with a molecular weight of 40.9 ku. The CD spectrum demonstrated that the protein displayed a favorable α-Helix structure, and the contents of α-Helix, β-sheet, turn, and random coil were 80 aa, 121 aa, 101 aa and 80 aa respectively. The identical ratios of α-Helix, β-sheet, turn, and random coil between MBP-DRA-(G4S)3-DRB and DRA-(G4S)3-DRB were 95%, 96.7%, 91.1% and 93.0%, respectively. The results also suggested that the reconstructed SLA-II complex presented an ideal conformation and can be used for studying its structure and function *in vitro*.

Keywords pig, MHC, loci, secondary structure

Translated from *Journal of China Agricultural University*, 2006, 11(3): 21–26 [译自: 中国农业大学学报]

Fengshan GAO (✉)
Bioengineering College, Dalian University, Dalian 116622, China

Fengshan GAO (✉), Lei WANG, Xinsheng LI, Yungang LI, Qinmei FANG, Huifang HAO, Chun XIA (✉)
College of Veterinary Medicine, China Agricultural University, Beijing 100094, China
E-mail: gfsh0626@126.com; Xiachun@cau.edu.cn

Huifei WANG
The Fire Engineering Department, the Chinese People's Armed Police Forces Academy, Langfang 065000, China

1 Introduction

The major histocompatibility complex (MHC) II is a cluster of genes which participate in antigen presentation. Swine MHC II complex is named SLA-II, mainly including DRa, DRb, DQa, DQb, DOb, DPa, TAP and LMP, etc. Among them, only SLA-DR and SLA-DQ belong to functional genes and they all have a 29 ku α-chain and a 34 ku β-chain which happens to be a heterogenous dimer. Similar to SLA-I, SLA-II also has a kind of extracellular protein which is divided into extracellular domain in the N-terminus, the transmembrane domain, and cytoplastic domain in the C-terminus. Different from SLA-I, SLA-II gene has two functional domains, α_1 and α_2 in α chain, β_1 and β_2 in β chain. The α_1 and β_1 forms four strands of β-fold and a strand of α-helix, separately, and eight strands of β-fold form a bottom structure which sustains two α-helixes from α-chain and β-chain, separately. These two α-helixes compose an open peptide-binding groove, which can bind 13–25 amino acids (Srinivasan et al., 1993; Patrick et al., 1999).

It has been a growing trend in molecular immunology to reconstruct MHC complex *in vitro* and to identify the amino acid sequences of bound peptides to MHC and to study their three-dimensional (3D) structures. Presently, the human and rat MHC class I complexes has been established *in vitro* (Uger and Barber, 1998; White et al., 1999; Denkberg et al., 2000; Wang et al., 2005) but there are few reports of reconstruction of MHC class II complex *in vitro*. The swine is an important economical animal and closely related to humans. As a model animal, swine SLA-II complex has been established *in vitro*, which will provide a research platform for screening B-cell or T-helper epitopes for some important viruses. In addition, it will be beneficial in improving the research of molecular immunology for swine and also in supplying the reference

model to study limited virus epitodes of human MHC II complex. In this experiment, we constructed a SLA-II complex contained α-chain and β-chain of DR locus in prokaryotic expressing system of pMAL-p2X. The reconstructed complex was analyzed by circular dichroism (CD) and the primary results indicate that the reconstructed complex can fold correctly *in vitro*, suggesting the complex has a correct conformation. Therefore, it is deduced that the complex may be used to identify the epitodes and to further study their 3D structure.

2 Materials and methods

2.1 Reagents, instruments and software

AMV reverse transcriptase, Ex Taq PCR kit, IPTG and X-gal, T4 DNA ligase and *Eco*R I was bought from the TaKaRa corporation in Dalian; Trizol was from the Invitrogen corporation; The GLASS MILK KIT was from the BODATAIKE corporation; pGEM-T Easy and *E. coli* TB1 vectors were from the Promega corporation; p2X vector and Factor Xa were from the NEB corporation. PCR geneAmp PCR System 9700 was from the Hybaid corporation; The Electrophoresis system was supplied by the Bio-RAD corporation.

