UNIVERSIDADE FEDERAL DE UBERLÂNDIA
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Imunodetecção de polipeptídios de 128 e 51kDa relativo às proteínas da Geléia Real no extrato de cabeça de abelhas *Apis mellifera L.*

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Monografia apresentada à Coordenação do Curso de Ciências Biológica, da Universidade Federal de Uberlândia, para a obtenção do grau de Bacharel em Ciências Biológicas.

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Resumo:

As principais proteínas hidrossolúveis da Geléia Real (PPGR 1- 5) são secretadas pelas glândulas cefálicas das abelhas operárias nutridoras *Apis mellifera* L. Seqüências de cDNA e mRNA das PPGR1 e PPGR2 foram encontradas no cérebro de *A. mellifera.* No presente estudo, identificamos por Western blot, três polipeptídios de 51, 70 e 128kDa (p51, p70 e p128, respectivamente) no homogeneizado da cabeça de *A. mellifera* usando anticorpos produzidos por imunoafinidade contra a PPGR1. Não houve detecção destes polipeptídios no tórax e abdômen desta abelha. No cérebro de operárias nutridoras detectou-se o p51 e o p128 e na glândula hipofaringeal o p70. Estes polipeptídios encontram-se presentes apenas nas operárias e apresentam uma distribuição diferente no homogeneizado da cabeça de operária nutridora e campeira. A nutridora apresenta o p51, o p70 e o p128 e a campeira apresenta o p51. Análise do homogeneizado de cabeça de outros himenópteros, não detectou a presença destes polipeptídios. A distribuição do p128 e do p51 nas regiões do cérebro mostrou que o primeiro encontra-se presente em todas as regiões investigadas e o segundo não está presente apenas na região denominada corpo de cogumelo. Estes resultados sugerem que estes polipeptídios são correlacionados com as proteínas hidrossolúveis da GR (PPGR1 e PPGR2) e que o p128 possivelmente é um novo polipeptídio desta família de proteínas.

Key words: inseto, imunodetecção, Western blot, PPGRs.

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Introdução:

A geléia real (GR) é um composto secretado pelas glândulas cefálicas, hipofaringeal e mandibular, das abelhas operárias jovens *Apis mellifera* L, entre o sexto e o sétimo dia de vida (Kubo et al., 1996). A constituição da GR é de 60 a 70% de água, 12 a 15% de proteínas, 10 a 16% de açúcar, 3 a 6% de lipídeos e os 2 a 3% restantes são vitaminas, aminoácidos livres, moléculas de baixo peso molecular e sais minerais (Albert et al., 1999 e Schimitzová et al., 1998).

Entre as proteínas da GR, destacam-se as proteínas hidrossolúveis (WSP). As proteínas WSP melhor caracterizadas são denominadas de principais proteínas da GR (PPGR). A família de PPGR consiste de cinco membros (PPGR1 – PPGR5) de cDNA expressos na cabeça de *A. mellifera* (Hanes e Simuth, 1992; Ohashi et al., 1997; Schimitzova et al., 1998; Simuth, 2001). Albert, 2003 encontrou três novas PPGRs em bibliotecas de cDNA de cérebro de *Apis mellifera*. As PPGR1 (57kDa), PPGR2 (49kDa), PPGR3 (60-70kDa), PPGR4 e PPGR5 (77-87kDa), representam 82% do total das WSP e cerca de 90% do total de proteínas da GR (Hanes e Simuth, 1992; Ohashi et al., 1997; Schimitzova et al., 1998; Simuth, 2001).

A GR é o único alimento da rainha de *A. mellifera* desde seu estágio larval, sendo importante na diferenciação das castas (Schmitzová et al.,1998). Usando técnicas de *microarray*, *Northern blot* e *Southern blot*, seqüências de DNA complementar (cDNA) e RNA mensageiro (mRNA) das PPGR1 e PPGR2 foram encontradas no cérebro de *A. mellifera*, estas proteínas podem ter funções fisiológicas ainda desconhecidas (Kucharski & Maleszka et al., 1998, Kucharski & Maleszka, 2002).

Por outro lado, algumas aplicações da GR foram descritas, dentre as quais d estacam-se: fatores r egulatórios no c ontrole do desenvolvimento da abelha (Malekova et al., 2003), aumento na taxa de crescimento (Kawamura 1961), atividade vasodilatadora e hipotensora (Shimoda et al., 1978), atividade antihipercolesterolêmica (Nakajin et al., 1982), ação desinfetante (Yatsunami et al., 1985), atividade antitumoral (Tamura et al., 1987), atividade antiinflamatória (Fuji et al., 1990), potencial bactericida (Fujiwara et al., 1990), efeito de antifadiga em ratos (Kamakura et al.,2001), atividade antioxidativa (Takeshi Nagai et al., 2001) e modulação de respostas imunes (Okamoto et al., 2003).

Neste trabalho produzimos anticorpos policlonais contra as PPGRs em coelho. Identificamos, por Western blot, no homogeneizado de cabeça da *A. mellifera* três polipeptídios de 51, 70 e 128kDa. Encontramos, nas regiões analisadas do cérebro da abelha operária, uma diferente distribuição destes polipeptídios. Sendo que estes polipeptídios são detectados apenas no homogeneizado de cabeça nesta casta. Mostramos que nos cérebros das operárias nutridoras e campeiras há uma diferente distribuição dos polipeptídios analisados. E pela comparação da expressão dos polipeptídios em homogeneizados de cabeça da *A. mellifera* com outros himenópteros, evidenciamos que o anticorpo reconhece polipeptídios tecido especifico unicamente na abelha *A. mellifera.*

Material e métodos

Animais

Os coelhos e ratos foram mantidos no Laboratório de Experimentação Animal (LEA) da Universidade Federal de Uberlândia – MG. Os pintainhos

foram cedidos pela granja Planalto S/A Uberlândia – MG. As abelhas *Apis mellifera, Mellipona scutellaris* e *Scaptotrigona postica* e a formiga *Camponotus atriceps* foram coletadas no Jardim experimental do Instituto de Biologia da Universidade Federal de Uberlândia, Campus Umuarama.

Homogeneização dos tecidos

Os tecidos alvos das abelhas (cabeça, cérebro, glândula hipofaringeal) e da formiga (cabeça) foram dissecados e congelados em nitrogênio liquido e mantidos em ultrafreezer (Sanio ultra low) -80ºC. Trinta cérebros de *Apis mellifera* foram dissecados, isolando-se o lobos ópticos, lobos antenais, corpo de cogumelo e parte central do cérebro, cada região foi homogeneizada em 400µl de tampão. Os demais tecidos foram homogeneizados em 1 mL por 1 minuto no *Omni-mixer*, em gelo, no tampão (40 mM Hepes pH 7.7; 10 mM EDTA; 2 mM EGTA; 5 mM ATP; 2 mM DTT; 1 mM benzamidina; 0.1 mM aprotinina e 0.5 mM PMSF) (Cheney et al., 1993).

Em seguida, as amostras dos homogeneizados foram centrifugadas a 40.000g/ 40min a 4^0C (Hitachi). Da amostra de sobrenadante foi feita a estimativa da concentração protéica, segundo método de Bradford (Bradford,1976). Adicionou-se tampão desnaturante TD 10 vezes concentrado (10% SDS, 20% sacarose, 10 % β-mercaptoetanol, 5% EGTA-Na, azul de bromofenol) para uma concentração final de 2µg/µl de proteína.

Homogeneização da GR

A GR fornecida pelo Apiário Girassol LTDA, Uberlândia – MG, foi estocada em ultrafreezer (Sanio ultra low) –80ºC (Kamakura et al 2001).

Alíquotas de 2g de GR foram homogeneizadas em 12ml de tampão pH 8.0 (50Mm NaH_2PO_4/Na_2HPO_4; 100mM NaCl; 20mM EDTA) (Schimatzová et al.,1998). O extrato protéico foi formado deixando o homogeneizado durante 30 minutos em gelo, sendo agitado no vórtex a cada 5 minutos. Em seguida foi centrifugado a 10000g a 4°C por 10 minutos. O sobrenadante foi dializado em Tampão Tris pH 8.0 (20mM Tris HCl; 0.5mM EDTA), por 24 horas. Após a diálise a GR foi novamente centrifugada a 10000g a 4°C por 10 minutos.

Eletroforese em gel desnaturante (SDS-PAGE)

As amostras d os h omogeneizados f oram a plicadas em mini géis de poliacrilamida SDS-PAGE 5-16% de dimensões 7,1 x 10 x 0,0075cm (Mini Protean, Biorad) (Laemmili, 1973), com amperagem constante de 35mA. As amostras foram preparadas 1/10 v/w em tampão TD. O perfil eletroforético foi obtido mediante aplicação de 15 µg/linha de proteína. Os géis foram corados com Comassie brilhant Blue- R 250.

