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BIOCHEMISTRY AND MICROBIOLOGY OF POLLEN COLLECTED BY HONEY BEES (*APIS MELLIFERA* L.) FROM ALMOND, *PRUNUS DULCIS*. II. PROTEIN, AMINO ACIDS AND ENZYMES (1)

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SUMMARY

The protein content, amino acid composition, presence of selected enzymes and 10-hydroxy-Δ^2-decenoic acid were determined for almond *(Prunus dulcis)* pollen obtained from anthers of the flowers, from the corbiculae of foraging honey bees (*Apis mellifera* L.), and from brood comb cells after a sojourn of 7, 21, and 42 days. The difference in amino acid composition of corbicular pollen and of stored pollen appeared to be significant, and some amino acids decreased 20 %. Stored pollen had significantly less phosphatase and alpha amylase than corbicular pollen, but beta amylase activity was ca. the same. The *p*H of corbicular pollen was 4.2; that of pollen stored for 7 and 21 days was 4.5; and that of pollen stored for 42 days was 4.2. 10-hydroxy-Δ^2-decenoic acid was not detected in the floral, corbicular or stored pollen materials.

INTRODUCTION

Pollen is the natural food of honey bees, *Apis mellifera* L., and, except for water and the carbohydrates in nectar or honey, is the source of all nutrients bees require for

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(5) Mention of a commercial or proprietary product does not constitute an official endorsement of the product by USDA to the exclusion of others which may be suitable.

individual growth, development and reproduction of the colony. Nevertheless, pollen is rarely consumed by the bees until after it has been stored in the cells of combs. Also, pollen varies widely in protein content and amino acid composition (TODD and BRETHERICK, 1940; BARBIER, 1970), enzyme constitution (STANLEY and LINSKENS, 1974), microflora (GILLIAM, 1979 a; 1979 b), and in food value for bees (STANDIFER, 1967). Also, the activity of bees in collecting and storing pollen in comb cells may change the pollen somehow so the composition and characteristics after storage are acceptable and nutritious. HAYDAK (1958) reported that when the worker bee packs pollen in comb cells, she adds a regurgitated liquid from her proventriculus (honey stomach) that contains honey sugars and probably also other materials (i.e., cellular and glandular secretions, microorganisms, etc.). CASTEEL (1912) reported that the liquid added to pollen is chiefly honey or recently gathered nectar and that if any saliva is used, it is so small an amount as to be of no significance. The fact is that chemical and biochemical changes occurring in pollen after bees have stored it in comb cells are not clearly understood, though numerous investigators have offered suggestions as to possible mechanisms involved (see reviews by GILLIAM, 1979 a, 1979 b; LOPER *et al.*, 1980). Such information is an essential pre-requisite to the development of an adequate artificial protein diet for honey bees and to understand the complexities of the nutritional requirements. We thus need a full and detailed analysis of the chemical and biochemical complexities and physical characteristics of the pollen that honey bees store in the cells of the brood combs.

A study of the composition of stored pollen from almond, *Prunus dulcis* (Mill.) D. A. Webb, is therfore in progress at the Carl Hayden Bee Research Center. In nature, stored pollen is likely to include pollen from several species of plants, but it was decided to use a single species of pollen known to be a preferred, acceptable and adequately nutritious in our tests. The results of the analyses for microorganisms, lipids, vitamins and minerals have been published previously (GILLIAM, 1979 a, 1979 b; LOPER *et al.*, 1980). The present paper reports on the results of analyses of the same pollen for protein, amino acids and enzymes. Since we are here interested in pollen at three stages from collection to storage in the comb, we have used the following terminology throughout :

(1) *Floral pollen* is pollen taken directly from flowers without the intervention of bees.

(2) *Corbicular pollen* is the pelleted pollen removed from the corbiculae of foragers returning to the hive.

(3) *Stored pollen* is the pollen bees have packed into cells of brood combs and is commonly referred to as « bee bread ».

