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Change in the Expression of Hypopharyngeal-Gland Proteins of the Worker Honeybees (*Apis mellifera* L.) with Age and/or Role

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The roles of adult worker honeybees change with age; young workers nurse brood by secreting bee milk (royal jelly), and older workers forage for nectar and pollen and process nectar into honey. The electrophoretic profile of worker hypopharyngeal-gland proteins changes with age and/or role. Immunoblotting analysis using affinity-purified antibodies against three major proteins (50, 56, and 64 kDa) of the nurse-bee gland showed that they are synthesized selectively and secreted as bee-milk proteins. Immunofluorescence study showed that the proteins condense in the duct after secretion from acini. However, a major 70-kDa protein synthesized specifically in the forager-bee hypopharyngeal gland was identified as an α-glucosidase. Therefore, the hypopharyngeal gland seems to have two distinct states differentiated by synthesizing of different major proteins depending on the age-dependent role change.

Key words: honeybee, hypopharyngeal gland, role change, royal jelly, α-glucosidase.

Age-dependent division of labor (role change) is a characteristic feature of the honeybee, *Apis mellifera* L., society. The behavior (role) of worker bees changes with age; young workers (generally, less than 14 days) nurse brood by secreting and feeding bee milk (royal jelly). Older workers (more than 10 days) forage for nectar and pollen and process nectar into honey (*1-4*). The age-dependency of the role change is flexible and largely depends on colony demands (*5*).

Physiological changes occur in some organs with the role change. For example, the hypopharyngeal gland, which is believed to synthesize bee milk (*6-11*), is well developed in young bees. In older bees, it shrinks and hydrolysis activity of sucrose in nectar to glucose and fructose is detected (*4, 12*). Consequently, we assume that the gland synthesizes different proteins according to age and/or the role change. However, there has been no substantial biochemical analysis of the proteins synthesized in the hypopharyngeal gland.

Halberstadt suggested that the protein profile of the gland changes with the age-dependent role change based on isoelectric focusing (*6*). Some reports describe similarities in the electrophoretic patterns of the nurse-bee gland and bee-milk proteins (*8, 10, 11*), and antiserum prepared against whole royal jelly seemed to cross-react with homogenate of hypopharyngeal gland (*8, 9*). Hanes and Simuth identified a 57-kDa protein in royal jelly and suggested that it is synthesized in the hypopharyngeal gland (*7*). However, to analyze the age-dependent expression and functions of proteins synthesized in the gland, it is necessary to purify and directly analyze them.

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First, we purified four major hypopharyngeal-gland proteins and analyzed the change in the expression of these proteins by immunoblotting. Three major proteins (50, 56, and 64 kDa) synthesized selectively in the nurse-bee gland were identified as bee-milk proteins. We also found that the major 70-kDa protein synthesized specifically in the forager-bee was an α-glucosidase. Therefore, the hypopharyngeal gland seems to have two distinct states differentiated with the age-dependent role change. This may be a unique model of an organ expressing different proteins according to the animal's behavior.

MATERIALS AND METHODS

Insects and Preparation of Hypopharyngeal-Gland Proteins—European honeybees (*A. mellifera* L.) kept at Tamagawa University were used. Newly-emerged day-0 worker bees were marked on their thorax using paint, and introduced into a normal queenright colony. Marked bees were collected 6 and 29 days later. The life span of a honeybee is usually 30 to 40 days. Nurse bees were collected when they were feeding brood; forager bees were collected when they returned to the colony after foraging for nectar and pollen. The bees were anesthetized on ice, and the hypopharyngeal glands were dissected under a binocular microscope. The glands (50 glands/ml) were homogenized in buffered insect saline (10 mM Tris-HCl, pH 7.4, containing 130 mM NaCl, 5 mM KCl, and 1 mM $CaCl_2$), containing 1 mM phenylmethylsulfonyl fluoride, 0.1 μg/ml pepstatin and 100 μg/ml leupeptin in a glass homogenizer on ice. By this method, major proteins are extracted efficiently from the gland. The homogenates were centrifuged at $700 \times g$ for 10 min and the supernatant was stored at -20°C.