Produção de anticorpos

Os coelhos foram imunizados dorsalmente com GR em um intervalo de 15 dias, a primeira dose foi administrada com 500 µg/ml juntamente com Adjuvente Completo de Freud's (Sigma) v/w. As doses de reforço foram administradas com 250 µl/ml juntamente com Adjuvante Incompleto de Freud's (Sigma) w/v. Cada animal recebeu doses de 1 ml.

O sangue foi coletado pelo lobo da orelha do animal após três administração de reforço. O soro, separado por centrifugação a 600g/ 5minutos temperatura ambiente foi estocado à -20 °C. Em seguida, fez-se o teste de

ELISA, para comprovar a presença de anticorpos contra as proteínas da GR. Após o ELISA foram administradas doses de reforço BURST, sem o adjuvante. Após três dias da ultima aplicação retirou-se 4ml de sangue para purificação dos anticorpos.

Para a purificação do anticorpo contra a PPGR1 utilizou-se o método de imunoafinidade. Aplicou-se 15µg/linha de proteína total da GR em gel SDS-PAGE 5 -16%. O s p olipeptídios foram e letrotransferidos p ara membrana de nitrocelulose 0,45µm (Hybond ECL) com amperagem constante de 100mA overnight a 4°C. A banda de interesse, correspondente a PPGR1, foi cortada, colocadas em microtubos e incubadas com solução bloqueio (SB) contendo leite desnatado 5% em TBS-T (50mM tris-HCL pH 8.0, 150 mM NaCl, 0. 05% TWEEN-20) por 12 horas a 4°C e lavada 3 vezes 5 minutos com TBS-T. Posteriormente incubou-se as fitas com o soro coletado dos coelhos por 12 horas a 4°C. Os anticorpos foram eluídos contra as PPGRs de interesse com 1,4% trietilamina por 5 minutos sob agitação em temperatura ambiente. Em seguida a reação foi neutralizada transferindo-se a solução de trietilamina com o anticorpo purificado para microtubos contendo 100µl de Tris- HCl 1M pH8,5. A fração eluída foi dialisada contra TBS por 2 dias. Depois da diálise foi lida a absorbância da amostra em 280nm, zerando o espectrofotômetro (Hiachi, UV 2000) com TBS utilizado para dializar a mamostra. A concentração protéica do anticorpo purificado, correspondente a fração eluída com trietilamina, foi determinada dividindo-se a absorbância da amostra pelo coeficiente de extinção molar da IgG; 13,6. Os anticorpos foram acondicionados a -20°C.

Titulação do antígeno e do anticorpo

Para a titulação do anticorpo produzido contra a PPGR-1 fez-se uma diluição seriada do anti-PPGR1 nas concentrações 0.4μg (1/2251), 0.2μg (1/4510), 0.15μg (1/6000), 0.1ηg (1/9000), e 0.05ηg (1/18000). O anticorpo diluído foi incubado com as fitas de nitrocelulose contendo PPGR1 na concentração de 2μg/μl. Para imunodetecção foi usado o anticorpo secundário anti-coelho conjugado com fosfatase alcalina (AP) na diluição de 1: 5000. As membranas foram reveladas com NBT/BCIP (Promega) em tampão AP (0.1M Tris, 0.1M NaCl, 5mM $MgCl_2$).

Para a titulação da GR total, fez-se uma diluição seriada do antígeno, nas concentrações 2μg/μl, 0.2μg/μl, 0.02μg/μl, 0.002μg/μl, 0.2ηg/μl, 0.02ηg/μl e 0.002ηg/μl. O imunoblot foi realizado eletrotransferindo as diferentes diluições do antígeno para membrana de nitrocelulose 0,45 μm e sondadas com o anti-PPGR1 na concentração de 0.2μg (1/4510).

Detecção de polipeptídios por Western blot.

As proteínas separadas em SDS-PAGE foram eletrotrasferidas em corrente constante 100mA por 12 horas a 4°C para membrana de nitrocelulose 0,45μm, utilizando tampão de transferência (25 mM Tris-HCl, 190mM glicina, 20% metanol) (Towbin et al., 1979). A membrana foi corada com solução de 5% Ponceau em 0,3% ácido tricloroacético. Para bloqueio dos sítios inespecíficos, a membrana foi incubada com SB por 12 horas a 4°C. Lavou-se a membrana de nitrocelulose com TBS-T 3 vezes 5 minutos. Em seguida incubou a membrana com o anticorpo purificado contra a PPGR1 por 12 horas a 4°C e anti-Rabbit - AP por 3 horas a 4°C.

Pesquisa em bancos de dados e análise computacional das PPGRs

As seqüências das PPGRs analisadas em bancos de dados públicos foram PPGR1, PPGR2, PPGR3, PPGR4 e PPGR5: GenBank acessos nos AAM73637, AAL83702, AAL83703, AAL83704, AAL99272, respectivamente.

As seqüências peptídicas das PPGRs, disponíveis em bancos de dados públicos, foram comparadas com 35 proteínas encontradas nos bancos de dados on line, usando o algoritmo: Blast http://www.ncbi.nlm.nih.gov/blast. O software Clustal W do Instituto Nacional Europeu EMBL-EBI encontrado no algoritimo: http://www.expasy.ch/tools, foi usado para o alinhamento das seqüências protéicas das PPGRs.

Resultados:

Produção e titulação do Anti-PPGR1

O anticorpo produzido contra a PPGR1, numa concentração de 0,45µg/µl, foi denominado anti-PPGR1. A reatividade deste anticorpo foi examinada sondando homogeneizados de proteínas da GR imobilizadas em membrana de nitrocelulose. Nos W estern blots destes homogeneizados, o anticorpo obteve reação cruzada com as proteínas da GR correspondente a massa molecular relativa das proteínas PPGR1, 2, 3 e 5 em SDS-PAGE. A análise da titulação do anti-PPGR1 contra as PPGRs mostrou que este anticorpo nas diluições de 0.4µg a 0.05ηg de anti-PPGR1 foram capazes de reconhecer as proteínas da GR imobilizadas na membrana de nitrocelulose (15µg/linha). A diluição de 0.1µg do anti-PPGR1 diminuiu o *background* permitindo uma melhor visualização das bandas polipeptídicas (Fig-01A).

Através da titulação do homogeneizado protéico de GR determinou-se a melhor diluição da GR em concentração mínima de 0.02μg/μl.linha reconhecendo uma única banda de 57kDa (Fig-01B).

Análise de bioinformática das proteínas da GR

Foram encontradas 35 seqüências protéicas relacionadas com as proteínas da GR usando a palavra-chave "Royal Jelly" e o programa Blast (http://www.ncbi . nlm.nih.gov/entrez/query.protein). A análise do alinhamento das seqüências fornecidas pelo Blast mostrou que 15 seqüências estão correlacionadas com as PPGRs, 10 seqüências são de proteínas do tipo yellow da *Drosophilla melanogaster* e as 10 seqüências restantes são outras proteínas da GR. Investigando a identidade e a similaridade das PPGRs, observamos que o alinhamento destas proteínas apresentou uma quantidade total de 606 aminoácidos (aa), e uma identidade igual a 25,58% (155 aa). Entre as PPGRs, os aa com características bioquímicas similares mostraram identidade de 18,32% (111 aa) e os aa com fracas características bioquímicas equivalentes de 7,76% (47 aa), sendo que 48,35% (293 aa) não apresentaram identidade.

Porém, ao analisarmos as combinações dos pares de alinhamentos protéicos entre as PPGRs, observamos que a PPGR1 e PPGR2 possuem a maior identidade (62,64%) e a menor diferença entre os aa (15,82%). Além disso, a PPGR1 possui mais de 44% de identidade com todas as proteínas da GR, tendo maior diferença dos aminoácidos em relação a PPGR5 (37,38%) (Fig-02).

Western Blot dos homogeneizados

A análise das proteínas de homogeneizados da cabeça dos diferentes himenópteros, imobilizadas em filtros de nitrocelulose demonstrou marcação de polipeptídeos correlacionadas com as PPGRs unicamente nos tecidos de *A. mellifera*. No filtro contendo o homogeneizado de cabeça de abelha, o anti-PPGR1 reconheceu três polipeptídios com massa molecular aparente de 51, 70 e 128kDa (p51, p70 e o p128, respectivamente). Nos homogeneizados de cabeça de himenópteros (abelhas: *M. scutellaris* e *S. postica* e da formiga: *C. atriceps*) sondados com o anti-PPGR1 não houve detecção (Fig-03).

