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ARTICLE

Expression of Recombinant AccMRJP1 Protein from Royal Jelly of Chinese Honeybee in *Pichia pastoris* and Its Proliferation Activity in an Insect Cell Line

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Major royal jelly protein 1 (MRJP1) is the most abundant member of the major royal jelly protein (MRJP) family of honeybee. Mature MRJP1 cDNA of the Chinese honeybee (*Apis cerana cerana* MRJP1, or AccMRJP1) was expressed in *Pichia pastoris*. SDS-PAGE showed that recombinant AccMRJP1 was identical in molecular weight to the glycosylated AmMRJP1 from the Western honeybee (*Apis mellifera*). Western blots probed with anti-AccMRJP1 antibody demonstrated that recombinant AccMRJP1 and soluble protein of the Western honeybee RJ (AmSPRJ) contained immunoreactive MRJP1. The 57 kDa protein in AmSPRJ contained an N-terminal amino sequence of N-I-L-R-G-E, which is identical to that previously characterized in AmMRJP1. The molecular weight of recombinant AccMRJP1 was decreased from 57 to 48 kDa after deglycosylation, indicating that AccMRJP1 was glycosylated. The recombinant AccMRJP1 significantly stimulated Tn-5B-4 cell growth, similar to AmSPRJ and fetal bovine serum, and affected cell shape and adhesion to the substrate.

KEYWORDS: ***Apis cerana cerana*; royal jelly; AccMRJP1; eukaryotic expression; proliferation activity**

INTRODUCTION

Royal jelly (RJ), a principal food of the honeybee queen and larvae of worker bees and drones within three days of emergence, is secreted from the hypopharyngeal and mandibular glands of nurse honeybees (*1*). In addition to the important role in regulating caste determination, development, and reproduction, RJ has a wide range of other biological activities, including vasodilative and hypotensive activities (*2*), antitumor activity (*3*), anti-inflammatory activity (*4*, *5*), antibacterial and disinfectant activities (*6*, *7*), and antifatigue activity (*8*). Moreover, RJ can increase the average lifespan of mice (*9*). Due to its richness of a large number of bioactive substances, RJ has been widely used as a popular and traditional food for health promotion, dietary supplementation, and cosmetic applications (*10*).

Proteins constitute about 50% of the dry mass of RJ (*11*), whereas the major royal jelly protein (MRJP) family accounts for 80−90% of total protein content (*12*). Previous studies have identified nine members of the MRJP family in RJ of the Western honeybee (*Apis mellifera*) (*13*). The most abundant protein is MRJP1, which constitutes 48% of water-soluble proteins in RJ (SPRJ). AmMRJP1, previously named apalbumin-1 (*14*−*16*), represents the MRJP1 in RJ of the Western honeybee. AmMRJP1 is a glycoprotein with a molecular weight of 55−57 kDa (*14*). The open reading frame (ORF) of the AmMRJP1 gene is a fragment of 1299 bp, which encodes a protein of 422 amino acid residues with a predicted molecular weight of 48 kDa for a deglycosylated protein (*15*−*17*). Previous studies have shown that AmMRJP1 is also an acidic heat-resistant protein and forms oligomeric complexes of 290 kDa (*17*). AmMRJP1 oligomers consist of a 55 kDa AmMRJP1 monomer and a 5 kDa apisimin, which is a joining protein that bridges AmMRJP1 monomers to the AmMRJP1 oligomer (*17*). As it contains a relatively high content of essential amino acids (48%), MRJP1 is considered as a potential ingredient of functional foods (*18*). In addition to its nutritional role in honeybee larval development, AmMRJP1 was found to be expressed in mushroom bodies of adult honeybee brain and to possess important functions, possibly involved in the development of learning ability (*19*). The C-terminus of AmMRJP1 may encode a precursor form of the antimicrobial peptide jelleine (*20*). It has been found that AmMRJP1 can stimulate mouse macrophages to release tumor necrosis factor-α (*21*). It has been confirmed that natural AmMRJP1 can stimulate proliferation of rat hepatocytes (*14*), promote hepatocyte DNA synthesis, and increase albumin production in the absence of fetal bovine serum (FBS) (*14*, *15*). AmMRJP1 oligomer can enhance and sustain cell proliferation in human lymphoid Jurkat cells in a dose-dependent manner (*17*). Thus, MRJP1 is considered to be an important potential substitute of FBS (*14*, *16*).

Due to the important functions of MRJP1, studies on recombinant MRJP1 have received much attention. Recombinant AmMRJP1 was first expressed in *Escherichia coli* (*22*). However, as with most recombinant proteins expressed in a prokaryotic

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system, this was insoluble, present in inclusion bodies, and not glycosylated and postprocessed and, therefore, lacked biological activity, thereby limiting its applicability. The AmMRJP1 gene was also introduced into tobacco plants and successfully expressed in tobacco leaf (*18*). However, the low expression level of the glycosylated AmMRJP1 in these plant cells limits its utilization. The AciMRJP1 gene from Indian honeybee (*Apis cerana indica*), a subspecies of the Asian honeybee (*A. cerana*), was first cloned (*23*) and expressed in *E. coli* (*24*) in Thailand.

The Chinese honeybee is another common local Asian honeybee subspecies that is primarily distributed in China (*25*). The AccMRJP1 gene from the Chinese honeybee was first expressed in the larvae of silkworm, *Bombyx mori* (*26*). Recently, the gene was expressed in *E. coli* by our laboratory (*27*). However, it has been understood that most recombinant proteins expressed in prokaryotic systems are not glycosylated and remain insoluble in inclusion bodies. Thus, as we previously described, recombinant proteins expressed in *E. coli* lack critical biological activities (*27*).