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Electrophoresis and Immunoblotting Analysis—Electrophoresis on SDS-polyacrylamide slab gel was carried out by the method of Laemmli (*13*). Samples were denatured by heating for 20 min at 75°C in 1% (w/v) SDS and 2% (v/v) 2-mercaptoethanol. After electrophoresis, the gels were stained with Coomassie Brilliant Blue (CBB) by the method of Fairbanks *et al.* (*14*). Separated proteins were transferred electrophoretically from the gel to nitrocellulose filter papers (BA85, Schleicher and Schuell), and the filter papers were immersed in skim-milk solution (20 mM Tris-HCl buffer, pH 7.9, containing 5% skim milk) for 1 h. The filter papers were transferred to 10 ml of rinse solution (10 mM Tris-HCl buffer, pH 7.9, containing 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, and 0.25% skim milk) containing 1 μl of affinity-purified antibody. After incubation for 4 h at room temperature, they were washed well with rinse solution, and transferred to 5 ml of rinse solution containing radioiodinated anti-rabbit IgG antibody (2×10^6 cpm) for 2 h. Finally, they were washed well with rinse solution and subjected to autoradiography with Kodak XAR film.

Antibodies against 50-, 56-, 64-, and 70-kDa Proteins and Affinity Purification—Antiserum against 56-kDa protein was prepared by immunizing male albino rabbits with purified 56-kDa protein (25 μg) with complete Freund adjuvant, followed by a booster injection of the same amount of protein 14 days later. To prepare antisera against 50-, 64-, and 70-kDa proteins, homogenates of hypopharyngeal glands were subjected to SDS-polyacrylamide gel electrophoresis and the bands corresponding to each protein (100 μg of each) were excised from the gel. Each excised gel was homogenized in 1 ml of buffered insect saline and injected into a rabbit with complete Freund adjuvant followed by a booster injection.

Affinity purification of each antibody was performed with the final preparations of 50-, 56-, 64-, and 70-kDa proteins; purified 56- and 70-kDa proteins (50 μg of each) and HPLC fractions containing 56- and 64-kDa proteins (50 μg of each) were first subjected to SDS-polyacrylamide gel electrophoresis and then blotted onto nitrocellulose filter papers. The small portions where each protein was concentrated were excised and treated with 5% skim-milk solution. Each filter strip was incubated in 15 ml of rinse solution containing twofold diluted corresponding antiserum at 4°C for 15 h with gentle shaking. The strips were washed well with rinse solution and the specific antibody was extracted with 0.2 M glycine-HCl buffer, pH 2.8. The resulting extract was neutralized, and a final concentration of 0.1% bovine serum albumin was added.

Immunofluorescence Study of Hypopharyngeal Gland with Antibodies against 50-, 56-, and 64-kDa Proteins—Hypopharyngeal glands of nurse bees were dissected and frozen in embedding medium (Tissue-Tek, Miles), and 10 μm sections were prepared on a gelatin-coated fluorescence-free glass slide. Samples were fixed in 10 mM sodium phosphate buffer, pH 7.4, containing 157 mM NaCl and 5 mM KCl and 1.5% formaldehyde for 15 min, and washed with buffered insect saline. Then, they were incubated with 30 μl of affinity-purified antibody solution for 1 h at room temperature. Serial sections were treated with normal IgG as controls. The specimens were then washed well with buffered insect saline and incubated with 30 μl of 20-fold diluted FITC-conjugated goat anti-rabbit IgG antibody for 20 min. Finally, they were immersed in 90% fluorescence-free glycerol and mounted with coverslips for examination.

Enzyme Assay—The activity of α-glucosidase was determined by measuring glucose liberated from sucrose by the modification of Momose's method (*15*). In brief, 20 μl of 10 mM phosphate buffer, pH 7.0, containing 0.1 M sucrose was mixed with 10 μl of test sample, and incubated for 10 min at 30°C. After boiling for 3 min to stop the reaction, 50 μl of 0.3% (w/v) 3,6-dinitrophthalic acid and 50 μl of alkaline solution [5% (w/v) sodium thiosulfate and 25% (w/v) potassium carbonate], were added and the mixture was left for 10 min at 100°C. The mixture was diluted to 1 ml and the absorbance at 450 nm was measured. One unit of the enzyme activity was defined as the activity that hydrolyzed 1 μmol of sucrose in 1 min.