No Western blot da fração solúvel do cérebro da *A. mellifera* (Fig-03), o anticorpo anti-PPGR1 reconheceu o p 128 e o p 51. O anti-PPGR1 dirigido contra homogeneizado de glândula hipofaringeal, reconhecendo o p51 e o p70. O p70 não foi detectado na fração solúvel de cérebro desta abelha (Fig-03).

Para investigar a distribuição destes polipeptídios em regiões do cérebro, isolou, homogeneizou e sondou com anti-PPGR1 as seguintes regiões, os lobo óptico, o lobo antenal, os corpos de cogumelo e a parte central do cérebro (lobos α e β). A análise da distribuição do p128 e do p51 nestas regiões mostrou que o p128 encontra-se presente em todas as regiões analisadas e o p51 não está presente apenas na região dos corpos de cogumelo (Fig-04).

A distribuição de p128 e p51 em relação a casta e desesenvolvimento da abelha foi correlacionados com as PPGRs sondando os homogeneizados de cabeça de diferentes castas (rainha, zangão e operária) de *A. mellifera* com o anti-PPGR1. A figura 05 mostra que os polipeptídios detectados encontram-se presentes apenas em abelhas operárias, não sendo detectados em abelhas

rainhas e zangões. As operárias nutridora e campeira apresentam uma diferente distribuição destes polipeptídios, onde detectou-se o p51 apenas no homogeneizado de cabeça de campeira, enquanto que o p51, o p70 e o p128 foram detectados no homogeneizado de cabeça de nutridora.

Discussão

A produção do anticorpo, por imunoafinidade, contra ás proteína PPGR1 da GR foi eficiente, tendo reação cruzada com polipeptídios da GR imobilizados em filtros de nitrocelulose. Não há relatos de imunodetecção de proteínas da família das PPGRs em cérebro de *A. mellifera.* Porém, outros pesquisadore relataram que existe uma expressão diferencial do mRNA da PPGR1 em a belhas recém em ergidas (Kucharski e t a l.,1998), nutridoras e campeiras (Klaudiny et al., 1994b). A expressão de mRNA para PPGRs foi também observados na glândula hipofaringeal de *A. mellifera* (Ohashi et al., 1997) e em neurônios intrínsecos dos corpos de cogumelo de *A. mellifera* (Kucharski et al., 1998). A caracterização do anticorpo produzido no presente estudo foi alcançada numa primeira etapa através do uso de uma concentração ótima do anticorpo, a qual deveria diminuir a intensidade do sinal e permitir uma melhor visualização dos polipeptídios reativos. O uso de um título de anticorpo para análise dos filtros com proteínas imobilizadas da GR que reconhecesse uma única banda, permitiu também detectar polipeptídios correlacionados as PPGRs em tecidos da *A. mellifera*.

As análises das combinações das seqüências dos pares de alinhamento das PPGRs que revelaram alta identidade sustentam os dados de reações cruzadas dos anticorpos com os polipeptídios relacionados as PPGRs 1 e 2.

Estudos de filogenia da família da PPGRs/YELLOW demostraram oito PPGRs altamente homólogas (Albert, 2003 e Albert et al 1999a). Além do mais, a reação cruzada dos anticorpos com homogeneizado protéico da GR, subsidia a identificação de proteínas correlacionadas as PPGRs no cérebro de *A. mellifera*. Isto é, a detecção de polipeptídios com massa molecular relativa distinto das PPGRs no homogeneizado de cabeça desta abelha.

A imunodetecção do p128 e p51 sugere que a abelha *A. mellifera* apresenta em seu cérebro proteínas semelhante à família das PPGRs. Além disso, estes polipeptídios possuem uma distribuição distinta para algumas regiões do cérebro da abelha, neste caso p51 não foi imunodetectado nos corpos de cogumelo. É interessante observar que p51 comigra em SDS-PAGE na mesma faixa da PPGR1. Esta proteína, é a mais abundante dentre as PPGRs e possui massa molecular de 56-57kDa (Hanes e Simuth, 1992; Ohashi et al., 1997; Schimitzova et al., 1998; Simuth, 2001). O mRNA da PPGR1 está expresso nas glândulas encefálicas e no cérebro de abelha (Klaudiny et al., 1994b; Ohashi et al., 1997; Kucharski et al.,1998) incluindo os corpos de cogumelo (Kucharski et al.,1998).

Com relação à identificação de outra PPGR em tecidos diferente da glândula hipofaringeal de *A. mellifera*, Kucharski & Maleska (2002) mostraram por microarray que o cDNA da PPGR2 está presente no cérebro e ausente no tórax e abdômen. A PPGR2 migra em SDS-PAGE com um Mr próximo ao do p51 (49 KDa) e é secretada pela glândula hipofaringeal de abelhas nutridoras (Hanes e Simuth, 1992; Ohashi et al., 1997; Schimitzova et al., 1998). Apesar dos relatos de identificação de PPGRs no cérebro de abelha, não existem dados sobre as possíveis funções destas proteínas. Os dados aqui

apresentados mostram que p51 é uma proteína semelhante às PPGRs imunodetectado na cabeça de abelha e possivelmente distribuída na glândula hipofaringeal e no cérebro de abelhas operárias nutridoras e campeiras

Os p olipeptídios p 51, p 70 e p 128 d etectados n o homogeneizado d e cabeça de *A. mellifera* não foram encontrados nos tecidos dos outros himenópteros investigados no presetnte estudo. Albert (2003) reportou 03 novas PPGRs (PPGR6, PPGR7 e PPGR8) aumentando o número de proteínas membros da família das PPGRs de cinco (Hanes e Simuth, 1992; Ohashi et al., 1997; Schimitzova et al., 1998; Simuth, 2001) para oito proteínas e mudando a denominação para família de PPGRs/YELLOW. Além do mais, proteínas desta família foram identificadas na saliva de diferentes insetos parasitas como *Lutzomya longipalpis* (Charlab et al., 1999) e *Phlebotomus papatasi* (Valensuela et al., 2001). A possibilidade para interação das proteínas da saliva com as PPGRs, segundo Albert (2003) pode representar uma intermediação na evolução das proteínas Yellow como componentes da geléia real de *A. mellifera*.

Com relação à identificação diferenciada do polipeptídio p70 pelo anti-PPGR1, o qual está presente na glândula hipofaringeal de abelha operária nutridora e ausente na glândula hipofaringeal de abelha operária campeira, como demostrado no presente estudo; sugere-se que p70 detectado na glândula hipofaringeal de nutridora seja de fato relacionado com a família PPGR. Uma vez que, uma outra proteína com Mr de 70kDa desta glândula refira-se à enzima alfa-glicosidase que é expressa na glândula somente quando a abelha operária passa do estágio de nutridora para campeira (Kubo et al.,1996; Ohashi et al.,1996; 1999). Nossos dados estão de acordo com os

dados relatados que mostram mudanças na expressão de proteínas da glândula hipofaringeal de abelhas operárias e em relação à idade e/ou função (nutridora ou campeira). (Whitfield et al., 2003).

A glândula hipofaringeal de nutridoras secreta, principalmente, proteínas de 50, 56 e 64kDa. Enquanto que a glândula hipofaringeal de campeiras secreta proteínas com 56 e 70kDa (Kubo et al.,1996). A análise da seqüência da alpha-glucosidase com as PPGRs, revelou menos de 12% de identidade, sugerindo uma especificidade do anticorpo testado no presente estudo para reconhecer a PPGR somente na glândula hipofaringeal de abelha nutridora.

A análise das seqüências de PPGRs pelo BLAST revela que estas proteínas são filogenéticamente restritas a *A. mellifera* e *D. melanogaster* (proteínas Yellow). A obtenção de anticorpo policlonal capaz de reconhecer as PPGRs em amostra de GR e reconhecer proteínas relacionadas em tecidos específicos de abelha, traz uma contribuição original para o estudo desta interessante família de proteínas, podendo sugerir que estas tem uma distribuição diferenciada nos tecidos analisados, nas casta e na função das abelhas operárias *A. mellifera.*

Agradecimento:

Aos pesquisadores do Laboratório de Bioquímica e Biologia Molecular, Pablo Marques Veraz Peixoto, Letícia Borges Euqueres Partata e Renata Roland Teixeira pela ajuda na revisão to texto. Apiário Girassol Uberlândia-MG pela GR fornecida.