2.2 Amplifying, cloning of *DRA* and *DRB* gene by RT-PCR, sequencing and analyzing

As described earlier (Yan et al., 2005), total RNA was extracted from the spleens of a crossbred pig (Changbai-Dalan) using the Trizol kit and then the first strand cDNA (fcDNA) was synthesized from the extracted total RNA by AMV Reverse Transcriptase with the primer OligodT-15 (Sangon in Shanghai).

Referring to the sequence (AY191779) in the DDBJ/EMBL/GenBank library, i.e. a swine *SLA-DRA* gene, primers were designed using the Oligo version 6.0 software. The forward primer (RA1) was: 5′-ATGACCATACT TGGGGTCCCAG-3′, and the reverse primer (RA2) was: 5′-GTCCATTCCCTGCAAGCACCTC-3′. DRA was amplified with the pair of primers RA1 and RA2. The volume of the PCR reaction was 50 μL. The template of fcDNA was 0.5 μg. The PCR reaction was carried out under the following conditions: 94°C, 3 min for pre-denaturation, then 94°C, 1 min for denaturation, 65°C 1 min for annealing, 72°C 2 min for extension, in a total 30 cycles followed by a final extension at 72°C for 10 min. The product of PCR (*DRA01*) was analyzed by 1 g per 100 mL agarose electrophoresis. The interest fragment was purified by GLASS MILK KIT and ligated to pGEM-T Easy vector, then transformed to JM109 competent cell. The positive clones identified by *Eco*R I and PCR were sequenced in Sanbo Corporation.

DRB was cloned in the same method as mentioned above. Referring to AY135579, the forward primer (RB1) was: 5′-GTTCTCCAGCATGTTGCATCTGTG-3′, and the reverse primer (RB2) was: 5′-AGAGGAT GCTTGCTTGGAGTGTC-3′. The PCR reaction was performed as follows: 94°C, 3 min for pre-denaturation, then 94°C 1 min for denaturation, 65°C 1 min for annealing, 72°C 2 min for extension, in 30 cycles followed by 72°C for 10 min. The products of PCR (*DRB01*) were purified, and cloned to pGEM-T Easy and sequenced by the Sanbo corporation.

The sequences were analyzed by GENETYX Version 9.0 (Software Development Co., Ltd, Tokyo, Japan).

2.3 Linking extracellular domains of *DRA* and *DRB* by SOE PCR, construction of pMAL-p2X fusion expression vector

As templates of *DRA01* and *DRB01* sequences, respectively, primers for expressing genes were designed in extracellular domains and a 15 amino acids linker was added to link the two fragments following the method of Yang et al. (2005). The forward and reverse primers in extracellular domain of *DRA01* were listed as follows, respectively: AP1: 5′-TCAGAATTCATCGTAGAGAATCACGTGA TCATCC-3′; AP2: 5′-ACCGCCAGAGCCACCTCCGC CTGAACCGCCTCCACCCTCCCAGTGCTTGAGAA GAGGCT-3′. The forward and reverse primers in the extracellular domains of *DRB01* were shown as follows, respectively: BP3: 5′-GGTTCAGGCGGAGGTGGCTC TGGCGGTGGC GGATCGAGGGACACCCCACCG CATTTC-3′; BP4: 5′-AGTAAGCTT TTACCATTCC ACTGTGACGGGGC-3′. *Eco*R I site and *Hin*d III site were added to primers AP1 and AP4, respectively. A pair of primers AP1/AP2 was used to amplify extracellular domain of *DRA01* on the template of pGEM-T Easy/*DRA01*. The PCR reaction condition was as follows: 94°C, 1 min, 65°C, 1 min, 72°C, 1 min, for 30 cycles and the final extension was 72°C for 10 min. Another pair of primers BP3/BP4 was used to amplify extracellular domain of *DRB01* on template of pGEM-T Easy/*DRB01*. The PCR reaction condition was the same as above. The two fragments of PCR product were purified by the GLASS MILK KIT and were then mixed as a molecular ratio of 1:1 and amplified for 16 cycles in a final volume of 50 μL without any primer. After that, 1 μL immediate product was taken out as a template to amplify using AP1/BP4 primer in another new 50 μL reaction system. The PCR reaction condition was as follows: 94°C, 3 min, 94°C, 1 min, 65°C, 1 min, 72°C, 1.5 min, for 30 cycles. The products of PCR (*DRA-linker-DRB*) were purified by the GLASS MILK KIT after electrophoresis, cleaved by restricted endoenzyme *Eco*R I and *Hin*d III, ligated to p2X vector and transformed to competent cell TB1.

