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Changes in protein components and storage stability of Royal Jelly under various conditions

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The changes in quality of Royal Jelly (RJ) stored at −20°C, 4°C, and room temperatures under dark and light conditions for up to 7 months were investigated. The results showed that colour, viscosity, fractions of water-soluble protein (WSP) and simple sugars of RJ changed significantly during storage at room temperature, but not at −20°C. The viscosity and browning intensity increased with storage time, and were enhanced by the ambient temperature. Two major fractions in WSP of RJ were obtained by gel filtration, while four to five different protein components were found when subjected to SDS-PAGE. The relative amounts and molecular weight distributions of these WSP components varied during storage. The results indicated that the quality deterioration of RJ during storage was due to the Maillard browning reaction.

INTRODUCTION

Natural food products that have undergone the least processing are now popular. Among them, royal jelly (RJ) is one of the most attractive and lasting products. It has been a commercial product, especially in dietetics and cosmetics, in many countries for more than 30 years. RJ is a secretion from the hypopharyngeal and mandibular glands of worker bees (*Apis mellifera* L.). It is a yellowish, creamy and acidic material with a slightly pungent taste. In the society of honey-bees, RJ is fed temporarily (less than 3 days) to the brood of workers and drones, but it is the only food of the queen bee throughout her life (Townsend & Lucas, 1940; Lercker *et al.*, 1981). The queen bee is well known for her reproductive ability, large body size and long life span. RJ is thus generally regarded as the major reason for the significant morphological and functional differences between queen and worker bees (Karaali *et al.*, 1988). Commercially, RJ can be produced in significant quantities using the Doolittle queen-rearing technique (Lensky, 1971).

The composition of RJ varies with seasonal and regional conditions. The average moisture content of RJ is 60–70%, crude protein 12–15%, lipid 3–6%, total sugars 10–16% and pH 3·5–4·5 (Takenaka, 1982; Howe *et al.*, 1985). The storage conditions of RJ depend upon the types of product. Raw or natural RJ, which is sold and consumed as it is, is stored at −20°C or lower, while the lyophilised type, either alone or in combination with other ingredients, is generally in capsulated or tablet forms and is stored at cool or room temperature. However, RJ spoils or deteriorates and loses its commercial value eventually when it is stored improperly. It was reported that there was a gradual deterioration of protein components (Okada *et al.*, 1979) and an increase in its viscosity and titratable acidity (Takenaka *et al.*, 1986; Yatsunami, 1988). These changes might be due to the Maillard reaction or lipid oxidation. Proteins of fresh RJ consist of water-soluble and water-insoluble fractions. Depending upon the extraction conditions used, the water-soluble protein (WSP) was the major fraction and made up 46–89% of the total protein (Tomoda *et al.*, 1977; Takenaka & Echigo, 1983); consequently, the change of WSP fraction might play an important role in the quality deterioration of RJ. However, there are very few data available on the changes in the composition of RJ during storage, especially in protein components.

In this study, the changes in quality of raw RJ, particularly in WSP, during a storage period of seven months both in the refrigerator and at room temperature were investigated. Finally, the feasibility of applying results obtained to detecting quality changes of RJ during storage was evaluated.

MATERIALS AND METHODS

Preparation of royal jelly samples

Two freshly harvested RJ samples (RJ-1 and RJ-2) were

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supplied directly from two different beekeepers in middle and southern Taiwan during June 1993. Bees (*Apis mellifera* L.) were fed with nectar and pollen from two fruit trees, longan (*Nephelium longana*) and lychee (*Litchi chinensis*). Each RJ sample was dispensed into 28 sterile, air-tight glass bottles (50 ml). These bottles were then randomly divided into four groups; seven bottles for each group were stored at –20°C, 4°C, and at room temperature in bright and dark places of laboratory, respectively. The light source was a fluorescent lamp (280 lux) and samples were illuminated for 15 h a day. The average room temperature was about 24 ± 3°C, and all bottled samples were protected from light by wrapping with aluminium foil, except those at room temperature in the bright place. Sampling was performed monthly and each bottle was sampled once only, in order to avoid the potential repeated freeze-thaw damage. Cold or frozen samples, were allowed to equilibrate to room temperature and then stirred with a glass rod to homogeneity before analysis. All analyses were done in duplicate.