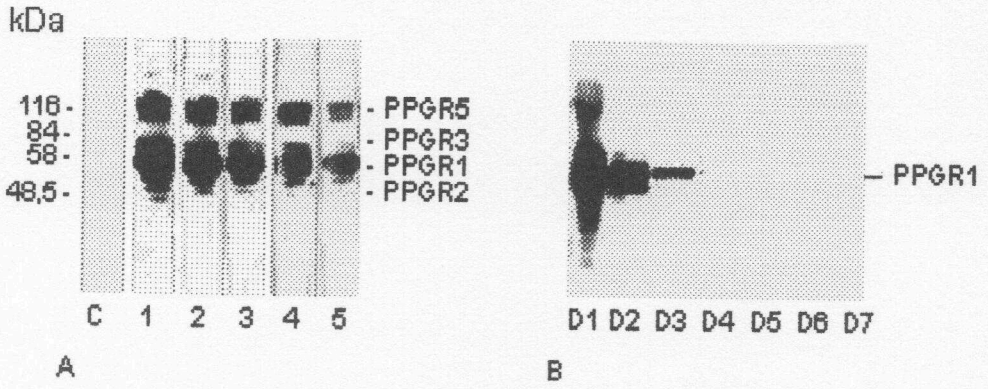

Fig-01A. Titulação do anti-PPGR1 através de Western blot em membrana de nitrocelulose 0,45μm. As diluições dos anticorpos foram feitas na proporção de 1- 0,4μg (1/2250), 2- 0,2μg (1/4500), 3- 0,15μg (1/6000), 4- 0,1ηg (1/9000), 5- 0,05ηg (1/18000) de anti-PPGR1, respectivamente. C-controle positivo (apenas anti-PPGR1). Evidenciamos que a diluição de 0,2μg melhor reconheceu os polipeptídios. **B.** Titulação da GR através de Western blot em membrana de nitrocelulose. As diluições do antígeno GR foram feitas nas proporções de D1- 2μg/μl, D2- 0,2μg/μl, D3- 0,02μg/μl, D4- 0,002μg/μl, D5- 0,2ηg/μl, D6- 0,02ηg/μl, D7- 0,002ηg/μl. O anti-PPGR1 reconhece no máximo a diluição de 0,02μg/μl (D3) de antígeno, sendo que nesta diluição não há um reconhecimento das demais PPGRs.

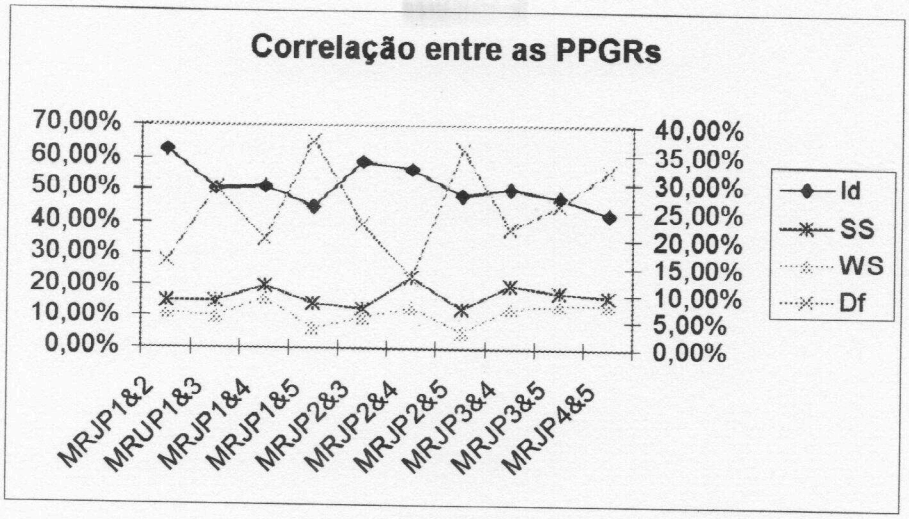

Fig-02. Correlação entre as combinações das proteínas da GR (PPGR1-PPGR5). As seqüências analisadas foram PPGR1, PPGR2, PPGR3, PPGR4 e PPGR5: A análise dos pares de alinhamentos mostrou que as PPGR1 e PPGR2 possuem a maior identidade (62,64%). A PPGR1 possui mais de 44% de identidade com todas as proteínas da GR. Id- identidade; SS- aminoácidos similares, WS- aminoácidos poucos similares, Df- aminoácidos diferentes.

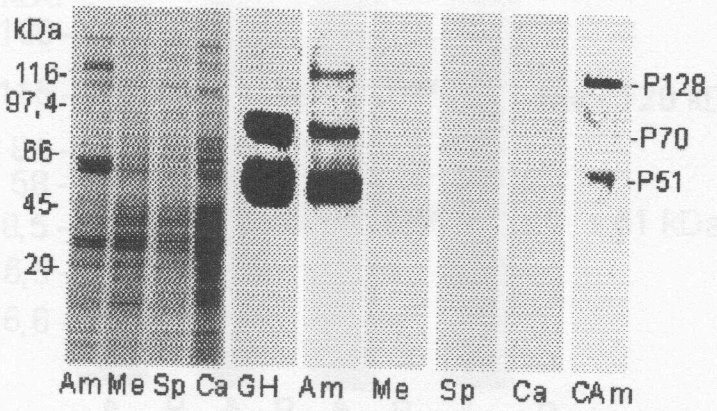

Fig-03. Imunodetecção de polipeptídios homólogos as PPGRs em cérebro da abelha *Apis mellifera* comparando com outros himenópteros através de Western Blot. Apresenta imunodetecção de três polipeptídios p51, p70 e p128 no homogeneizado de cabeça de *A. mellifera*, sondado com anticorpo anti-PPGR1 (Am) e Imunodetecção de dois polipeptídios p51 e p70 na glândula hipofaringeal de *A. mellifera* (GH). Detecção de dois polipeptídios, p51 e p128, em homogeneizado de cérebro de *A. mellifera*, sondados com o anti-PPGR1 (CAm). SDS-PAGE 5-16% dos homogeneizados de cabeça de *A. mellifera* S1A; *M. scutellaris* S1M; *S. postiça* S1P; *C. atriceps* S1C, corado com Coomassie blue.

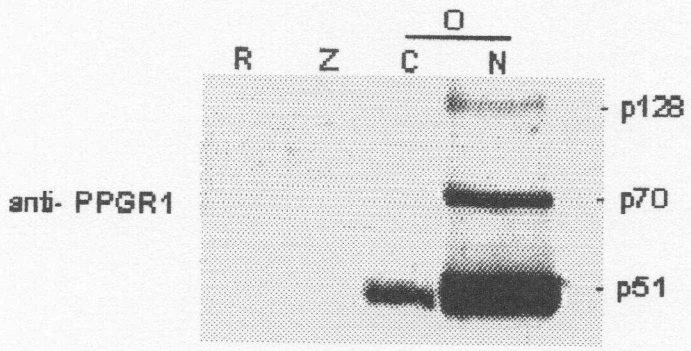

Fig-05. Imunodetecção dos p51, p70 e p128 em homogeneizados de cabeças de diferentes castas de *A. mellifera*, por Western Blot. No homogeneizado de campeira houve marcação apenas do p51, enquanto que no de nutridora os três polipeptídios foram detectados. R- Rainha; Z- Zangão; C- Campeira; N- Nutridora. Nas castas R e Z não houve marcação.

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Pergamon

Insect Biochemistry and Molecular Biology 29 (1999) 427–434

Insect Biochemistry and Molecular Biology

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Molecular characterization of MRJP3, highly polymorphic protein of honeybee (*Apis mellifera*) royal jelly

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Received 6 October 1998; received in revised form 27 January 1999; accepted 17 February 1999

Abstract

Major proteins of honey bee (*Apis mellifera*) royal jelly are members of the MRJP protein family. One MRJP protein termed MRJP3 exhibits a size polymorphism as detected by SDS-PAGE. In this report we show that polymorphism of the MRJP3 protein is a consequence of the polymorphism of a region with a variable number of tandem repeats (VNTR) located at the C-terminal part of the MRJP3 coding region. We present the characterization of five polymorphic alleles of MRJP3 by DNA sequencing. By PCR analyses, at least 10 alleles of distinct sizes were found in randomly sampled bees. Studies with nurse bees from a single honeybee colony revealed both Mendelian inheritance and very high variability of the MRJP3 genomic locus. The high variability and simple detection of the MRJP3 polymorphism may be useful for genotyping of individuals in studies of the honeybee.