2.4 Expression, SDS-PAGE, western-blot, purification and Xa factor cleavage

Recombinant *E. coli* TB1/p2X-DRA-linker-DRB was added to 100 mL LB medium and cultured at 37°C, 180 times per min until the OD_{600} was grown to 0.4–0.6, then induced by 0.3 mmol·L^{-1} IPTG and cultured for 6 h. One mL culture was collected and denatured with 2 × SDS and electrophoresed in SDS-PAGE. After electrophoresis, it was visualized using Coomassie Brilliant Blue (Xia et al., 2005). The expressed protein was named MBP-DRA-(G4S)3-DRB.

For Western-blot analysis, the protein bands were electrotransferred onto PVDF membrane at 100 V for 1 h. The filter membrane was blocked for 1 h with phosphate-buffered saline containing 5% bovine serum albumin, incubated with a rabbit anti-MBP antiserum (diluted at 1:3000) for 1 h and washed three times with PBS, each time for 10 min. The filter membrane was incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody diluted at 1:500, then washed twice with PBS, each time for 10 min. At last, proteins were visualized using a 4-chloro-1-naphthol/hydrogen peroxide substrate mixture.

The expressed product was purified according to the manufacture's instructions (NEB: pMAL™ Protein Fusion and Purification System Instruction Manual. Version 5.01. New England BioLabs Inc). The cells were collected by centrifugation at 5000 × g for 20 min and resuspended in a column buffer containing 20 mmol·L^{-1} Tris-HCl, 200 mmol·L^{-1} NaCl, 1 mmol·L^{-1} EDTA, then stored at −80°C. After sonication, the supernatant was recovered by centrifugation at 9000 × g for 20 min and was then loaded onto an amylose affinity resin column. The protein was eluted using a column buffer (20 mmol·L^{-1} maltose in buffer A). The result of purification was determined by SDS-PAGE. In order to harvest the protein with the wanted purity, a further purification using DEAE-sepharose ion exchange chromatography needed to be carried out.

The fusion protein has a MBP tag about 42.5 ku, and it could be cleaved and separated from the interest protein with factor Xa. One mg of the purified protein was incubated with 20 IU Factor Xa for 48 h, at 4°C. The cleavage mixture was separated and purified by DEAE-sepharose ion exchange chromatography and amylose affinity resin column, in turn. The purified protein was determined by SDS-PAGE.

2.5 Secondary structure

Protein concentrations were determined according to Bradford (1976), and diluted to 0.4 g·L^{-1}. Far UV (190–250 nm) spectrum spectra were recorded at 25°C for DRA-(G4S)3-DRB, MBP-DRA-(G4S)3-DRB and MBP using a Jasco model 715 spectrophotometer. The sample volume was 600 μL. Each reported spectra was the average of four scans and the mean residue molar ellipticity was used to express the CD data. The percentages of secondary structure elements were estimated using the software J-715 for Windows Secondary Structure Estimation (Version 1.10).

3 Results

3.1 RT-PCR of *DRA* and *DRB* genes, and sequencing

RT-PCR of *DRA* and *DRB* genes resulted in 779 bp and 836 bp fragment in an α-Imager after being determined on 1 g per 100 mL agarose electrophoresis, which was consistent with the theoretically calculated values (Fig. 1). *DRA01* and *DRB01* were sequenced, compared and analyzed, respectively. The results indicated that *DRA01* gene has 100% amino acid homology with other swine *DRA* alleles reported in GenBank. *DRB01* was shown to have a 90%–98% amino acid homology with other *DRB* alleles. The sequence of *DRB01* was submitted and published on DDBJ/EMBL/GenBank (AB205163).