Measurement of viscosity

The viscosity was measured using a Brookfield digital viscometer (Model DV-II, Brookfield Engineering Lab.) equipped with a small sample adapter (12 ml) and a no. 28 spindle. The RJ sample was previously equilibrated to 30·0 ± 0·1°C. The measurement was taken three times with the speed of spindle being set at 100 rpm.

Measurement of browning intensity

One gram of sample was diluted with distilled water to a final volume of 100 ml (Solution A). Ten millilitres of the Solution A was further diluted with an equal amount of distilled water and measured for absorbance at 420 nm using a spectrophotometer (Hitachi, U-2000). In addition, 50 ml of the above Solution A was withdrawn and mixed with 2·5 ml 10% trichloroacetic (TCA) solution and stirred for 5 min. The filtrate was then collected through a membrane filter (0·45 μm) to measure the absorbance at 420 nm.

Extraction of WSP

Two types of WSP, WSP-1 and WSP-2, were prepared from RJ. For the preparation of WSP-1, 5 ml of distilled water were added to 2·5 g sample; the supernatant after centrifugation (5000 × *g*, 30 min) was designated WSP-1. The extraction of WSP-2 was done as described by Takenaka and Echigo (1983) with modifications. Fifteen millilitres of 0·01 M phosphate buffer (pH 7·2) was added to 2·0 g of RJ samples and centrifuged (5000 × *g*, 30 min) at 4°C. The supernatant was withdrawn and saturated with 90% ammonium sulphate in an ice bath. The precipitate was collected after centrifugation (7000 × *g*, 50 min, 4°C) and redissolved with 10 ml of 0·01 M phosphate buffer (pH 7·2), followed by dialysing against distilled water in a cold room for about 2 days. The dialysed solution obtained, as WSP-2, was then lyophilised.

Gel filtration chromatography

The proteins in the WSP-2 were separated by gel filtration. The separation was carried out on a Sephacryl S-200 (Pharmacia, LKB) column (1·6 cm D × 90 cm H). The flow rate through the column was 15 ml/h using 0·01 M phosphate buffer, pH 7·2, as eluant. The relative amount of proteins in the eluted fractions was detected continuously with a UV-detector (ISCO, UA-6) at 280 nm. The area under a peak in the chromatogram was approximated by cutting the area out of the graph paper and weighing it (Robyt & White, 1987). The ratio of area of leading peak (peak 1) to that of the second peak (peak 2) was defined as R_a value.

SDS-PAGE analysis

The SDS-PAGE analysis of WSP-2 was performed according to Cooper (1977). 7·5% polyacrylamide gel was cast and run at 120 V using a Dual Mini Slab Kit (Atto, AE-6450). Protein bands were stained with Coomassie brilliant blue. The molecular weight markers were supplied the electrophoresis calibration kit for low-molecular-weight protein (Pharmacia LKB).

Sugar analysis

Sucrose, glucose and fructose were determined by HPLC (Ball, 1990). One gram of RJ sample was mixed with 5 ml of 80% (v/v) aqueous ethanol at 4°C for 30 min. The supernatant after centrifugation (7000 × *g*, 30 min) was filtered through a membrane filter (0·45 μm) before analysis. The column was a pre-packed column (4 mm i.d. × 250 mm L), packing material was Lichrosorb NH_2. Aqueous acetonitrile solution (acetonitrile/water = 87 : 13, v/v) was used as eluant, the flow rate was 1·0 ml/min.

Total nitrogen determination

The Kjeldahl method (AOAC, 1980) for the determination of organic nitrogen was used to determine the total nitrogen contents in the WSP-1 and RJ. One gram of RJ sample or aliquots of WSP-1 solution were mixed with 15 ml conc. sulphuric acid, and placed in a digestion tube, digested in a microwave digestion system (Maxidigest MX-350) for 15 min, followed by distilling and titrating with a Kjeltec System (Tecator, model 1026). The nitrogen content (%N, w/w) was then calculated, and the ratio of total nitrogen content for WSP-1 solution to that of RJ sample was defined as R_n value.