Keywords: *Apis mellifera*; Polymorphism; VNTR; Tandem repeats; Gene duplication; Royal jelly; Nutrition; cDNA

1. Introduction

The larval food of honeybee (*Apis mellifera*) queen larvae called royal jelly (RJ) is believed to play a key role in honeybee queen development (Moritz and Southwick, 1992). An important component of royal jelly are proteins which form about 50% of the dry mass of RJ (Rembold, 1987). Major proteins of RJ, (MRJPs) have been characterized by cDNA cloning and sequencing (Klaudiny et al., 1994; Albert et al., 1996; Ohashi et al., 1997; Schmitzova et al., 1998). It was found that these proteins are expressed in the hypopharyngeal glands of nurse bees (Kubo et al., 1996; Lensky and Rakover, 1983), they are present in similar amounts in royal and worker jellies and belong to one protein family (Schmitzova, et al., 1998). The composition of MRJPs indicates that they are nutritive components of RJ serving as:

1. A supply of essential amino acids due to their high relative content in the structure of some MRJPs, such as MRJP1, MRJP5, MRJP4.
2. A storage of biologically accessible nitrogen required for the fast developing organism, which is supported by the presence of extensive repetitive regions consisting of high amounts of nitrogen-rich amino acids in several MRJPs (MRJP3, MRJP5, MRJP2, Albert, Bhattacharya, Klaudiny and Simuth, unpublished observations).

A recent report of Kucharski et al. (1998) suggests that some of the MRJPs could also have another function in bee physiology. They found that in addition to its expression in hypopharyngeal glands, MRJP1 is also expressed in a subset of neuronal cells of the honeybee brain. Its function there remains unknown.

Several proteins with molecular masses of 60–70 kDa were shown to have the identical N-terminal sequences, which were identical with the protein sequence inferred from MRJP3 cDNA (Schmitzova et al., 1998). This suggested that they might be variants of the MRJP3 protein. MRJP3 (named originally RJP57-1) was the first MRJP characterized by cDNA cloning and sequencing (Albert

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PII: S0965-1748(99)00019-3

et al., 1996; Klaudiny et al., 1994). The protein contains a repetitive region which is composed of a 20-fold regularly repeating motif of five amino acid residues.

In this publication we provide evidence that the 60–70 kDa proteins are variants of MRJP3 protein which is the product of the highly polymorphic MRJP3 gene. By PCR and DNA sequencing analyses we show that different alleles of MRJP3 gene differ in their lengths as a result of the variable number of basic repeat units in the repetitive region of MRJP3. Five different alleles are characterized at the molecular level.

2. Material and methods

2.1. Biological samples

Commercial samples of royal jellies were obtained from the Apicultural Institute Liptovsky Hradok, Slovakia and originate from different localities in Slovakia.

Royal and worker jellies from one colony were collected from the private apiary of Dr J. Simuth, Sebechleby, Slovakia. They were removed from the queen larval cell and worker larval cells (at a larval age of three days) located on the same comb in a area with a diameter of 7 cm. Worker jellies were obtained (after removing the larvae) by homogenizing the content of each brood cell with 30 µL of phosphate buffer (50 mM NaH_2PO_4/Na_2HPO_4, pH 7.0; 100 mM NaCl; 20 mM EDTA, pH 8.0). They were frozen and stored at −20°C.

Nurse bees and drones *Apis mellifera carnica* used in the comparative studies of MRJP3 polymorphism were gained from the private apiary of Mr E. Gottschalk located on the outskirts of Göttingen, Germany. The inner frame of the colony was shaken above a table. Fallen bees remaining on the table were collected as nurse bees. Collected animals were anesthetized on dry ice and stored at −20°C until use.

2.2. Preparation of head protein extracts and genomic DNA

Single honeybee heads were homogenized with a microhomogenizer in Eppendorf vials in 100 µL of SDS loading buffer (Laemmli, 1970) and centrifuged. The supernatant obtained was boiled prior to gel electrophoresis.

Genomic DNA from the thorax was isolated according to Fondrk et al. (1993).

2.3. Cloning of the repetitive region of MRJP3, its expression and purification

The original plasmid pRJP57-1 (Klaudiny et al., 1994; Albert et al., 1996) was digested with *Sma*I (cuts in polylinker upstream of the 5′ end of cDNA insert) and *Eco*RV (located just at the beginning of the repetitive region) and then religated to remove the 5′ part of cDNA. The deleted plasmid termed pBrep was cleaved with *Bam*HI + *Xho*I and the resulting fragment recloned into the *Bam*HI + *Xho*I-cleaved vector for Glutathione S-transferase (GST) fusion proteins expression, pGEX-TT (Albert, unpublished results) to give pGEX-rep1. To shift the reading frame, the construct pGEX-rep1 was digested by *Bam*HI, filled-in by Klenow enzyme + dNTPs and religated. The resulting plasmid was named pGEX-rep2. The integrity of the reading frame was checked by DNA sequencing of the final construct. For the expression of GST-rep fusion protein, an overnight culture of E.coli DH5α containing pGEXrep2 was diluted 1:10 in 1 liter of fresh LB+ampicillin medium, and after 1.5 hours shaking at 37°C induced by IPTG added to 0.1 mM final concentration. The culture was further shaken at 37°C for three hours and centrifuged, 15 min, 5000 ***g***. The cell pellet was resuspended in 40 mL of lysis buffer (50 mM Tris-HCl pH 7.5, 100 mM KCl, 1 mM PMSF). For the purification of the GST-rep fusion protein, the cells were disintegrated by sonication 3×1 minute by Cell Disrupter B15 (BRANSON) on ice. Cell debris and inclusion bodies were pelleted by centrifugation at 17 000 ***g***, 15 min. The supernatant was mixed with equilibrated glutathione-Sepharose4B (Pharmacia) and incubated for three hours at 4°C. The beads were washed extensively on the column by the lysis buffer until $A_{280}<0.01$.

For the analysis of GST-rep protein expression, a small aliquot (1 µL) of affinity beads was boiled in SDS loading buffer and separated by SDS PAGE.

2.4. Preparation of polyclonal antiserum against the repetitive region of MRJP3

The affinity purified GST-rep2 fusion protein was further purified by preparative SDS-PAGE. The gels were stained by 0.3 M $CuCl_2$ for 5 min (Lee et al., 1987). The part of the gel with the fusion protein was cut, destained 10 min in 0.25 M EDTA, 0.25 M Tris-HCl pH 9 and washed twice for 8 min in water. The gel piece containing around 130 µg of protein was fragmented, ground, homogenized with PBS buffer and Freund's adjuvant passing it repeatedly through a syringe and injected subcutaneosly into a rabbit. The boost followed three weeks after the primary injection and then twice at 6–7 week intervals. Polyclonal antiserum was prepared from blood collected 12 days after the fourth injection.

2.5. Polyacrylamide gel electrophoresis and immunoblot analysis

Protein electrophoreses in 7% polyacrylamide gels were performed according to Laemmli (1970). Honeybee

head protein extracts were diluted 1:10 in SDS loading buffer, boiled for 5 minutes and electrophoresed. The gels were either stained with Coomasie blue or electrophoretically transferred on nitrocellulose membrane (BA85, Schleicher and Schuell, Germany) using the tank method of transfer. The membranes were first blocked for 1 hour by 5% skim milk in Tris-buffered saline, 0.05% Tween20 (TBST), then incubated with an antiserum against the GST-repeat fusion protein diluted 1:2000 in the same buffer, for 1 hour. After extensive washing, the membranes were incubated with secondary anti-rabbit HRP-linked antibodies (Amersham, dilution 1:10 000) in TBST-skim milk, for 1 hour. Bound antibody was detected by ECL detection kit and exposed to Hyperfilm ECL detection films (Amersham).

2.6. PCR amplifications

PCR reactions were performed using the following templates: 0.1 μg honeybee genomic DNA, 0.01 μg plasmid DNA encoding MRJP3 cDNAs, 0.01 μg of the total cDNA used for the preparation of the λ Uni-ZAP cDNA library or 2 μL of amplified cDNA library (8×10^6 pfu/μL). The reaction volume was 100 μL, containing 50 pmol of primers flanking the sequence of the repetitive region of pMRJP3 (primer #1: 5′-ATG TAA TTT TGA AGA ATG AAC TTG-3′ primer #2: 5′-TGT AGA TGA CTT AAT GAG AAA CAC-3′). The amplifications were done over 30 cycles using 1U of *Taq* polymerase (AmpliTaq, Perkin Elmer) per reaction using PTC 100 thermal cycler from MJ Research. The process was initiated by denaturation for 2 min at 94°C (the polymerase was added at this step) and amplification was performed as follows: 30 sec at 94°C, 30 sec at 54°C and 1 min at 72°C. The PCR products were size-fractionated in a 2% agarose gel (Low DNA, FMC Bioproducts) and visualized by staining with ethidium bromide. To sequence the PCR product, the appropriate band was isolated from the gel using QIAQUICK kit (Qiagen), ligated into the multiple cloning site of the PCRII TA cloning vector (Invitrogen) and the isolated plasmid DNA was sequenced.