Here, we report the cloning of the AccMRJP1 gene from heads of the Chinese honeybee nurse and expression of purified mature peptide in *Pichia pastoris*. In addition, we have confirmed that recombinant AccMRJP1 expressed in *P. pastoris* was glycosylated and have measured the proliferation activity of the recombinant glycosylated AccMRJP1 in the Tn-5B-4 insect cell line.

MATERIALS AND METHODS

Bacterial Strains and Chemicals. The bacterial strains *E. coli* TG1 and DH5a and the cDNA library of the head of Chinese honeybee nurses constructed by James D. Watson Institute of Genome Sciences, Zhejiang University, are maintained in our laboratory. *P. pastoris* strain GS115 and expression vector pPIC9K were purchased from Invitrogen Corp. (Carlsbad, CA). Ethidium bromide (EB) and DL-2000 and DL-12000 markers were purchased from TakaRa (Dalian, China). *Taq* polymerase, PCR purification kit, and ampicillin were purchased from Sagon (Shanghai, China). T4 DNA ligase was purchased from Fermentas International Corp (Burlington, Canada). PCR purification, plasmid DNA extraction, and yeast DNA extraction kits were purchased from OMEGA Bio-Tek Corp (Doraville, GA). Anti-glutathione *S*-transferase (GST)−AccMRJP1 rabbit polyclonal antibody prepared with recombinant GST-AccMRJP1 fusion protein expressed in *E. coli* was preserved in our laboratory (*27*). Goat anti-rabbit IgG-linked horseradish peroxidase (IgG/HRP) and diaminobenzidine (DAB) were purchased from Boster Biological Technology, Ltd. (Wuhan, China). Mouse anti-histidine monoclonal antibody was purchased from Novagen, Inc. (Madison, WI). Goat anti-mouse IgM-linked horseradish peroxidase (IgM/HRP) was purchased from Shanghai Sino-American Technology Co., Ltd. (Shanghai, China). PVDF membrane was purchased from Millipore Corp. (Billerica, MA). SinoBio protein marker and enterokinase (EK) were purchased from Shanghai Sino Biotech Co., Ltd. Prepacked ready-to-use HisTrap FF crude affinity columns (1 mL) were purchased from GE Healthcare Bio-Sciences Corp (Piscataway, NJ). Fresh RJ of the Western honeybee was provided by Hangzhou Biyutian Health-care Production Co. Ltd. (Hangzhou, China) and stored at −70 °C until used. *Trichoplusia ni* cell line (Tn-5B-4 cell) was maintained in our laboratory. TNM-FH medium was purchased from Sigma Corp. of America (Ronkonkoma, NY). Fetal bovine serum (FBS) was purchased from GIBCO BRL (Grand Island, NY). A Bradford Method Protein Assay Kit was purchased from Shanghai Generay Biotech Co., Ltd. (Shanghai, China). An *N*-Glycosidase F deglycosylation kit was purchased from New England Biolabs, Inc. (Ipswich, MA). All kits were used according to the instructions of the individual manufacturer.

DNA Sequencing and Data Analysis. Expression sequence tags (ESTs) were assembled into contigs using sequence assembly programs CAP3 and PHRAP (www.genome.washington.edu/UWGC). All ESTs in the AccMRJP1 contigs were filtered with the DNASTAR program Lasergene (Madison, WI), and only those ESTs containing a complete 5′-terminus of the AccMRJP1 gene sequence were chosen for further analysis. The selected clones were sequenced by Shanghai Sangon Biotechnology Corp. Homologous sequences were then searched with BLASTN (www.ncbi.nim.nih.gov), aligned with ClustalW (http://www.ebi.ac.uk/Tools/clustalw/), and visualized with GenDoc (http://www.psc.edu/biomed/gendoc). The aligned DNA sequences were converted to protein sequences with Genetyx-Win version 5 (Software Development Co., Tokyo, Japan). The location of signal peptide cleavage sites in the amino acid sequence of AccMRJP1 was predicted using SignalP 3.0 (http://www.expasy.org/tools).

Amplification of AccMRJP1 cDNA and Construction of AccMRJP1-pPIC Expression Vector. The pBluescript II XR plasmid with the entire AccMRJP1 sequence served as a template. Primers of mature AccMRJP1 were constructed from the cDNA of AccMRJP1. A *Eco*RI restriction site (underlined), $(His)_6$-Tag encoded nucleotides (boldface), and an enterokinase cleavage site (italic) were incorporated in a forward primer F1 (5′-AGAATTC**CATCATCATCATCAT CAT***GATGACGACGACAAG*AGCATTCTTCGAGGA-3′), whereas a *Not*I restriction site (underlined) was incorporated in primer R1 (5′-CCGGCGGCCGCTTA CAGATGTATTGAAATTTTG-3′). Primers F1 and R1 were then used to amplify the AccMRJP1 gene from AccMRJP1 cDNA. Polymerase Chain Reaction (PCR) was performed in a programmable thermal controller (BOER, China) with one cycle at 94 °C for 4 min, followed by 30 cycles of denaturation (20 s at 94 °C), annealing (20 s at 57 °C), and extension (40 s at 72 °C) and ending at 72 °C for 10 min. PCR products were visualized on a 2.5% agarose gel with EB staining. The purified AccMRJP1 fragment was inserted into an *Eco*RI/*Not*I digested pPIC9K vector to construct pPIC9K−AccMRJP1, which was then transformed into DH5a cells. The resulting construct, pPIC9K−AccMRJP1, was used to transform DH5a cells. The plasmid was subjected to PCR, restriction digestion, and DNA sequencing to confirm the correct in-frame fusion between the sequences encoding the *a* factor secretory signal of pPIC9K and the AccMRJP1 coding region.