RESULTS

Change in Electrophoretic Profile of Hypopharyngeal-Gland Proteins with the Age-Dependent Role Change—To determine whether the function of the hypopharyngeal gland changes with the age-dependent role change, first, we identified proteins selectively expressed in the glands of nurse and forager bees using SDS-polyacrylamide gel electrophoresis. Nurse bees and forager bees are collected according to their behavior. Figure 1A shows that three major proteins with molecular masses of 50, 56, and 64 kDa were found in the nurse-bee glands, whereas two major proteins with molecular masses of 56 and 70 kDa were detected in glands of forager bees. We also analyzed homogenates from 6-day old and 29-day old workers whose roles were not identified (Fig. 1B). The same electrophoretic profile as that in Fig. 1A was obtained, coinciding with the previous observation that the role change is a function of age; young workers nurse brood, whereas older workers

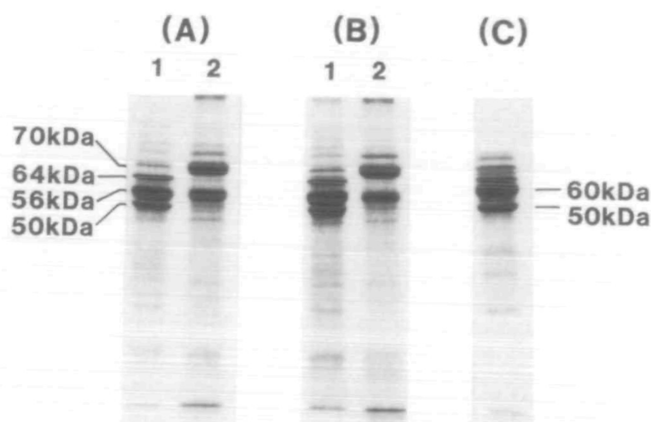

Fig. 1. **SDS-polyacrylamide gel electrophoresis of hypopharyngeal gland proteins of worker bees.** Homogenates (15 μg of each protein) of hypopharyngeal glands from nurse bees (Panel A, lane 1), forager bees (Panel A, lane 2), 6-day worker bees (Panel B, lane 1), and 29-day worker bees (Panel B, lane 2) were subjected to SDS-polyacrylamide (12.5%) gel electrophoresis followed by staining with CBB. In panel (C), 50 μg of bee milk (royal jelly) proteins were analyzed. The positions of the bands for 50-, 56-, and 64-kDa proteins of the nurse-bee gland, and 70-kDa protein of the forager-bee gland are shown on the left. The positions of the bands for the two major proteins (50- and 60-kDa proteins) of bee milk are shown on the right.

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forage for and process nectar (*1-4*). Thus, the change in the expression of hypopharyngeal-gland proteins should be due to the age-dependent role change. The protein profile of nurse-bee glands is similar to that of bee milk (Fig. 1C), as reported previously (*6, 8, 10, 11*), suggesting that the gland of younger bees synthesizes bee-milk proteins.

Purification of 50-, 56-, and 64-kDa Proteins of Hypopharyngeal Gland and Identification as Bee-Milk Proteins—We purified 50-, 56-, and 64-kDa proteins from the hypopharyngeal gland of nurse bees and analyzed the expression by immunoblotting. The homogenate of nurse-bee glands was subjected to HPLC with a reverse-phase column of Synchropak RP-R (C18); bound materials were eluted with a linear gradient of 0 to 60% acetonitrile. Figure 2A shows two major peaks (1 and 2) and some minor peaks. Peak 1 gave a single 56-kDa protein at SDS-polyacrylamide gel electrophoresis as shown in Fig. 2B, lane 1. Peak 2 contained a mixture of 50- and 64-kDa proteins, as shown in Fig. 2B, lane 2. Therefore, we excised these three proteins from the SDS-polyacrylamide gel and used them as antigens to prepare affinity-purified antibodies.