2.7. DNA sequencing

Plasmid DNA was sequenced either by the Sanger dideoxy termination method using Sequenase II kit (Unites States Biochemical), or by the cycle sequencing method using the Prism Ready Reaction Dyedeoxy Terminator kit (Perkin Elmer) on an ABI 373A sequencing device according to the manufacturer's instructions.

3. Results

3.1. Antibodies raised against the repetitive region of MRJP3 react with several MRJP bands

Immunoblot analyses of three different samples of royal jelly proteins, size-separated by SDS-PAGE, were performed with antiserum raised against the GST-rep fusion protein. Several discrete bands with apparent molecular mass in the 60–70 kDa range were resolved (Fig. 1(a)). These immunoreactive proteins have been found to possess the identical N-terminal sequences: AAVNHQR(K/R)SANNLAH (Schmitzova et al., 1998)

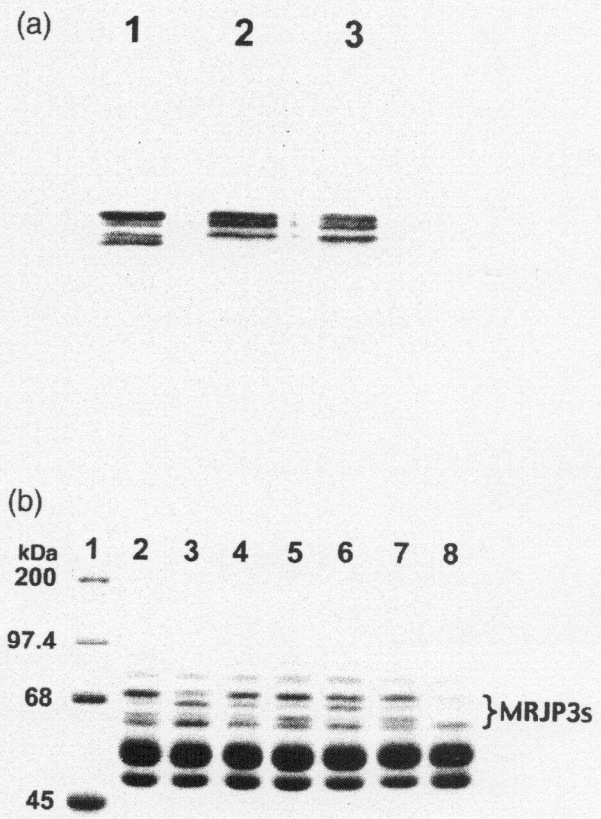

Fig. 1. Identification of MRJP3 protein in royal and worker jellies. (a) Detection of MRJP3 protein with antiserum against its repetitive region. 20 μg of three different samples of royal jelly were electrophoresed, blotted and immunodetected with antibodies against the repetitive region of MRJP3. In each case, several immunoreactive bands of different molecular masses were recognized. Different samples of royal jelly differ in their MRJP3 banding profile. (b) Electrophoretic profiles of royal jelly and 6 different worker jellies from the same colony. Royal jelly (lane 2) and worker jellies from 6 different cells on the same frame (lanes 3–8) were separated by SDS PAGE, the gel was stained with Coomasie blue. The region, where the immunoreactive MRJP3 proteins appear is shown on the right side. The amounts of distinct forms of MRJP3 differ among worker jellies.

which correspond to the sequence inferred from MRJP3 cDNA (Albert et al., 1996). Interestingly, banding patterns differed among jellies of different origin. On 7% SDS-PAGE, three to four protein bands could be distinguished in different samples of royal jelly (Fig. 1(a)).

We also compared royal and worker jellies within one honeybee colony obtained from different cells of one comb. Quantitative representation of different MRJP3 forms not only differed between royal and worker jelly, but also jellies provided to different worker cells differed mutually (Fig. 1(b)).

3.2. Identification of size variability of MRJP3 repetitive region

Immunological studies revealed that there are several MRJP3 forms in royal jelly. Amounts and molecular masses of MRJP3 forms varied among different royal jellies.

A hypothesis has been worked out, according to which MRJP3 forms are products of polymorphic alleles of the MRJP3 gene. It is based on the results of Southern analysis indicating that there is only one copy of MRJP3 in the genome of haploid drone (Albert, Bhattacharya, Klaudiny and Simuth, unpublished observations). This hypothesis could explain the differences observed among different samples of royal jelly as well as differences among worker jellies provided to different worker larvae. The repetitive region of MRJP3 has been chosen as a possible site causing protein polymorphism because some repetitive genomic regions were previously described as highly polymorphic variable number tandem repeat (VNTR) markers (Wolff et al., 1991). To test this hypothesis, two primers flanking the repetitive region of MRJP3 (see Section 2) were designed. These primers were used for PCR amplifications with an original cDNA preparation used for the construction of the nurse honeybee heads cDNA library (Klaudiny et al., 1994) or phages of the amplified cDNA library. In both cases, three dominant products could be seen on the agarose gel electrophoresis (Fig. 2, lane 2 shows the PCR profile of the cDNA preparation). This analysis shows that in the pool of cDNAs from nurse honeybee heads, there are cDNAs that differ in the length of the repetitive region.

3.3. Characterization of cDNAs encoding polymorphic alleles of MRJP3

The PCR approach was used to analyse the cDNA clones obtained by immunoscreening of the nurse heads cDNA library with antibodies against water-soluble proteins of royal jelly. Twenty plasmids with cDNA inserts larger than 1600 bps were amplified employing primers described above. Three cDNA clones were identified that yielded PCR products with sizes different from that of

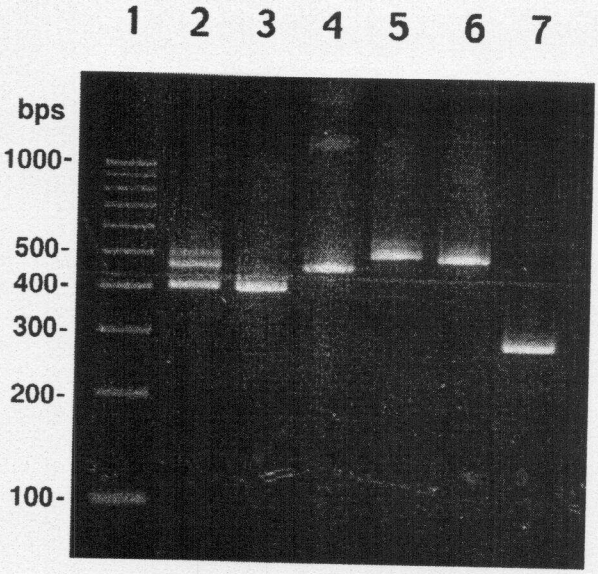

Fig. 2. PCR amplification of the MRJP3 repetitive region from total cDNA and plasmids harboring different MRJP3 forms. PCR amplification of the MRJP3 repetitive region has been performed with different templates: lane 2: total cDNA preparation, lane 3: pMRJP3a, lane 4: pMRJP3c, lane 5: pMRJP3d, lane 6: pMRJP3e, lane 7: pMRJP3b. Lane 1: 100 bps DNA Ladder (BIOMOL). Three DNA bands were amplified from the preparation of the total cDNA, whereas PCR with plasmids harboring different MRJP3 cDNA yielded single bands of distinct sizes.

RJP57-1, previously characterized form of MRJP3 (Fig. 2, lanes 4,5,7). The original cDNA preparation yielding three different PCR products was used as a size marker (Fig. 2, lane 2). cDNA termini as well as repetitive regions of all these cDNAs were sequenced. Two cDNAs, those giving the shortest and the longest PCR products, were sequenced completely. They were identical at each nucleotide, except in the repetitive region. They both show a high degree of identity with MRJP3 cDNA. We termed these new cDNAs, MRJP3b and MRJP3d respectively. The cDNA of the third plasmid giving PCR product of medial size was termed MRJP3c. The original MRJP3 cDNA was renamed MRJP3a. Comparison of the MRJP3b and MRJP3d sequences with that of MRJP3a revealed several single base differences leading to silent or conservative, but in some cases also to non-conservative substitutions (Table 1). However, the most remarkable were the differences in the size of the repetitive regions. These differed predominantly in the number of the repetitive units but also in the sequences near the end of the repetitive region (see Fig. 3, Table 2).