Transformation of *P. pastoris*. The AccMRJP1−pPIC9K vector was linearized with *Sac*I and electroporated (1.5 kV, 25 μF, and 200 Ω, as described in the published manual of (Invitrogen) into *P. pastoris* GS115 genome at the alcohol oxidase 1 (*AOX1*) locus. After electroporation, 1 mL of ice-cold 1 M sorbitol was immediately added to the GS115 cells. The cells were then spread on MD plates [1.34% yeast nitrogen base (YNB), 4×10^{-5}% biotin, 2% dextrose, and 2% agar] and incubated at 30 °C for 3 days. The His^+ transformants were selected among GS115 cells according to the published method of Invitrogen. The His^+ and MutS phenotypes of transformants were evaluated by spotting them onto minimal medium (MM) agar plates (1.34% YNB, 4×10^{-5}% biotin, 0.5% methanol, 1.5% agar) as the primary carbon source.

Selection of Secreting Colonies and Small-Scale Expression. Genomic integration of AccMRJP1 clones at the *P. pastoris* AOX1 loci was screened by PCR on individual colonies, using forward primer 5′*AOX1* (5′-GACTGGTTCCAATTGACAAGC-3′) and reverse primer 3′*AOX1* (5′-GCAAATGGCATTCTGACATCC-3′). Amplified product was then analyzed by agarose electrophoresis and sequenced. Each positive colony was inoculated into 2 mL of buffered minimal glycerol BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 0.4 mg/L biotin, 1% glycerol) and incubated at 30 °C in a shaking incubator (250 rpm) for 24 h followed by addition of methanol (1% at 12 h intervals) with further incubation for 48 h. Culture supernatants were harvested by centrifugation at 24, 48, and 72 h after induction for analysis by sodium dodecyl sulfate−polyacrylamide gel (12%, v/v) electrophoresis (SDS-PAGE) according to a described protocol (*28*).

The electrophoresed protein samples of the recombinant AccMRJP1 from different transformants were transferred from the SDS-PAGE gel to a PVDF membrane for 15 min at 0.2 A in a Bio-Rad transblot apparatus. The membranes were incubated with the anti-His antibody (1:3000 dilution). After a rinse to remove unbound primary antibody, the membranes were exposed to the 1:5000 dilution of goat anti-mouse IgM/HRP (secondary antibody), followed by DAB substrate for colorimetric detection. The colonies with clear possible target protein band on SDS-PAGE profile and confirmed by the Western blot will be selected as higher level expression transformants.

Production, Purification of AccMRJP1. The selected colonies were inoculated in 100 mL of BMGY in a 500 mL flask and incubated at 30 °C in a shaking incubator (250 rpm) for 2 days (OD_{600} of 2−6). Cells were harvested and resuspended in 500 mL of BMMY medium until the value of OD_{600} dropped to 1.0 and then incubated at 28 °C and 250 rpm. Scaling

this procedure to five additional flasks (500 mL) provided enough sample for purifying AccMRJP1 using prepacked ready-to-use HisTrap FF crude affinity columns. For induction, methanol (2%) was added to the culture at 24, 48, and 72 h during incubation to maintain a concentration of 1% (v/v).

Histidine-containing, yeast-derived AccMRJP1 was purified from induced yeast culture supernatant using prepacked ready-to-use HisTrap FF crude affinity columns with minor modifications. The culture supernatant was cleared by two rounds of centrifugation (4000*g* for 20 min, 10000*g* for 20 min), filtered through a 0.2 μm low protein binding membrane filter (Corning Inc., Corning, NY), and subsequently applied to 1 mL prewashed and pre-equilibrated purification columns using a peristaltic pump. HisTrap FF columns were washed with 3−5 column volumes of 1× phosphate-buffered saline (PBS) containing 20 mM imidazole, and bound protein was eluted in 1 mL fractions with 1× PBS containing 500 mM imidazole. Eluted fractions were dialyzed against 1× PBS overnight at 4 °C. HisTrap FF crude columns allowed for rapid purification by using only partly clarified extracts and eliminating the need for the 10000*g*/20 min centrifugation and filtration.

Aliquots of the purified product and the culture supernatant were subjected to SDS-PAGE, using the culture supernatants of GS115 transformed with pPIC9K and GS115 transformed with pPIC9K−AccMRJP1 as positive and negative controls, respectively. The purified recombinant AccMRJP1 was confirmed by using the Western blot with the anti-His antibody again. The concentration of the recombinant protein was measured by the dye-binding assay method of Bradford with bovine serum albumin as standard. After purification, the proteins were placed in the sealed dialysis bag and dipped in double-distilled water to remove imidazole over 24−48 h, with at least 6six changes of water, and then lyophilized, weighed, and stored at −70 °C.

Deglycosylation of AccMRJP1. Deglycosylation of the purified recombinant AccMRJP1 was done with an *N*-glycosidase F deglycosylation kit. Purified recombinant AccMRJP1 (20 μg) was denatured by heating at 100 °C for 10 min and then incubated with 1000 U of *N*-glycosidase F for 1 h at 37 °C. An identical treatment with *N*-glycosidase F mixture without the purified recombinant AccMRJP1 was used as a negative control. The undigested recombinant AccMRJP1 was used as a positive control. The resulting products were analyzed by SDS-PAGE.

Extraction of the Soluble Proteins of RJ. Soluble proteins (AmSPRJ) were extracted with 1× PBS from the Western honeybee RJ as described by Salazar-Olivo and Paz-Gonzalez (*1*). After removal of salt by dialysis, the AmSPRJ and purified His-AccMRJP1 were lyophilized, weighed, and stored at −70 °C until used.