RESULTS AND DISCUSSION

Changes in the viscosity of RJ during storage

The viscosity of fresh RJ-2 was about 2060 cp and it remained almost unchanged up to 7 months when the sample was stored at –20°C (Table 1). However, the

Table 1. Changes in viscosity of royal jelly (RJ-2) stored under different conditions

Storage conditions	Viscosity[a] at storage period (month)				
	0	1	3	5	7
−20°C	2060	2070	2100	2020	2300
4°C	2060	2350	2790	3300	3630
Room temperature and					
Bright Place	2060	*E*[b]	*E*	*E*	*E*
Dark Place	2060	*E*	*E*	*E*	*E*

[a]Unit in centipoise (cp).
[b]The actual viscosities exceeded the upper limit (5000 cp) of the viscometer used under the specified condition as described in the Materials and Methods section.

viscosity increased linearly with time as storage temperature increased to 4°C. Furthermore, a remarkable change was observed for those samples stored at room temperature, the increase of viscosity was so sharp that it failed to be measured by the instrument. The viscosity was thus a sensitive parameter in tracing the storage stability of RJ.

Browning reaction of RJ during storage

The yellowish white colour of freshly harvested RJ became darkened gradually as storage time increased. Figure 1 shows how the browning reaction occurred in fresh RJs when stored under different conditions. The brown intensities increased rapidly and reached maxima within 2 months, when RJ were stored at room temperature in the presence or absence of light. On the other hand, the extent of the browning reaction increased to a much lower level when RJ were at 4°C, and was almost negligible at −20°C. Apparently, the browning reaction was stimulated by the higher temperatures, and little by the presence of light. Similar results were also observed in colour difference values (ΔE) of RJ during storage at room temperature (Takenaka *et al.*, 1986).

If portions of RJ samples stored at room temperature for different periods were further treated with TCA in order to remove the proteins, the TCA treatment resulted in a drastic decrease of absorbance to nearly zero, despite the storage time. This indicated that brown pigments were either tightly absorbed in the protein fractions or associated with molecules such as glycoproteins. Takenaka and Echigo (1983) reported that both the water-soluble and water-insoluble proteins in a fresh RJ were actually glycoproteins; the carbohydrate moieties were composed of glucose and mannose.

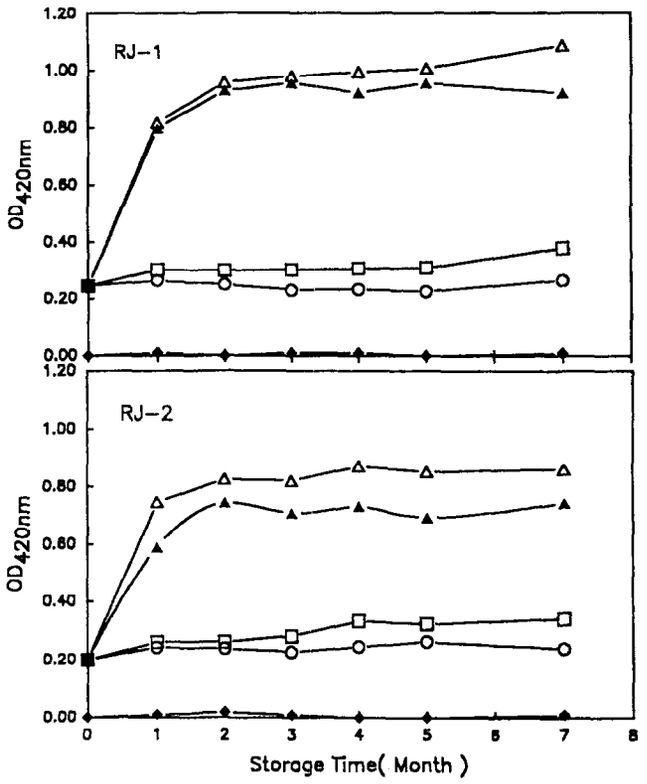

Fig. 1. Changes in browning intensity of royal jelly during storage. Samples from two origins (RJ-1 and RJ-2) were stored under different conditions; (○) −20°C; (□) 4°C; (△) room temperature and bright place; (▲) room temperature and dark place. Samples stored at room temperature and bright place were also measured with a pretreatment of TCA (♦).