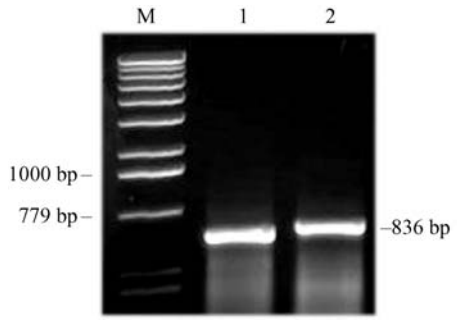

Fig. 1 Amplification of *DRA* and *DRB* by PCR
Note: M: 1 kb DNA ladder; 1: *DRA* gene, 779 bp; 2: *DRB* gene, 836 bp.

3.2 Reconstruction of pMAL-p2X fusion expression vector by SOE-PCR

After SOE PCR, a fragment of 1146 bp was amplified, which was identical to the theoretical value of the designed recombinant *DRA-linker-DRB* gene (Fig. 2). The reconstruction of pMAL-p2X/DRA-(G4S)3-DRB was shown in Fig. 3.

3.3 SDS-PAGE, Western-blot, purification and cleavage of the fusion protein

After induction of the recombinant DRA-(G4S)3-DRB/p2X, the expressed protein MBP-DRA-(G4S)3-DRB shows a size of about 80 ku band in SDS-PAGE electrophoresis which is consistent with the calculated value of 83.4 ku (Fig. 4a). The result of the Western-blot shows that the size of antibody hybrid band was about 80 ku

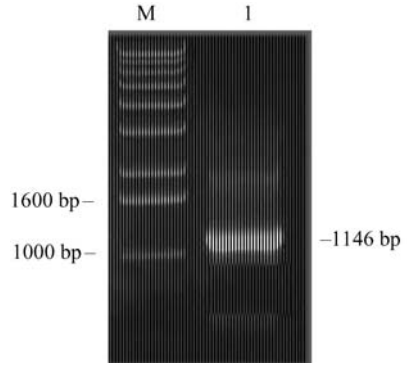

Fig. 2 Amplification *DRA-linker-DRB* by SOE-PCR
Note: M: 1 kb DNA ladder; 1: *DRA-linker-DRB*.

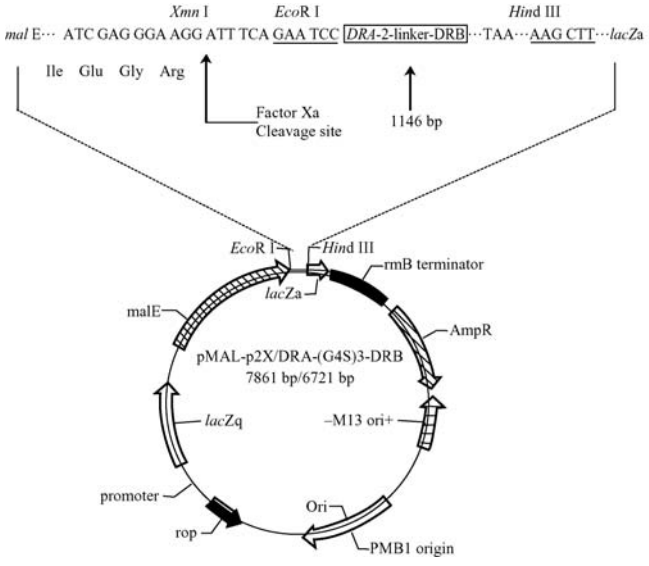

Fig. 3 Construction of pMAL-p2X/DRA-(G4S)3-DRB recombinant vector with *Eco*R I at carboxyl site of vector and *Hin*d III at amino site of vector
Note: The left arrow indicates Factor Xa cleavage site close to *Eco*R I and the right arrow indicated the inserted fragment and its length.

which was consistent with the size of expressed band (Fig. 4b). The expressed protein was purified with amylose affinity resin column and DEAE-sepharose ion exchange chromatography and its purity was about 90% (Fig. 4c). After a cleavage of the fusion protein by factor Xa, a single band with about 40 ku was shown and the concentration of single-band was increased more and more as the cleavage time was extended which proves that the fusion protein was cleaved specifically (Fig. 4d).