Western Blot Assay with Anti-GST−AccMRJP1 Polyclonal Antibody and N-Terminal Amino Acid Sequence. We used anti-GST−AccMRJP1 polyclonal antibody (1:1000 dilution) as the primary antibody to further confirm the existence of recombinant AccMRJP1 and AmSPRJ. The samples of recombinant AccMRJP1 and AmSPRJ were separated by SDS-PAGE and electroblotted onto PVDF membranes, the treatment membranes were exposed to the 1:5000 dilution of goat anti-rabbit IgG/HRP (secondary antibody) and reacted with DAB substrate as described above. The 57 kDa bands of AmSPRJ on PVDF without treatment with antibody were excised and subjected to sequencing by automated Edman degradation on a gas phase sequencer [ABI Procise 492cLC (GC320078)].

Assay of Proliferation Activity in Insect Cells. The purified His−AccMRJP1 was cleaved by enterokinase at the concentration 10 μL/mL in 1× PBS at 37 °C for 24 h. The AccMRJP1 fraction was isolated with the HisTrap FF crude columns as described above under Production, Purification of AccMRJP1. After removal of salt by dialysis, the purified AccMRJP1 was lyophilized, weighed, and stored at −70 °C. We followed a published procedure (*1*) with minor modifications to test the proliferation activity of AccMRJP1 in Tn-5B1-4 cells. The protein samples were diluted with sterilized double-distilled water until the protein concentrations of the samples reached 8.09 μg/mL (AmSPRJ) and 8.24 μg/ML (AccMRJP1), respectively. The samples were then filtered with a Millipore syringe-driven filter (Billerica, MA) to remove microorganisms. Tn-5B1-4 cells were plated (1×10^6 cells/dish) in 12 culture dishes supplemented with 10% FBS for 2 days. The 12 dishes were divided into 4 groups (3 dishes per group). The four groups of dishes were then twice-washed with FBS-free medium and fed with either 10% FBS-supplemented TNM-FH or serum-free TNM-FH containing AmSPRJ, serum-free TNM-FH containing purified recombinant AccMRJP1, and serum-free TNM-FH (CK). Culture dishes were maintained at 28 °C for 48 h and later photographed at 200× magnification at the dish center under the fluorescence and inverted microscope at 24 and 48 h after changing culture media. The cell number in each picture was measured by display on a computer screen. The polyhedral cells adhering to the substrate were considered as living cells; the rounded and stratified cells, as well as cells floating on the culture medium, were considered as the dead cells. The average proportion of living cells across the three dishes in each group was calculated as the measure of cell survival rate for each group.

Statistical Analysis. Data analysis was performed with the statistical software SPSS 16.0 (SPSS, Inc., Chicago, IL). One-way ANOVA with Duncan's post hoc tests was adopted to determine if there were any significant differences between any two treatment groups. Values are reported as mean ± SD. p values are two tailed, and a p value of < 0.05 is considered to be significant.

RESULTS

Sequence Analysis of AccMRJP1 cDNA. A total of 3574 ESTs were assembled into one contig encoding AccMRJP1. The fact that 41.7% of all ESTs (8569) of the cDNA library from the head of Chinese honeybee nurses encoded AccMRJP1 indicates that AccMRJP1 is the most abundantly expressed gene in this sample. Eight clones containing the 5′ end of the complete AccMRJP1 cDNA were sequenced. The nucleotide sequence of AccMRJP1 was deduced from the sum of these clones, which yielded an open-reading frame (ORF) of 1302 base pairs (**Figure 1**) with the predicted protein molecular weight of 47.6 kDa. AccMRJP1 shares 99.80 and 90.5% amino acid similarity with the previously reported AccMRJP1 sequence from the Chinese honeybee (accession AY279539) (*29*) and AmMRJP1 (accession NM_001011579) from the Western honeybee, respectively. AccMRJP1 contained a putative signal peptide of 20 residues and a mature peptide of 413 residues with a theoretical molecular weight of 43.6 kDa containing three potential N-linked glycosylation sites (**Figure 1**). These structural features are identical to those in AciMRJP1 from the Indian subspecies of *A. cerana* (*23*, *24*) and AmMRJP1 from the Western honeybee (*30*).

Construction of Expression Vector and Transformation of *P. pastoris*. After cloning of the 1302 bp cDNA into pBluescript II XR, sequencing showed that the inserted DNA fragment is 1275 bp including 1239 bp encoding the mature peptide of AccMRJP1 and an inserted 36 bp sequence encoding the $(His)_6$ tag. This protein showed an EK cleavage site for enterokinase, which would cleave the AccMRJP, and so the DNA fragment encoding 423 amino acids was identical to our design. Transformation of the AccMRJP1−pPIC9K vector into GS115 *P. pastoris* was confirmed by PCR from the His^+/Mut^+ transformants (containing *AOX1* conferring growth on methanol as the sole carbon source) (**Figure 2**). We selected seven transformants that contain the vector GS115−pPIC9K−AccMRJP1 to express AccMRJP1 in the next step.

Small-Scale Expression Studies. SDS-PAGE profiles from the culture supernatant of cells 3 days postinduction showed an expressed product of 57 kDa (**Figure 3A**), > 10 kDa above the predicted molecular weight (47.8 kDa) of the recombinant protein fused to $(His)_6$-EK. The difference of expression level for recombinant protein among seven selected transformants was also shown on the SDS-PAGE profile. The 57 kDa protein band was absent in the positive and negative controls. Three transformants possessing higher expression levels (**Figure 3A**, lanes 1, 2, and 4) were selected on the basis of SDS-PAGE profile. Western blot analysis indicated the 57 kDa protein bands expressed by these recombinants could be recognized by the anti-His antibody (**Figure 3B**). These transformants were used to prepare purified recombinant protein.