We examined the expression of the 50-, 56-, and 64-kDa proteins by immunoblotting analysis. Figure 3, A, B, and C, shows that 50-, 56-, and 64-kDa proteins were detected in the nurse-bee gland, but not in the forager-bee gland, coinciding with the result in Fig. 1. All these proteins were also detected in bee milk. These results suggested that these three proteins are synthesized in the nurse-bee gland

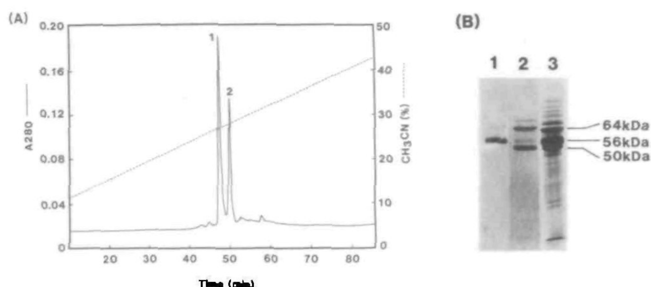

Fig. 2 **(A) Elution profile of hypopharyngeal-gland proteins of nurse bees on C_{18} reverse-phase HPLC, and (B) SDS-polyacrylamide gel electrophoresis of fractions.** (A) A homogenate of the hypopharyngeal gland of nurse bees was subjected to a column of Synchropak RP-R (C18) reverse-phase HPLC (250×4 1 mm, Gilson) equilibrated with 0.05% trifluoroacetic acid. Bound materials were eluted with a linear gradient of 0 to 60% acetonitrile. Proteins were detected by measuring absorbance at 280 nm (B) SDS-polyacrylamide gel electrophoresis of peak-1 (2 μg, lane 1) and peak-2 (5 μg, lane 2) fractions of Mono Q FPLC, and homogenate of nurse-bee gland (15 μg, lane 3). Proteins were detected by staining with CBB. The positions of 50-, 56-, and 64-kDa proteins are shown on the right.

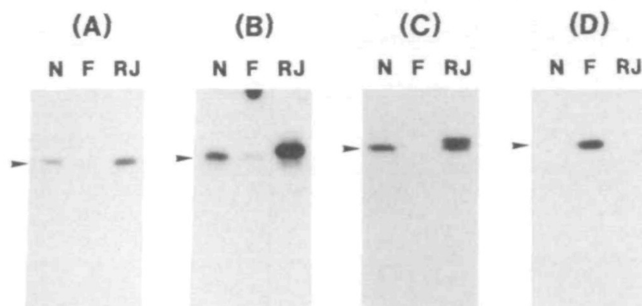

Fig 3. **Immunoblotting analysis of homogenates from nurse-bee and forager-bee hypopharyngeal glands, and bee-milk proteins with antibodies against 50-, 56-, 64-, and 70-kDa proteins.** Homogenates (15 μg of each) of the hypopharyngeal gland from nurse bees (lane N), forager bees (lane F), and bee-milk (royal jelly) proteins (lane RJ) were analyzed by immunoblotting analysis using affinity-purified antibodies against 50- (Panel A), 56- (B), 64- (C), and 70-kDa proteins (D). The positions of the band for each protein are shown by arrowheads.