MATERIALS AND METHODS

The floral pollen for the study was obtained from a commercial source that harvests specific pollens and markets them for use in mechanical (artificial) pollination of high-value crops. It was collected from

the anthers of the flowers by hand in March 1977, refrigerated for several days, and then frozen in liquid nitrogen and held at 0 °C until analyzed. The corbicular almond pollen pellets for the study were obtained February 27 and March 5, 1976 by fitting six colonies of bees in hives with pollen traps. The pellets in the traps were collected hourly, frozen in liquid nitrogen, and then stored at 0 °C until analyzed or until they were supplied to the colony of bees selected to produce the stored pollen. The pellets consisted of 99.8 % almond pollen based on color and light microscope examination.

The stored pollen for the study was obtained by managing and manipulating a colony containing ca. 7.7 kg of adult bees, a fertile young laying queen, and unsealed and sealed brood on September 13-15, 1976. Three empty combs were placed in the hive for bees to pack and store the pollen pellets. The colony was confined in a polyethylene-covered greenhouse regulated to maintain 31 °C during the day, 18 °C during the night, and 45-55 % R. H. A sucrose-water solution (2 : 1 v/v) and water were continuously available in the greenhouse. Bees in the colony were induced to store the corbicular pollen pellets in cells of the empty combs by placing two pollen pellets in each cell on both sides of each of the combs. Four hours later, we opened the hive and examined the combs to see whether the pollen pellets had been packed tightly in the cells. Two more pollen pellets were put in those comb cells containing packed pollen, and the frames were put back in the hive for another four hours. This process was repeated for 2 or 3 days by which time most of the comb cells were at least 1/2-3/4 full. At this time, the combs of stored pollen were removed from the hive and held in a room maintained at 35 °C and 55-60 % R. H., the average temperature and relative humidity of a honey bee colony. Quantities of the pollen stored in the comb cells that were adequate for the analyses were removed after 7, 21 and 42 days, immediately frozen in liquid nitrogen, and held at 0 °C until analyzed. We could perceive no visible gross differences between this stored pollen and pollen that was stored normally by free-flying colonies of bees.

Analysis for Crude Protein

The microkjeldahl technique for nitrogen was used to determine the crude protein (N × 6.25) content of the pollen materials according to the procedure of KIRK (1950). Pollen materials of sufficient size were analyzed in duplicate. Values are reported on the basis of air-dried samples. (Moisture content of the pollen used for these analyses was 3-7 %).

Analysis of Amino Acids

Samples were subjected to acid hydrolysis, taken up in *p*H 2.2 buffer, and analyzed on a Beckman amino acid analyzer (5) (SPACKMAN, 1963). Samples were analyzed in duplicate where possible. Values are reported on the basis of air-dried samples.

Analyses for Enzymes

The floral, corbicular and stored pollen materials were analyzed for cellulase, glucose oxidase, phosphatase (acid phosphatase), and amylase (alpha and beta) by an independent testing laboratory, WARF Institute, Inc., Madison, Wisconsin (WARF Institute Report, 1977). The methods used for these analyses were :

1. *Cellulase :* (Worthington Enzyme Manual, 1972)

A 30-mg sample was incubated for 2 hours at 37 °C with 200 mg microcrystalline cellulose, *p*H 5. Limits of detection for floral pollen and for corbicular and stored pollens were : 0.17 mg glucose and 5 mg glucose, respectively, produced per gram sample from 200 mg cellulose.

2. *Glucose oxidase :* (BERGMEYER *et al.*, 1974)

Limit of detection for floral pollen and for corbicular and stored pollens : six units per gram; one unit oxidizes 1 mole glucose per minute to gluconic acid at 35 °C, *p*H 5.1. Absorbance changes were recorded for 2-4 minutes at *p*H 7 at 25 °C.

3. *Phosphatase [acid phosphatase]* : (BERGMEYER, 1974)

Corbicular and stored pollens : *p*-nitro phenol produced from *p*-nitro phenyl phosphate per gram pollen at 37 °C for 30 minutes, *p*H 4.8. Samples of floral pollen were not available for this analysis.

4. *Amylase, Alpha and Beta :* (BERGMEYER *et al.*, 1974)

Grams of maltose produced per gram of corbicular and stored pollen for 5 minutes at 25 °C. Samples of floral pollen were not available for this analysis.

*The p*H

The *p*H's of corbicular and stored pollen samples were analyzed by the procedures of the A.O.A.C. (1975) (WARF Institute Report, 1977). Samples of floral pollen were not available for this analysis.