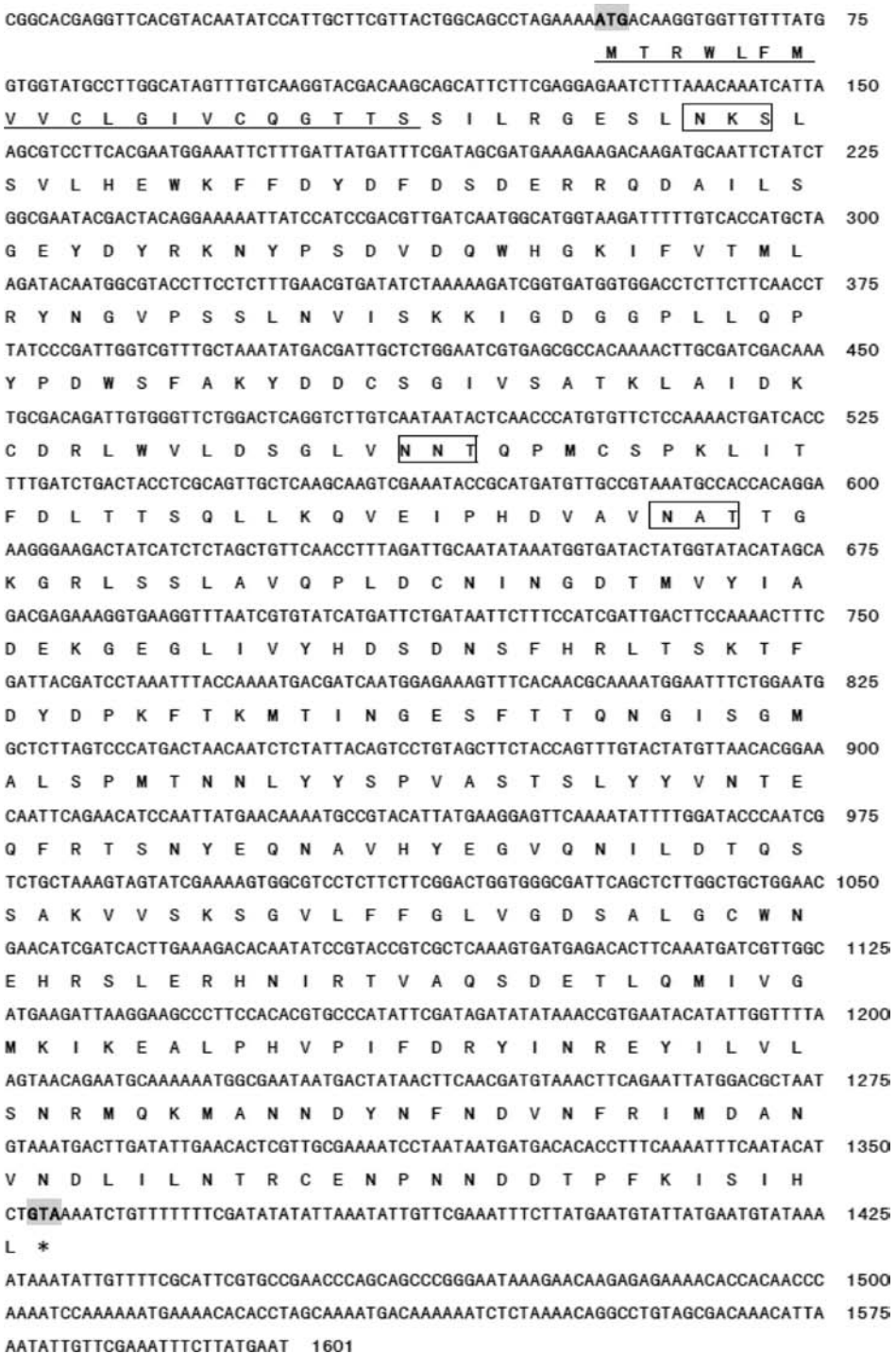

Figure 1. Nucleotide and deduced amino acid sequence of AccMRJP1 in Chinese honeybee. Start and stop codons are shaded and bold-faced. Potential N-linked glycosylation sites are boxed. The signal peptide sequence is underlined.

Purification of Recombinant AccMRJP1 and Preparation of AmSPRJ. Under the optimal growth conditions determined above and after 72 h of induction, 2 L of cell-free culture medium containing AccMRJP1 were collected. The fusion protein was purified with HisTrap affinity columns and yielded a single band of ~57 kDa by SDS-PAGE (**Figure 4A**, lanes 1 and 2). It could be recognized by the anti-His antibody again on Western blot profile (the result is identical to **Figure 3B**). The yield of purified recombinant AccMRJP1 was 0.11 mg/mL in culture broth. AmSPRJ was extracted from RJ of Western honeybee with PBS. The SDS-PAGE profile showed four protein bands with estimated molecular weights of 25, 57, 67, and 80 kDa (**Figure 4B**, lane 1), similar to those previously reported (*1*). Western blot analysis utilizing anti-GST−AccMRJP1 polyclonal antibody demonstrated that affinity-purified recombinant AccMRJP1 samples contained immunoreactive 57 kDa proteins (**Figure 4C**, lanes 2−4), which were identical to the molecular weights of the glycosylated AmMRJP1 from natural RJ (**Figure 4C**, lane 1) and a previous study (*31*). N-Terminal amino acid sequence analysis showed that the 57 kDa band of AmSPRJ contained a sequence of N-I-L-R-G-E, which is identical to that previously characterized in AmMRJP1 (*15*).

Deglycosylation of AccMRJP1. The purified recombinant AccMRJP1 was digested with *N*-glycosidase F and subjected to

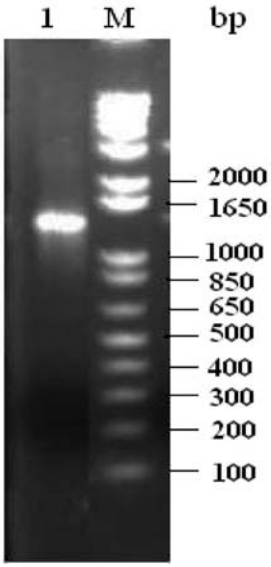

Figure 2. Detection of PCR amplification product of AccMRJP1. Lanes: 1, AccMRJP1 PCR product; M, DL12000 DNA marker.