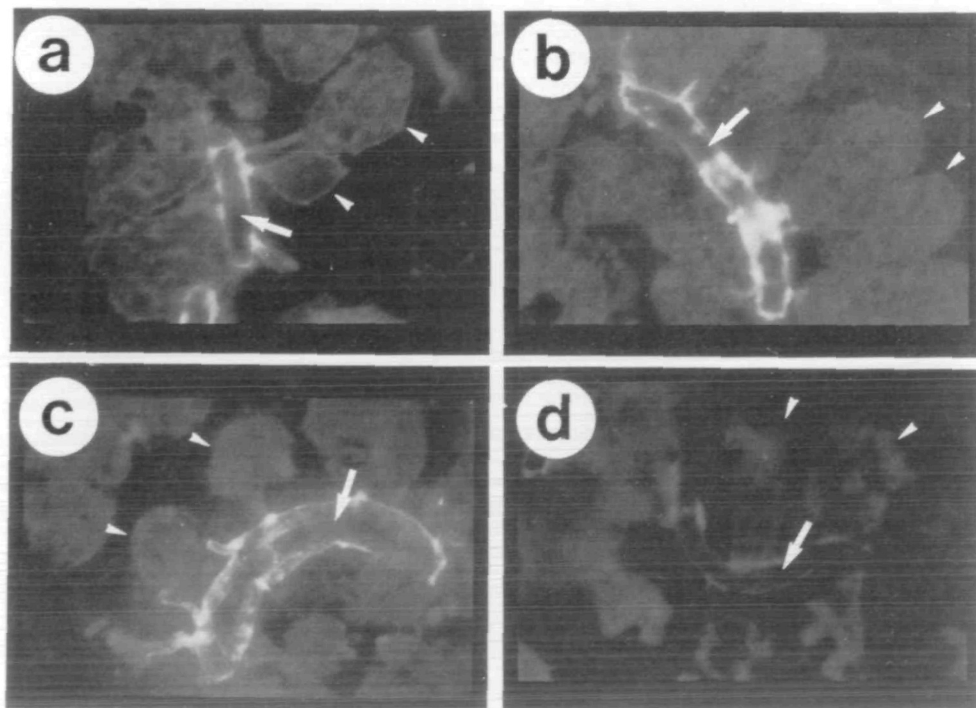

Fig. 4. **Immunofluorescence study of 50-, 56-, and 64-kDa proteins in hypopharyngeal gland of nurse bees.** Sections of the hypopharyngeal gland from nurse bees were subjected to immunofluorescence staining with affinity-purified antibodies against 50- (Panel a), 56- (b), and 64-kDa proteins (c) (d) Background fluorescence of section obtained with normal IgG A duct (indicated by an arrow) and some secretory cells (acini) attached to the duct (indicated by arrowheads) can be seen in each panel.

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and are secreted as bee-milk proteins. However, the molecular mass of the protein detected in the bee milk with the anti-56-kDa protein antibody was slightly larger (60 kDa), and two bands with molecular masses of 64 and 72 kDa were detected with the anti-64-kDa protein antibody. Possibly, the 56- and 64-kDa proteins are stored in the bee milk in modified forms.

We also localized the 50-, 56-, and 64-kDa proteins in the nurse-bee gland by immunofluorescence studies. As shown in Fig. 4, antibody-specific fluorescence was detected in the duct of the gland, suggesting that the proteins condense in the duct to be secreted as bee-milk proteins.

Purification of 70-kDa Protein and Identification as α-Glucosidase—To purify the 70-kDa protein, we subjected the homogenate of forager-bee glands to a column of Mono Q FPLC equilibrated with 10 mM Tris-HCl buffer, pH 7.4, containing 130 mM NaCl, 5 mM KCl, and 1 mM $CaCl_2$. The bound materials were eluted with a linear gradient of 0 to 0.3 M NaCl. As shown in Fig. 5B, 70-kDa protein was a major component of the elutant from fractions 25 to 29. Previous reports indicate that the α-glucosidase activity in the hypopharyngeal gland, which converts sucrose in nectar to glucose and fructose, remains at a very low level in glands of young bees, and reaches a higher level in glands of older bees (*12, 16, 17*). Therefore, we hypothesized that the major 70-kDa protein synthesized in the forager-bee gland is an α-glucosidase. To test this idea, we measured the α-glucosidase activity of the fractions. Figure 5A shows that the activity corresponded to 70-kDa protein, suggesting that this protein is an α-glucosidase. However, a minor 78-kDa protein was also detected in the fractions.

To remove this 78-kDa protein, we subjected the Mono Q fractions to Superose 12 gel filtration FPLC. As shown in Fig. 6, A and B, the 78-kDa protein disappeared, and the α-glucosidase activity and 70-kDa protein was coeluted again. The recoveries of both the 70-kDa protein and the α-glucosidase activity were more than 50% in the Mono Q and Superose 12 steps. Consequently, we conclude that the 70-kDa protein is an α-glucosidase. The protein is thought to be a monomer as a result of calibrating the Superose 12 gel with molecular mass markers.