3.4. Characterization of another MRJP3 repetitive region obtained from genomic DNA

PCR amplification was performed with the genomic DNA of a single worker honeybee. The bee used in this

Table 1
Nucleotide and inferred amino acid alterations between MRJP3a and MRJP3d/MRJP3b sequences. The numbers refer to the sequence of the MRJP3 cDNA which is deposited in the international databases (accession number: Z26318). The differences in repeat number are discussed in Table 2. Non-silent mutations are bold

Position	Nucleotide mutation	Inferred amino acid mutation
785	A→G	**Thr→Ala**
804	A→G	**Tyr→Cys**
853	TCT→CTC	**Leu→Ser**
895	C→G	**Ser→Arg**
979	G→C	Ser→Ser
1000	G→A	Leu→Leu
1146	G→A	**Arg→Lys**
1799	T→deletion	non-coding region

experiment originated from a colony other than the colony used for nurse heads cDNA library preparation. Two different PCR products were obtained, sizes of which matched the size of MRJP3a (lower band) and MRJP3d (upper band). The larger PCR product was cloned and sequenced. The sequence of this fragment differs from the repetitive region of MRJP3d or any other MRJP3 characterized so far. (Fig. 3, Table 2). It consists of 28 tandemly repeated units. In repeat unit 9, the always 100% conserved asparagine residue is replaced by serine. The corresponding difference at the DNA level is an A→G transition. Sequenced DNA was obtained by PCR amplification, therefore a possibility exists that this difference occurred during the amplification process. Taken together, cloning of the PCR product of a single bee led to identification of another variation of the MRJP3 repetitive region. We termed this allele, MRJP3e (Fig. 3, Table 2).

3.5. Studies of polymorphism of the MRJP3 repetitive region within a single honeybee colony

The genomic DNAs isolated from 10 individual nurse honeybees and 5 individual drones of the same colony were used in the PCR analysis of polymorphism of the MRJP3 repetitive region (see Section 2).

In the set of five drones, only one PCR product from DNA of each individual was always amplified. The same has been observed by PCR amplifications of other DNAs obtained from drones of diverse origin (not shown). It shows that the genome of each haploid drone contains only one copy of the MRJP3 gene as has previously been suggested by Southern blot experiments (Albert, Bhattacharya, Klaudiny and Simuth, unpublished observations). PCR products of two different sizes were detected among the analyzed drone genomic DNAs, which indicates that the queen of this colony is heterozygous at the MRJP3 locus and the bands obtained represent the two alleles of her genotype.

In the set of 10 nurse bees, 5 different banding patterns were observed, showing that the MRJP3 locus is very polymorphic in the population of this colony (Fig. 4(a)). The number of PCR bands was either 1 or 2 showing that some nurse bees are homozygous at the MRJP3 locus. In each case (except bee 8), at least one of the bands fits by its size with one of the two queen alleles identified by drone analysis. The second alleles that can differ from the two maternal alleles come from father drones which mated with the queen during her mating flight. Fig. 4, lane 8 suggests that individuals might be present that possess two alleles which were not found in the queen, but the resolution is too poor to be certain. If, such individuals are found, they could be due to drifting, and this issue could be addressed by comparing repeat lengths from individuals taken from brood combs with those from older bees from the same hive.

Western blot analysis of the protein extracts from the heads of the bees used in PCR analysis (Fig. 4(b)) shows that the molecular mass of expressed MRJP3 proteins differs among nurse bee individuals. The banding patterns of proteins correlate perfectly with those obtained by PCR (compare Figs. 4(a) and (b)). However, the resolution is lower than that of PCR analysis. Aligning the immunoblot with the stained gel showed that individual MRJP3 forms are strongly expressed in nurse bee heads. Therefore the MRJP3 protein polymorphism can be easily detected by staining the proteins after their size-separation on SDS PAGE (Fig. 3(c)).

4. Discussion

4.1. Overall characterization of the repetitive region of MRJP3 and its variations

The repetitive region is located at the C-terminal part of MRJP3 open reading frame, but does not form the terminus by itself; it is followed by invariant residues HHSSKLH in all clones sequenced so far. The nucleotide sequence encoding these invariant residues was involved in the sequence of the 3′ terminal PCR primer (see Section 2). The sequence of the repetitive region consists of two distinguishable segments (Fig. 3). Both segments, although partially different, originated from a common ancestral sequence. The first segment located more N-terminally is more conserved and consists of tandemly arranged NQNA(D/N/G) pentapeptide units. They are encoded by AATCAGAATGCT(A/G)A(C/T) pentadecanucleotide, where 13 nucleotides are conserved to 100%. This motif appears in 6 to 8 copies in characterized MRJP3s. The interesting feature of this segment is that each pentadecanucleotide coding for the basic unit contains a recognition site for *Bsm*I restriction endonuclease (GAATGCN). This feature can be used to distinguish the alleles where the overall length of the

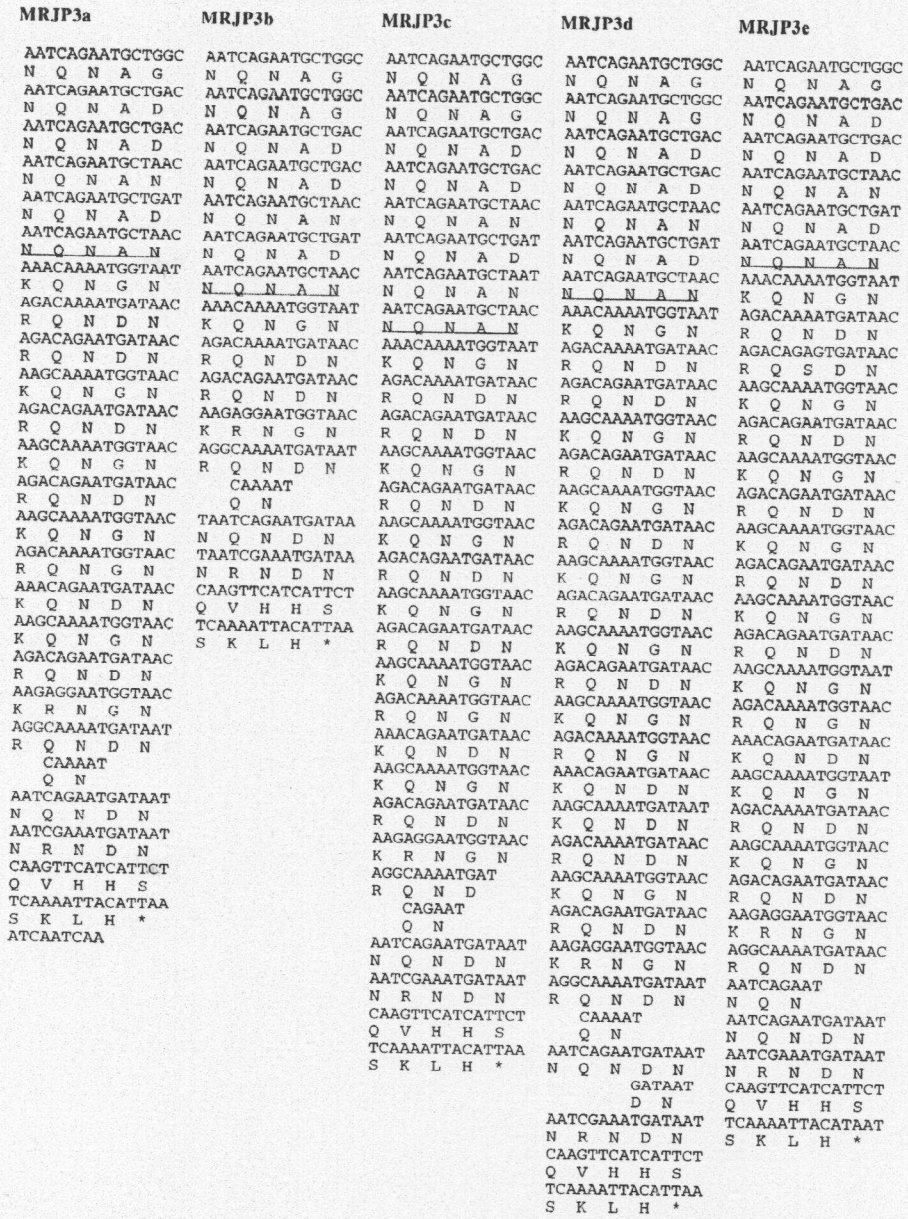

Fig. 3. Nucleotide and inferred amino acid sequences of the repetitive regions of five characterized MRJP3 alleles. One row represents the length of a basic repetitive unit. The lines separate the N-terminal *Bsm*I-cleavable segment from the C-terminal lysine/arginine-rich segment. Gaps close to the termini indicate the sites where short variable sequences occur.

repetitive region is identical, whereas the sizes of its two segments differ. The second segment is less conserved. It consists of (K/R)QN(D/G)N pentapeptides. The nucleotide sequence encoding the basic unit is A(A/G)(A/G)CA(A/G)AATG(A/G)TAA(C/T). Also in this segment, the invariant glutamine-asparagine (QN) motif is conserved, but units end with an invariant asparagine at the 5th position, which is the only position variable in the first segment. Interestingly, each unit in this segment begins with positively charged lysine or arginine which gives this part of the repeat region a basic character. The unit copy number of this segment varies from 5 to 21.