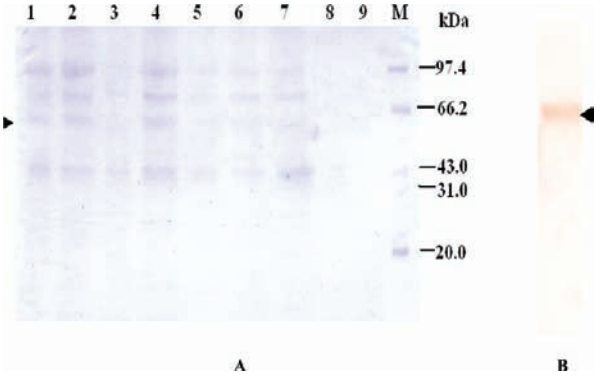

Figure 3. SDS-PAGE analysis of recombinant protein and Western blot analysis of recombinant AccMRJP1 fusion protein. (**A**) SDS-PAGE analysis of recombinant protein. Lanes: 1−7, induced supernatant of seven GS115 pPIC9K-AccMRJP1 strains (lanes 1, 3, and 4 indicate higher expression level for recombinant protein); 8, noninduced supernatant of strain GS115 pPIC9K-AccMRJP1; 9, induced supernatant of strain GS115 pPIC9K; M, protein marker. (**B**) Western blot analysis of recombinant AccMRJP1 fusion protein with anti-His antibody. The positions of the recombinant AccMRJP1 band are indicated with arrows.

SDS-PAGE to examine its molecular weight change. We found that the band of the positive control, undigested recombinant AccMRJP1 (57 kDa, **Figure 5A**, lane 1), which had a molecular weight equal to that of glycosylated AmMRJP1 in natural RJ (**Figure 5A**, lane 3), shifted to 48 kDa (**Figure 5A**, lane 2) after enzyme treatment, indicating that the recombinant AccMRJP1 expressed in yeast is a glycoprotein and that the oligosaccharide chains were removed by glycosidase F. We found that the protein band of *N*-glycosidase F (36 kDa) also appeared in the negative control without recombinant AccMRJP1 (**Figure 5B**, lane 1) and in the *N*-glycosidase F-digested recombinant AccMRJP1 (**Figure 5A**, lane 2).

Effects of AccMRJP and AmSPRJ on Cell Growth, Cell Shape, and Cell Adhesion of Tn-5B-4 Insect Cells. The results show that the cell densities of the treatment groups, serum-free medium supplemented with FBS, AmSPRJ, and AccMRJP1 (**Figure 6A−C**) are higher than that of the control group, serum-free TNM-FH (CK, **Figure 6D**), whereas there is no significant difference among the

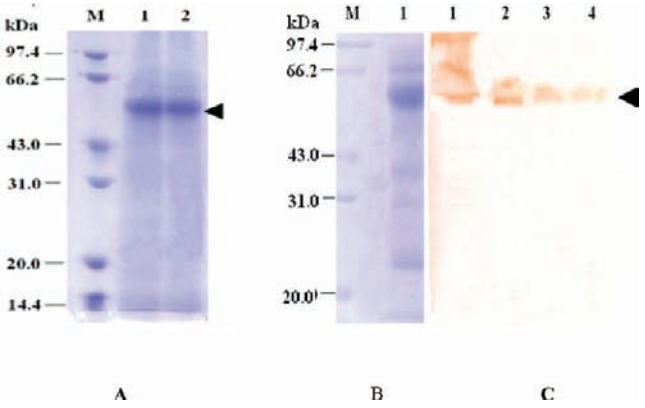

Figure 4. SDS-PAGE and Western blot analysis of purified recombinant AccMRJP1 fusion protein and AmSPRJ. (**A**) SDS-PAGE pattern of the purified recombinant AccMRJP1 protein. Lanes: M, protein marker; 1 and 2, recombinant AccMRJP1 proteins. (**B**) SDS-PAGE pattern of the AmSPRJ. Lanes: M, protein marker; 1, AmSPRJ. (**C**) Western blot analysis of AmSPRJ and the purified recombinant AccMRJP1 protein with anti-GST−AccMRJP1 antibody. Lanes: 1, AmSPRJ; 2−4, recombinant AccMRJP1 proteins. The positions of the natural AmMRJP1 and recombinant AccMRJP1 protein bands are indicated with arrows.

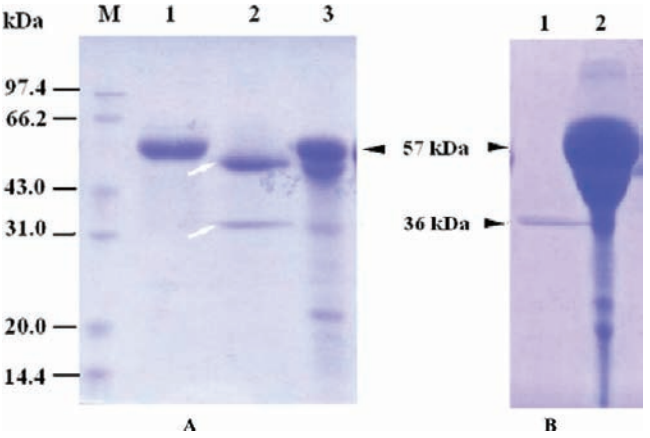

Figure 5. Change of recombinant AccMRJP1 on the SDS-PAGE gel after *N*-glycosidase F treatment. (**A**) Lanes: M, protein marker; 1, positive control, the undigested recombinant AccMRJP1 (57 kDa band); 2, glycosidase F-digested recombinant AccMRJP1 (48 kDa band) and remaining glycosidase F (36 kDa band) (the positions of the two bands are indicated with white arrows, respectively); 3, natural AmSPRJ (AmMRJP1 is indicated with black arrow). (**B**) Lanes: 1, negative control (glycosidase F); 2, natural AmSPRJ. The positions of 57 and 36 kDa protein bands are indicated with black arrows, respectively.