The 70-kDa protein is expressed selectively in the forager-bee gland as shown by immunoblotting analysis

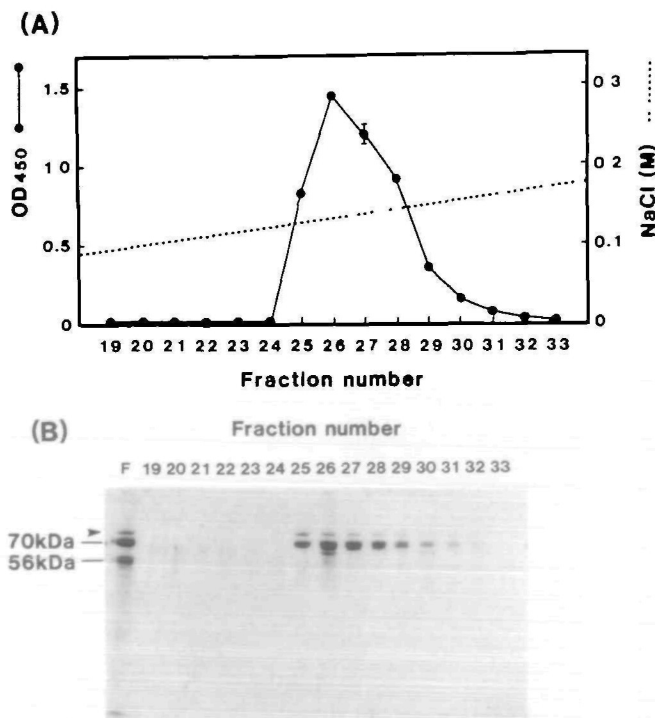

Fig 5 **Elution profiles of α-glycosidase activity and 70-kDa protein of hypopharyngeal gland on Mono Q FPLC.** (A) A homogenate of the hypopharyngeal gland of forager bees (120 μg) was subjected to a column of Mono Q FPLC (0.5×5 cm, Pharmacia). Bound materials were eluted with a linear gradient of 0 to 0.3 M NaCl in each 0.5 ml fraction. α-Glycosidase activity of each fraction was measured as described Points indicate mean values of duplicate measurements. (B) SDS-polyacrylamide gel electrophoresis of each fraction (10 μl) The homogenate of forager-bee glands (10 μg) is in lane F. The positions of 56- and 70-kDa proteins are shown on the left. The position of contaminant 78-kDa protein is indicated by an arrowhead