3.4 Determination and analysis of the secondary structure

The results of the secondary structure of the DRA-(G4S) 3-DRB, MBP- DRA-(G4S)3-DRB, and, MBP are shown in Figs. 5a, b, c. For DRA-(G4S)3-DRB, MBP-DRA-(G4S)3-DRB, and MBP, double minima were displayed at 208 and 222 nm by far UV CD spectrum analysis, indicating a conventional α-helical structure. The results of the calculation of the secondary structure elements of DRA-(G4S)3-DRB are listed in Table 1 using the CONTIN procedures and Yang-Chen's formula (Chen et al., 1972). In DRA-(G4S)3-DRB, a direct estimate reveals the contents of α-helix, β-sheet, turn, and random coil was 80 aa, 121 aa, 101 aa, and 80 aa, respectively. While the calculated contents of the α-helix, β-sheet, turn, and random coil of DRA-(G4S)3-DRB are 76 aa, 117 aa, 103 aa, and 89 aa, respectively.

4 Discussion

So far, no SLA-DR locus sequence cloned from the Changbai-Dalan hybrid pig has been reported in China. This experiment determined the sequences of *DRA* and *DRB* of the Changbai-Dalan pig by molecular cloning methods and analyzed them with the GENETYX software. The homology between *DRA* and other published *DRA* sequence (AY19177) on GenBank are 100%, while

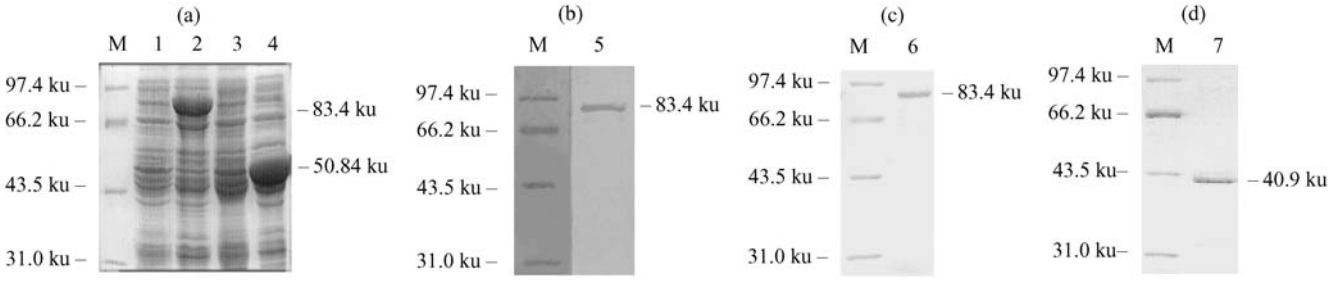

Fig. 4 SDS-PAGE, purification, cleavage and western-blot of MBP-DRA-(G4S)3-DRB
Note: (a) SDS-PAGE for MBP-DRA-(G4S)3-DRB in TB1; (b) Western-blot for MBP-DRA-(G4S)3-DRB; (c) Purification of MBP-DRA-(G4S)3-DRB; (d) Cleavage of MBP-DRA-(G4S)3-DRB. M: molecular weight standards. 1: TB1; 2: induced cells for recombinant MBP-DRA-(G4S)3-DRB; 3: uninduced cells for recombinant MBP-DRA-(G4S)3-DRB; 4: induced cells for recombinant p2X; 5: MBP-DRA-(G4S)3-DRB protein band visualized by western-blot; 6: purification of MBP-DRA-(G4S)3-DRB; 7: mixture of MBP (42.5ku) and DRA-(G4S)3-DRB as 40.9 ku.