Analysis for 10-Hydroxy-Δ^2-decenoic Acid

Analyses for 10-hydroxy-Δ^2-decenoic acid in the floral, corbicular and stored pollens were done by the Plant Protection Phytochemistry Research Unit, Western Regional Research Center, AR, SEA, USDA, Berkeley, California. Both published methods (CALLOW *et al.*, 1959) and methods developed by the Unit were used in the search for this acid in the pollens.

RESULTS

Protein Content and Amino Acid Composition of Floral, Corbicular and Stored Almond Pollens

The results of the analyses for the protein content and amino acid composition of almond floral and corbicular pollen and of almond pollen stored 42 days in comb cells are reported in Table 1. Because the stored pollens did not differ significantly after 7, 21 and 42 days storage in comb cells, either in the content of the individual amino acids or the total amino acids, we present data only for the pollen stored 42 days. Values shown are percentages of each amino acid in the air-dried samples rather than percentages of each in the protein fraction because we wished to consider the changes during storage. When one compares the amino acid values in Table 1 for corbicular pollen with those for pollen stored 42 days, losses of between 5 and 20 % are observed, and the average loss was about 10 %. The percentage of crude protein was about the same in the floral pollen (22.6 %) and corbicular pollen (22.2 %) and also in pollen stored for 7 days (22.1 %) and 21 days (20.4 %), but dropped slightly in pollen stored 42 days (18.9 %). However, the totals for amino acid content of pollen stored 7, 21 and 42 days were 21.8, 21.3 and 21.5 % respectively. Total amino acid content was higher in the floral pollen (37.3 %) than in the corbicular pollen (24.5 %) or in the stored pollen for 42 days (21.5 %).

Our results do not indicate that the differences in crude protein and amino acid content of the pollen materials we analyzed are significant in the nutrition of bees. This can only be determined by feeding tests made to establish the comparative

TABL. 1. — *Percentages of crude protein and amino acids in samples (a) of almond pollen (1) hand-collected from flowers, (2) trapped from the corbicula of bees, and (3) stored 42 days in cells of brood comb.*

Protein % Amino Acid	Percent of Pollen Sample		
	Hand-Collected Floral Pollen	Bee-Collected (b) Corbicular Pollen	Stored Pollen in Comb Cells for 42 Days
Crude Protein	22.6	22.2	18.9
Amino Acid (c)			
Lysine	2.86	1.84	1.28
Histidine	0.84	0.53	0.48
Arginine	1.91	1.23	1.13
Threonine	1.45	0.96	0.84
Valine	2.32	1.56	1.42
Methionine	0.76	0.53	0.58
Isoleucine	1.76	1.21	1.07
Leucine	2.79	1.93	1.78
Phenylalanine	1.76	1.16	1.07
Aspartate	4.77	2.98	2.81
Serine	1.34	1.00	0.96
Glutamate	4.41	3.02	2.68
Proline	5.13	2.90	2.20
Glycine	1.83	1.26	1.12
Alanine	2.23	1.51	1.35
Cysteine	0.03	0.06	0.03
Tyrosine	1.10	0.75	0.68
Totals	37.36	24.53	21.55

(a) Samples air-dried; moisture content 3-7 %.
(b) Removed from the bees' legs with pollen traps.
(c) First 9 required by honey bees. Tryptophan is also essential for bees.

value of the pollen materials in promoting growth, development and brood-rearing in bee colonies.

The observed 40 % increase of cysteine in corbicular pollen could be either the result of microbial activity or the synthesis of cysteine from methionine since methionine decreased in corbicular pollen. Also, as noted in the footnote to Table 1, analysis was not done for tryptophan though it is essential for the bee. Determination for tryptophan in protein hydrolysates is frequently omitted because : (1) it is hardly ever a limiting essential amino acid, and (2) its isolation from protein requires a separate alkaline hydrolysis with an additional amount of sample needed.