FBS, AmSPRJ, and AccMRJP1 groups. In **Figure 7**, the survival rate of the serum-free TNM-FH group is significantly lower (p value < 0.0001) than those of the other three groups at 24 and 48 h after changing the medium (**Figure 7**). There is, however, no significant difference among the survival rates of the FBS, AmSPRJ, and AccMRJP1 groups (**Figure 7**).

AccMRJP1 and AmSPRJ can affect cell shape and adhesion to the substrate, as shown in **Figure 6**. Tn cells maintained in a medium supplemented with recombinant AccMRJP1 (**Figure 6C**) were similar in shape to those grown in medium supplemented with AmSPRJ (**Figure 6B**). Tn cells maintained in AccMRJP1 and AmSPRJ also formed a homogeneous and continuous monolayer of flattened polyhedral cells, scarcely stratified and strongly adhered to the substrate, and did not exhibit notable differences in appearance with cells maintained in FBS (**Figure 6A**). However,

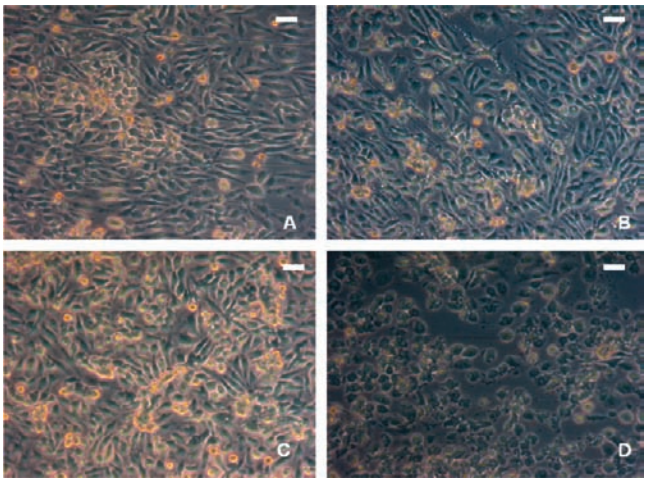

Figure 6. Effects of AccMRJP1 on Tn-5B1-4 cell shape and adhesion. Tn-5B1-4 cells were inoculated in (**A**) serum-free TNM-FH supplemented with FBS, (**B**) serum-free TNM-FH supplemented with AmSPRJ, (**C**) serum-free TNM-FH supplemented with AccMRJP1, and (**D**) serum-free TNM-FH (CK). Cell monolayers were photographed under a fluorescence and inverted microscope. Bar = 100 μm.

cells maintained in serum-free TNM-FH (CK) exhibited a stratified and round shape and adhered poorly to the substrate, where most of the cells eventually floated free of the substrate (**Figure 6D**).

DISCUSSION

In this study, we cloned the AccMRJP1 gene from the cDNA library of the Chinese honeybee worker head. Purified AccMRJP1 was expressed in *P. pastoris* using the pPIC9K expression system and yielded a molecular weight of 57 kDa, >10 kDa above the theoretical molecular weight of 46.3 kDa. The recombinant AccMRJP1 expressed in yeast is a glycosylated protein similar to the naturally glycosylated AmMRJP1. We also demonstrated that the enterokinase-cleaved AccMRJP significantly stimulated cell growth or sustained cell proliferation in the Tn-5B-4 insect cell line, similar to AmSPRJ and FBS, and affected cell shape and cell adhesion to the substrate. Thus, AccMRJP can be used as a substitute for FBS in cell culture.

Increasingly, different yeasts have been developed as highly successful systems for producing a variety of heterologous proteins. Specifically, we chose the methylotrophic yeast *P. pastoris* because it possesses the capability of performing many types of post-translational modifications, such as glycosylation, disulfide bond formation, and eukaryotic proteolytic processing, and secretes heterologous eukaryotic proteins in their native, biologically functional form (*32*). Currently, many types of FDA-approved therapeutic proteins including insulin and hepatitis B surface antigen are produced using yeasts (*33*, *34*). Moreover, yeasts and higher eukaryotes share many metabolic pathways, one of which is the asparagine-linked glycosylation pathway, which is similar in yeast and animal cells to the first stage of glycan processing. Thus, yeast is a good model system in which to study protein glycosylation (*35*).

N-Glycosylation is one of many important post-translational modifications that are crucial in a number of physiological and biochemical properties of a protein, such as folding, stability, intracellular trafficking, or activity (*36*, *37*). Glycoproteins are fundamental to biological processes such as fertilization, immune defense, viral replication, parasitic infection, cell growth, cell−cell adhesion, degradation of blood clots, and inflammation (*38*). Natural AmMRJP1 is a glycoprotein with a molecular weight of