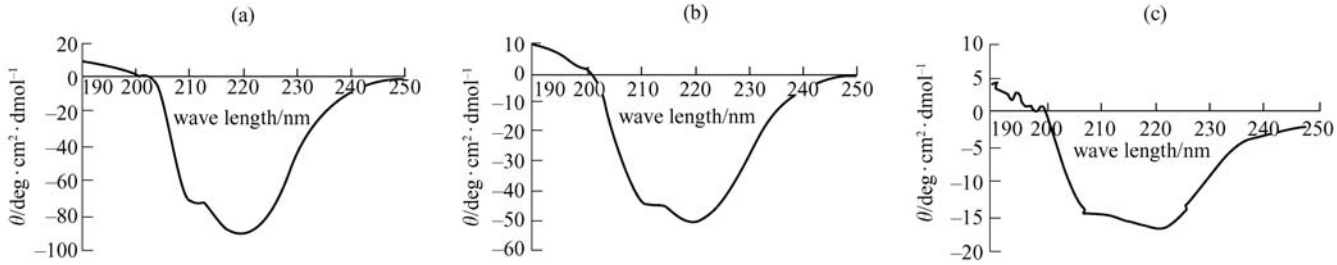

Fig. 5 Far UV CD spectrum of the DRA-(G4S)3-DRB, MBP-DRA-(G4S)3-DRB and MBP proteins

Table 1 Detecting result of secondary structure of elements of proteins

elements of secondary structure	CD estimation of DRA-(G4S)3-DRB (382aa)		CD estimation of MBP (392aa)		calculation of the secondary structure elements of DRA-(G4S)3-DRB	CD estimation of MBP- DRA-(G4S)3-DRB(774aa)	
	percentage/%	aa[1]	percentage/%	aa	aa[2]	percentage/%	aa
α-helix	20.2	80	18.9	74	76	19.4	150
β-sheet	38.5	121	19.1	75	117	24.8	92
turn	17.3	101	23.5	92	103	25..2	195
random coil	24.0	80	38.5	151	86	30..6	237

Note: 1: Denoted amino acids in every elements of secondary structure; 2: Denoted calculated amino acids which equal to the difference between the fusion protein MBP- DRA-(G4S)3-DRB and the monomer protein DRA-(G4S)3-DRB.

DRB genes manifest a homology range from 92.7% to 98.0%, compared with the other published allele genes (AY102479, AY1024801, AY102481, AY191776, AY243103 and AY243107, etc.).

According to early descriptions by Yang et al. (2005), a glycine-serine rich linker (G4S)3 could link two genes such as the extracellular regions of *DRA* and *DRB* to a new combined fragment by SOE-PCR and this would be in favor of studying the mutual action between *DRA* and *DRB*. In addition, since glycine is a simple amino acid in structure because of a lack of a side chain (only 1 H), it can easily regulate the space of other residues when β-turn is formed and it regulates the distortion and curvature of the β-layer while no collision will be caused. In short, it will basically not affect the structure of β-sheet. Serine often appears in the β-turn or random coil and the space between glycine and serine is only 3.5 nm. The secondary structure can not form in the process of folding which will not affect the correct folding and function of proteins. In our experiment, extracellular regions of *DRA* and *DRB* were found linked with a linker (G4S)3 and then amplified by SOE-PCR to obtain the combined gene, i.e., α and β chain of DR locus are linked into a complex by means of artificial manipulation, while the middle linker (G4S)3 favors formation of the correct folding between DRA protein and DRB protein and will not affect the natural structure of proteins (Wang et al., 2002).

In this experiment the prokaryotic expression system pMAL/p2X contained *malE* in the C-terminal of p2X and coded maltose binding protein (MBP), which promoted foreign protein to express as a soluble state (NEB: pMAL™ Protein Fusion and Purification System Instruction Manual. Version 5.01. New England BioLabs Inc). Compared with other expression systems such as His6 or GST system, the pMAL system could produce soluble foreign proteins more easily while soluble protein would form correct folding more easily (Kapust and Waugh, 1999). Because of a specific affinity to maltose, fusion protein labeled with MBP could be purified by Amylose Affinity Resin Column (NEB Inc.) and the operation was quite simple and feasible. There was a specific sequence Ile-Glu-Gly-Arg between *malE* and multiple cloning site, specifically recognized by Xa factor, so MBP protein could be cleaved and the interest protein could be separated from MBP. In this experiment, the fusion protein MBP-DRA-(G4S)3-DRB and the monomer protein DRA-(G4S)3-DRB, were all favorably soluble as determined by SDS-PAGE and this will be beneficial to the study of the structure and function of proteins.