Cellulase, Glucose Oxidase, Amylase and Phosphatase Analyses

The tests for cellulase and glucose oxidase were negative in all pollen materials examined for these enzymes. The content of alpha amylase and beta amylase, expressed as grams of maltose produced per gram pollen material, of corbicular pollen

was 0.22 and 0.22, that of pollen stored 7 days was 0.12 and 0.24, that of pollen stored 21 days was 0.14 and 0.24, and that of pollen stored 42 days was 0.14 and 0.22, respectively. The content of phosphatase (acid phosphatase), expressed as milligrams of *p*-nitro phenol produced from *p*-nitro phenyl phosphate per gram of pollen material, of corbicular pollen was 2.16, that of pollen stored 7, 21, and 42 days was 1.02, 1.62, and 1.20, respectively. Thus, the stored pollen had considerably less phosphatase and alpha amylase than the corbicular pollen, but corbicular pollen and stored pollen had about the same content of beta amylase. Futhermore, the corbicular pollen and the stored pollen differed only in that the amount of phosphatase decreased with storage. As noted in the methods, floral pollen was not analyzed for phosphatase or the amylases because the quantity of pollen material available was not adequate for the analyses.

*p*H *of Floral, Corbicular and Stored Pollen*

The *p*H of the corbicular pollen was about the same order (4.2) as the *p*H of the stored pollen, 4.5 after 7 and 21 days of storage and 4.2 after 42 days. The floral pollen was not analyzed for *p*H because the quantity of pollen materials available was not adequate for the analyses.

10-Hydroxy-Δ^2-decenoic Acid

10-hydroxy-Δ^2-decenoic acid was not detected in the floral, corbicular or stored pollen materials. Detection of this acid in the corbicular pollen and the stored pollen could indicate participation of the mandibular gland of the bees during the collecting of pollen from flowers and packing of the pollen in comb cells. Since 10-hydroxy-Δ^2-decenoic acid is a very stable material, these results suggest that the mandibular gland did not contribute this chemical to corbicular or stored pollen. However, mandibular gland secretion may have contributed other materials to the pollen.

DISCUSSION

Published reports are inconclusive and contradictory concerning the relative composition and nutritive value of pollen before and after it is stored by bees. For example, STANDIFER *et al.* (1960) reported that a mixture of stored pollen (several pollens of unknown sources) and honey did not stimulate hypopharyngeal gland development in newly emerged bees more than 10 % protein mixtures made of sucrose solution (2 : 1) plus mustard pollen *(Brassica rapa* L.), cherry pollen *(Prunus avium* L.), or almond pollen. MAURIZIO (1954) reported differences in the food value of pollen hand-collected directly from the flowers of plants and pollen collected by bees from the same plants. NIELSEN *et al.* (1955) speculated that differences in nutritive value of floral and corbicular pollen are probably caused by a mixing of the pollen with

other substances by the bee. STROIKOV (1963, 1967) reported that stored pollen has greater food value than fresh pollen and that stored pollen deteriorates during storage. HERBERT and SHIMANUKI (1978) reported little differences in the levels of protein and the nutritive value for bees of corbicular pollen pellets removed from the bees' legs with pollen traps and stored pollen.

Our analysis did not detect glucose oxidase in the floral, corbicular, or stored pollen, though it is a component of hypopharyngeal secretion (GAUHE, 1940). The presence of this enzyme in the corbicular pollen or in the stored pollen would have indicated hypopharyngeal gland participation when the bees collect pollen from flowers or when they pack pollen in comb cells. (Glucose oxidase is present in honey and is probably derived from the salivary system of bees). It is also absent from many angiosperm pollens, which suggests that it is not involved in the chemical and microbial changes that occur in corbicular pollen after stored by bees in comb cells.

The situation in regard to the analyses for the other enzymes is somewhat contradictory. RINAUDO *et al.* (1973) reported that amylase is present in the hypopharyngeal gland secretion of honey bees. HERBERT and SHIMANUKI (1978) reported that they found starch present in corbicular pollen but not in stored pollen obtained from seven locations in the U.S.A. In the present study, amylase was found in both corbicular pollen and stored pollen. However, we detected ca. 2 × more alpha amylase in corbicular pollen than in stored pollen (beta amylase was ca. equal). The decrease of amylase (g maltose produced/g pollen) and also of phosphatase (mg *p*-nitro phenol produced from *p*-nitro phenyl phosphate/g pollen) from 0.22 and 2.6 in stored pollen, respectively, could be the result of hydrolysis by proteolytic enzyme activity induced by changes in microflora, inhibition of enzyme activity, or dilution. Cellulase was absent, which again is characteristic of many angiosperm pollens. Therefore, it too, may not be involved in the conversion of corbicular pollen to stored pollen.