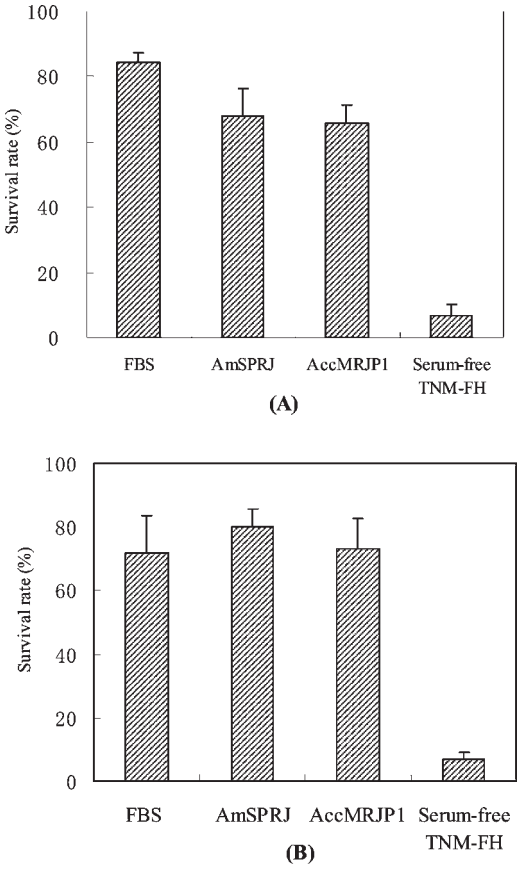

Figure 7. Effects of AccMRJP1 on insect cell growth. (**A**) Comparison of survival rates of cells for four culture medium groups, 10% FBS-supplemented TNM-FH, serum-free TNM-FH with AmSPRJ, TNM-FH serum-free TNM-FH with purified recombinant AccMRJP1, and serum-free TNM-FH (CK) at the 24th h after changing medium. (**B**) Comparison of survival rates of cells for the same four medium groups as in **A** at 48 h after changing the medium. The survival rate of cells for each group is equal to the average proportion of living cells across three dishes in each group. Bars represent standard errors. The survival rate of the serum-free TNM-FH group is significantly lower (p value < 0.0001) than those of the other three groups at 24 and 48 h after changing medium.

55−57 kDa (*14*) and possesses many nutritional functions and diverse biological activities. Others have reported that the N-glycosylated characteristics of AmMRJP1 are closely related to its molecular structure and biological activity (*31*). SDS-PAGE analysis showed that the bands of native AmRJP1 (57 kDa) shifted to 48 kDa after the oligosaccharide chains were removed by *N*-glycosidase F (*31*). It was also confirmed here that the N-terminal amino acid sequence of the 57 kDa moiety of AmMRJP1 protein, separated from AmSPRJ, contained N-I-L-R-G-E, which was identical to that deduced from the ORF of the AmMRJP1 gene and previously characterized in AmMRJ1 (*14*). Moreover, the 57 kDa protein band from the Western honeybee worker brain was shown to be AmMRJ1 by identification of peptide mass fingerprinting (PMF) and a database search (*39*). However, the molecular weights of both recombinant unglycosylated AmMRJP1 and AciMRJP1 expressed in *E. coli* were only 48 kDa (*15*, *23*) because bacteria are not able to perform this type of post-translational processing (*32*). A similar result was shown when our recombinant AccMRJP1 was expressed in *E. coli*. We found that the molecular weight of recombinant fusion protein of GST−AccMRJP1 expressed in *E. coli* was about 65 kDa. The molecular weight of

AccMRJP1 was estimated at about 48 kDa when the GST was reduced from the fusion protein GST−AccMRJP1. We also found that most of the recombinant protein existed in the inclusion body and was insoluble (*22*). Thus, we decided to express AccMRJP1 in a yeast system to improve its biological activity.

Our results using both Western blot analysis and N-terminal amino acid sequencing of the natural AmMRJP1, together with our previous papers (*25*), confirmed that the 57 kDa protein band of AmSPRJ was indeed a glycosylated protein. Meanwhile, it can be concluded from our result that the recombinant AccMRJP1 was glycosylated in yeast.

The proliferation activity of RJ protein in cell lines derived from human, rat, and insect has received a fair amount of recent attention. Previous studies suggested that RJ proteins can stimulate proliferation of rat and human myeloid cell (*40*) and monocytes (*41*) and of mouse osteoblast-like NC373-E1 cells (*42*). Moreover, RJ proteins can facilitate the neurogenesis and restoration of rat neuronal cells damaged by trimethyltin (*43*). Interestingly, RJ proteins also inhibit the bisphenol A-induced proliferation of a human breast cancer cell line (*44*). It has been found that soluble RJ protein (AmSPRJ) can stimulate proliferation of Tn-5B-4 insect cells more efficiently than FBS, suggesting that AmSPRJ contains growth factors or hormones promoting cell growth (*1*). Our results show that the capability of the recombinant AccMRJP1 in promoting cell proliferation of Tn-5B-4 insect cell line is similar to those of AmSPRJ and FBS. These results provide further evidence that MRJP1 may serve as the main growth factor in SPRJ.

Recent studies have shown that natural AmMRJP1 can promote cell proliferation of animal and human cell lines in place of FBS-supplemented culture (*14*, *15*, *17*). In this paper, we have demonstrated that AccMRJP1 expressed in yeast is capable of promoting proliferation of insect cells in vivo. Our results provide fundamental knowledge for further studies on the genetic engineering of AccMRJP1 and its application in the food and pharmaceutical industries as a potential ingredient of functional foods and as a substitute for FBS.

ABBREVIATIONS USED

RJ, royal jelly; MRJPs, major royal proteins; AmMRJP1, MRJP1 of *Apis mellifera*; AccMRJP1, MRJP1 of *A. cerana cerana*; AciMRJP1, MRJP1 of *A. cerana indica*; EST, expression sequence tags; ORF, open-reading frame; YNB, yeast nitrogen base; EK, enterokinase; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; HRP, horseradish peroxidase; goat anti-rabbit IgG/HRP, goat anti-rabbit IgG-linked horseradish peroxidase; goat anti-mouse IgM/HRP, goat anti-mouse IgM-linked horseradish peroxidase; DAB, diaminobenzidine; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; PCR, Polymerase Chain Reaction; SPRJ, soluble protein of RJ; AmSPRJ, SPRJ of RJ from *A. mellifera*; FBS, fetal bovine serum.

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