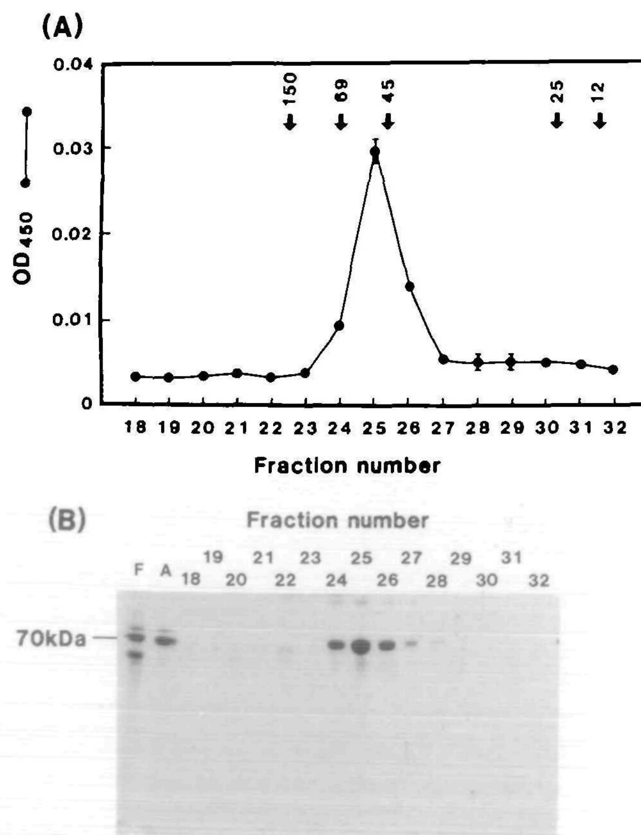

Fig. 6 **Elution profiles of α-glycosidase activity and 70-kDa protein on Superose 12 FPLC.** (A) Mono Q active fractions (12 μg) were combined and subjected to a column of Superose 12 FPLC (1.2× 30 cm, Pharmacia) Half milliliter of each fraction was collected and α-glycosidase activity was measured Points indicate mean values of duplicate measurements. Arrows indicate the positions of molecular-mass markers: IgG (150 kDa), bovine serum albumin (69 kDa), ovalbumin (45 kDa), α-chymotrypsinogen (25 kDa), and chytochrome c (12 kDa). Molecular masses are given in kDa. (B) 170 μl of each fraction was precipitated with trichloroacetic acid and subjected to SDS-polyacrylamide gel electrophoresis The homogenates of forager-bee glands (10 μg) and Mono Q fractions (1 μg) are in lane F and A, respectively. The position of 70-kDa protein is shown on the left.

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(Fig. 3D). It is known that the protein synthesis of the hypopharyngeal gland of nurse bees is more than 10 times higher than that of forager bees (*8*). Therefore, it is possible that the expression of 70-kDa protein in the forager-bee gland did not increase substantially. To investigate this point, we measured the α-glucosidase activity of nurse- and forager-bee glands. We found that the α-glucosidase activity of the forager-bee gland (0.10 unit/gland) is seven times higher than that of the nurse-bee gland (0.014 unit/gland), indicating that the expression of α-glucosidase is activated.

DISCUSSION

We purified four major proteins from the hypopharyngeal gland of worker bees, and demonstrated that the expression pattern changes drastically with the age-dependent role change. Three proteins (50, 56, and 64 kDa) selectively synthesized in the nurse-bee gland were identified as bee-milk (royal jelly) proteins. The nurse-bee gland is a highly-differentiated tissue synthesizing and secreting bee-milk proteins. These proteins showed no cross-immunoreactivity, suggesting that they are different.

We assume that the 56- and 64-kDa proteins are stored in bee milk in modified forms, explaining the different electrophoretic profiles of the nurse-bee gland and bee-milk proteins. Recently, Hanes and Simuth identified a major 57-kDa protein in bee milk, and detected a 55-kDa immuno-crossreactive band in the hypopharyngeal gland (*7*). Our results seem to coincide with their results. They also isolated a cDNA for a royal-jelly-related protein from the whole head of the honeybee (*18*). The relationship between the protein encoded by the cDNA and the hypopharyngeal gland proteins we identified remains unknown at present.

Previous reports indicate that the α-glycosidase activity in the hypopharyngeal gland remains at a very low level in young worker bees, reaching a higher level in older bees (*4, 6, 12*). We purified a major 70-kDa protein selectively expressed in the forager-bee gland and identified it as an α-glucosidase. Consequently, the forager-bee gland is also highly differentiated in synthesizing this enzyme for processing nectar. Takewaki *et al.* purified two isoforms of α-glycosidase from the whole body of the worker bee. The 70-kDa protein could be an isoform of the enzyme (*19*).

At present, we cannot definitely exclude the possibility that the change is simply due to age and not to behavior. However, since older forager bees can redevelop degenerated glands to work as nurse bees when all younger worker bees are removed from a colony (*5*), it is reasonable that the change is due to the role change.

In general, differentiated cells express characteristic major proteins required for cellular functions. The cells constituting the acini of the hypopharyngeal gland express different major proteins with the age-dependent role change, and these proteins are required for the gland cellular functions: bee-milk proteins for the nurse-bee gland, and α-glycosidase for the forager-bee gland. The hypopharyngeal gland seems to have two discrete differentiation states according to the role of the worker bee. This could be a unique and excellent model of behavior-dependent expression of different proteins by an organ.

We suggested that juvenile hormone may be a key substance in inducing the changes in both the worker-bee behavior and the cellular functions of the gland (*4, 17, 20*). Juvenile hormone may also modulate the expression of these proteins in the hypopharyngeal gland.

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