Finally, the data obtained in the present study (Tabl. 1) provide no evidence for any significant addition of proline to pollen by bees when they pack it into cells of comb. Nevertheless, proline is present in honey (derived from the bee), pollen, and secretions of the hypopharyngeal glands of nurse bees (WHITE and RUDYJ, 1978; and see references cited in DAVIES, 1978). Also, foraging bees add proline to nectar when it is collected from plants (DAVIES, 1978).

The small differences in *p*H between almond corbicular pollen (4.2) and stored pollen (4.5) after 7 and 21 days of storage in comb cells suggest that neither the liquid materials bees add to pollen when packing pollen into comb cells nor the activities of microflora produce any appreciable change in the *p*H value during the conversion of corbicular pollen to stored pollen. Nevertheless, even the comparatively small amount of this liquid that is used in the pollen packing process by bees may somehow produce significant changes in the chemical, biochemical and microbiological properties of the stored pollen.

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RÉSUMÉ

BIOCHIMIE ET MICROBIOLOGIE DU POLLEN RÉCOLTÉ PAR LES ABEILLES (*APIS MELLIFERA* L.) SUR L'AMANDIER *(PRUNUS DULCIS)*. II. PROTÉINES, ACIDES AMINÉS ET ENZYMES.

On a analysé du pollen d'amandier *(Prunus dulcis)* provenant soit des anthères des fleurs (récolté à la main), soit des pattes des abeilles (*Apis mellifera* L.) butineuses, soit des réserves dans les rayons (pollen stocké), du point de vue de la teneur en protéines, de la composition en acides aminés et de la présence de cellulase, glucose oxydase, alpha-amylase, beta-amylase, phosphatase et acide hydroxy-10-Δ^2-décène-2 oïque. Le pollen des rayons a été analysé après avoir été stocké 7 jours, 21 jours et 42 jours dans les cellules du rayon. Le pourcentage de protéines brutes dans le pollen varie de 18,9 % dans le pollen stocké en rayons pendant 42 jours à 22,6 % dans le pollen récolté à la main. La différence de composition en acides aminés entre les pelotes de pollen prises sur les pattes des butineuses et le pollen stocké dans les cellules des rayons se révèle significative. La celluase, la glucose oxydase et l'acide hydroxy-10-Δ^2-décène-2 oïque n'ont été détectés dans aucun des échantillons de pollen examinés. Le pollen stocké dans les cellules des rayons possédait significativement moins de phosphatase et d'alpha-amylase que les pelotes de pollen provenant des pattes d'abeilles, mais l'activité de la bêta-amylase était sensiblement la même.

ZUSAMMENFASSUNG

BIOCHEMIE UND MIKROBIOLOGIE VON BIENENGESAMMELTEN POLLEN AUS MANDELBLÜTEN *(PRUNUS DULCIS)*. II. PROTEIN, AMINOSÄUREN UND ENZYME.

Mandelpollen *(Prunus dulcis)* verschiedener Herkunft (handgesammelt von den Antheren der Blüten, Höschenpollen von den Beinen der Bienen und aus Brutwaben im Volk-Vorratspollen-) wurde auf Proteingehalt, Zusammensetzung der Aminosäuren, Zellulase, Glukoseoxidase, Alpha-Amylase, Beta-Amylase, Phosphatase und 10-Hydroxy-Δ^2-decensäure untersucht. Der Vorratspollen aus Brutwaben wurde nach 7, 21 und 42 Tagen Aufenthalt in den Wabenzellen untersucht. Der prozentuelle Anteil von Rohprotein im Pollen schwankte von 18,9 % im Vorratspollen, 42 Tage in Wabenzellen gelagert, bis 22,6 % bei frischem handgesammelten Pollen. Der Unterschied in der Zusammensetzung der Aminosäuren von Pollenhöschen aus der Pollenfalle und von Vorratspollen aus Wabenzellen war signifikant. In keiner der Pollenproben wurde Zellulase, Glukoseoxidase oder 10-Hydroxy-Δ^2decensäure nachgewiesen. Vorratspollen aus Wabenzellen hatte signifikant weniger Phosphate und Alpha-Amylase als Pollenhöschen von den Beinen; die Aktivität der Beta-Amylase hingegen war bei beiden ungefähr gleich.

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