Circular dichroism (CD) spectrum is a technology that applies asymmetric molecules which had different absorption in circular polarization to produce elliptically polarized light in analyzing secondary structure of the protein in solution. The content of α-Helix, β-sheet, turn and random coil can be calculated by means of quantifying the molar ellipticity in the secondary structure of a protein. In this experiment, elements of the secondary structure of the fusion protein MBP-DRA-(G4S)3-DRB, the monomer protein DRA-(G4S)3-DRB and MBP were determined by CD. By comparing the determined and the calculated CD value of DRA-(G4S)3-DRB, the identical ratios of α-helix, β-sheet, turn, and random coil are shown in high consistency as 95% (76/80), 96.7% (117/121), 91.1% (92/101) and 93.0% (80/86), respectively. In addition, the

circular dichroism spectrum of the fusion protein and the monomer protein both display similar alpha helix with each other, suggesting the two proteins have the same favorable conformation and the reconstructed complex DRA-(G4S)3-DR can be used for further peptide binding or crystal structure analysis.

References

Bradford M N (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem, 72: 248–254

Chen Y H, Yang J T, Martinez H M (1972). Determination of the secondary structures of proteins by circular dichroism and optical rotary dispersion. Biochemistry, 11(22): 4120–4131

Denkberg G, Cohen C J, Segal D, Kirkin A F, Reiter Y (2000). Recombinant human single-chain MHC-peptide complexes made from *E. coli* by *in vitro* refolding: functional single-chain MHC-peptide complexes and tetramers with tumor associated antigens. Eur J Immunol, 30(12): 3522–3532

Kapust R B, Waugh D S (1999). *Escherichia coli* maltose-binding protein is uncommonly effective at promoting the solubility of polypeptides to which it is fused. Protein Sci, 8(8): 1668–1674

Patrick M R, Buchbinder D, Jolley X, Dennett, Buchanan R (1999). Incidence of inflammatory myopathies in Victoria, Australia, and evidence of spatial clustering. J Rheumatol, 26(5): 1094–1100

Srinivasan M, Domanico S Z, Kaumaya P T, Pierce S K (1993). Peptides of 23 residues or greater are required to stimulate a high affinity class II-restricted T cell response. Eur J Immunol, 23(5): 1011–1016

Uger R A, Barber B H (1998). Creating CTL targets with epitope-linked beta 2-microglobulin constructs. J Immunol, 160(4): 1598–1605

Wang J Y, Zhu S G, Xu C F (2002). Biochemistry. 3rd ed. Beijing: Higher Education Press, 197–252 (in Chinese)

Wang Y, Rubtsov A, Heiser R, White J, Crawford F, Marrack P, Kappler J W (2005). Using a baculovirus display library to identify MHC class I mimotopes. Proc Natl Acad Sci USA, 102(7): 2476–2481

White J, Crawford F, Fremont D, Marrack P, Kappler J (1999). Soluble class I MHC with beta2-microglobulin covalently linked peptides: specific binding to a T cell hybridoma. J Immunol, 162(5): 2671–2676

Xia C, Wu D, Wu W X, Wan J Q, Wang L, Yang T Y, Wang Q, Ning Y B (2005). Cloning and expression of interferon-alpha/gamma from a domestic porcine breed and its effect on classical swine fever virus. Vet Immunol Immunopathol, 104(1–2): 81–89

Yan R Q, Li X S, Yang T Y, Xia C (2005). Characterization of BF2 and beta(2)m in three Chinese chicken lines. Vet Immunol Immunopathol, 108(3–4): 417–425

Yang D F, Zhu H F, Wang Z H, Shen G X, Tian D Y (2005). Construction of single chain Fv antibody against transferrin receptor and its protein fusion with alkaline phosphatase. World J Gastroenterol, 11(21): 3300–3303