Near the terminus of the repetitive region, short sequences disturbing the tandem repetitive structure appear. They vary in their lengths and location between the repetitive regions (Fig. 3) and seem to originate from the repetitive units. Four different variations of this short sequence were detected among five MRJP3 alleles. Interestingly, the length differences are always in complete triplets. It seems that selection worked against the shift of the reading frame which would also change the

Table 2
Overview of the characterized repetitive regions of the MRJP3

Clone name	BsmI repeat[a]	XQNXN repeat[b]	Total repeats	Total base pairs in repetitive region
MRJP3a	6	16	22	336
MRJP3b	7	7	14	216
MRJP3c	8	17	25	393
MRJP3d	7	22	29	447
MRJP3e[c]	6	22	28	429

[a] Corresponds to the first segment of the repetitive region.
[b] Corresponds to the second segment of the repetitive region.
[c] This sequence was obtained by sequencing of the cloned PCR product obtained from the genomic DNA of one honeybee.

sequences behind it. This phenomenon could indicate the importance of conserved amino acid residues behind the MRJP3 repetitive region. Short variable sequences in the variable region seem to undergo their rearrangements independently from the fate of the 'great' repetitive region, as can be seen in MRJP3a and MRJP3b which have identical sequences of this short region, whereas the repetitive regions differ by 120 base pairs.

4.2. Function and origin of the MRJP3 repetitive region

Besides MRJP3, two other MRJPs contain repetitive regions. A similar pentapeptide repeat appears in the C-terminal region of MRJP2 protein and a totally different tripeptide repetitive motif appears at a different position in another member of the family, MRJP5. The repetitive region apparently evolved independently twice in MRJP protein family. All repetitive regions in MRJPs contain high amounts of nitrogen-rich amino acids. Their presence significantly increases the nitrogen content of the MRJP proteins. It seems therefore that the repetitive regions are domains storing nitrogen in biologically processable form (Albert, Bhattacharya, Klaudiny and Simuth, unpublished observations).

4.3. MRJP3 gene/protein polymorphism, its applications

Six different MRJP3 alleles were identified within 10 bees of a single honeybee colony. In experiments with different colonies and with bees sampled randomly from different locations, we were able to identify more than 10 different PCR products (alleles) ranging from ~380 to ~550 bp in their size. Evidently, MRJP3 gene/protein is highly polymorphic in the honey bee unlike the observation of Metcalf et al. (1975) with allozyme frequency in Hymenoptera. These authors argue that genetic variation which is not neutral becomes exposed and subsequently eliminated in the haploid organism (drone). Further, the variability is expected to be lower in haplo-diploid Hymenoptera, as well as in sex-linked loci, due to smaller effective population size and subsequent lower recombination rate (Crozier, 1976). The large

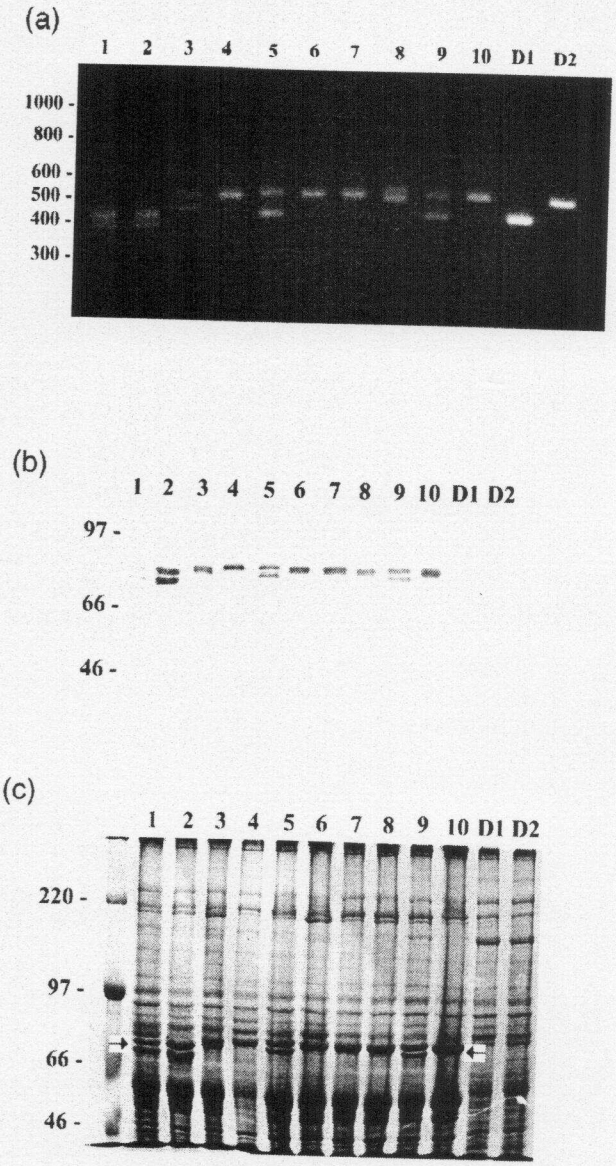

Fig. 4. Study of MRJP3 polymorphism within single honeybee colony. (a) Polymorphism at the genomic level. Genomic DNAs from 10 nurse bees (1–10) and two drones, each representing one queen allele (D1, D2), were subjected to PCR amplification and the products were size-separated in 2% agarose gel and stained by ethidium bromide. (b, c) Polymorphism of the MRJP3 protein in honeybee head extracts. Protein extracts from the heads of the same nurse bees and drones as above were separated on 7% SDS PAGE, electroblotted and probed with antiserum against MRJP3 repetitive region (b) or stained with Coomasie Blue (c). The arrow points to the polymorphic MRJP3 proteins. Polymorphic MRJP3 protein belongs to the dominant proteins of nurse honeybee head extract but is not synthesized by drones.

number of variations in the MRJP3 repetitive region can be explained in three ways:

1. The sequence rearrangements are generated via replication slippage or gene conversion, mutation rate of these is not compromised by male haploidy (Wolff et al., 1991).
2. We show that MRJP3 is not expressed in haploid drones (Fig. 4(b), lanes D1, D2). This eliminates selection against possible non-neutral alleles in the population during their passage through the haploid organism.
3. The variability of the MRJP3 repetitive region may be genetically neutral.

The observation that some alleles of MRJP3 contain, besides the polymorphism of the repetitive region, also several other single base differences in the coding region which do not always lead to conservative amino acid substitution (see Table 2), could support this argument. We also observed a phenomenon in some nurse bees, where the allele present in PCR analysis was not expressed at the protein level (Klaudiny, unpublished observations). Whether the polymorphism of the MRJP3 locus is due to the inherent ability of the MRJP3 repetitive region to generate new alleles or there is some selection keeping this locus highly polymorphic will be the subject of further investigation.

The highly polymorphic character of the MRJP3 repetitive region described here can be used as a highly informative locus in the genetic studies of honeybee colonies (Beye et al., 1998). Large size differences among MRJP3 alleles enable detection of the various alleles by PCR and routine gel electrophoresis in 2–3% agarose gel.

The polymorphism of the MRJP3 locus can also be detected at the protein level by SDS polyacrylamide electrophoresis of nurse bee head extracts, although the sensitivity is lower than with PCR. Polymorphic MRJP3 proteins are discernible in stained 7–8% polyacrylamide gels. However, immunological detection with the antiserum against the MRJP3 repetitive region gives more clear-cut results (Fig. 4). The polymorphism of MRJP3 protein provides a novel possibility of gentle, and simple, albeit only rough, genotyping of individuals, which requires neither the death nor removal of part of the body of the tested subject. As little as 1 microliter of larval food obtained from a nursing honeybee is sufficient to detect the MRJP3 protein polymorphism by Western blot analysis. This is a great advantage over present methods (except phenotypical methods which are not always neutral).

Acknowledgements

This work was supported by the Scientific Grant Agency of the Ministry of Education of the Slovak Republic and the Slovak Academy of Sciences VEGA 2/1098/95. Authors wish to thank Mrs. Jane Marsh for critical reading of the manuscript.

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