Changes of WSP-1 in RJ during storage

The changes of WSP-1 in RJ during storage were investigated. The ratio of WSP-1 to total nitrogen content, R_n, indicates the extent of hydrolysis of proteins in RJ. R_n values were between 0·5 and 0·6 for freshly harvested RJs (Fig. 2). Townsend and Lucas (1940) and Tomoda *et al.* (1977) found almost equal amounts of

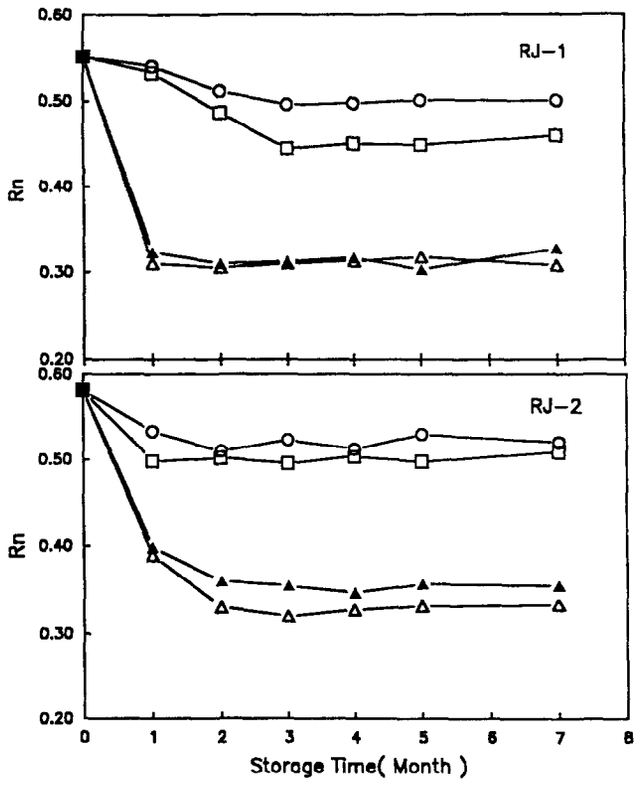

Fig. 2. The ratios (R_n) of water-soluble nitrogen to total nitrogen of two royal jelly samples stored under different conditions. For an explanation of the symbols, see Fig. 1.

water-soluble and water-insoluble proteins in RJ. However, up to 89% of water-soluble nitrogen in the total nitrogen content of RJ was also reported (Takenaka & Echigo, 1983). The R_n values decreased during the first 2–3 months, and reached constant values depending upon the storage temperature. The total amount of free amino acids of RJ stored at room temperature was also found to be decreased with time (Takenaka *et al.*,

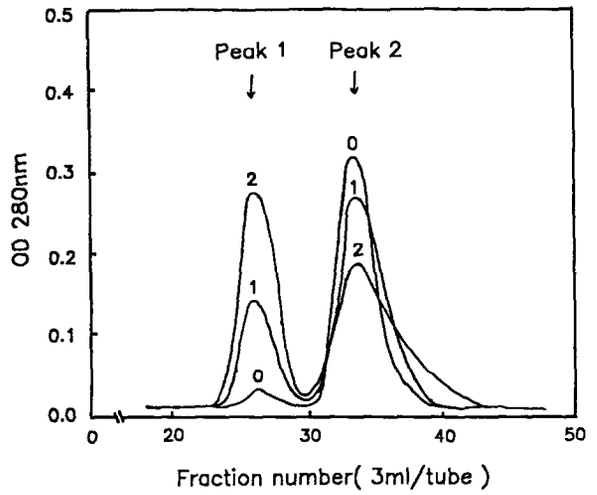

Fig. 3. Representative gel filtration chromatograms of water-soluble protein (WSP-2) from royal jelly during storage. WSP-2 was extracted from royal jelly, RJ-2, which has been stored at –20°C for 0–2 months. Numbers 0–2 indicate the storage period from 0 to 2 months, respectively.

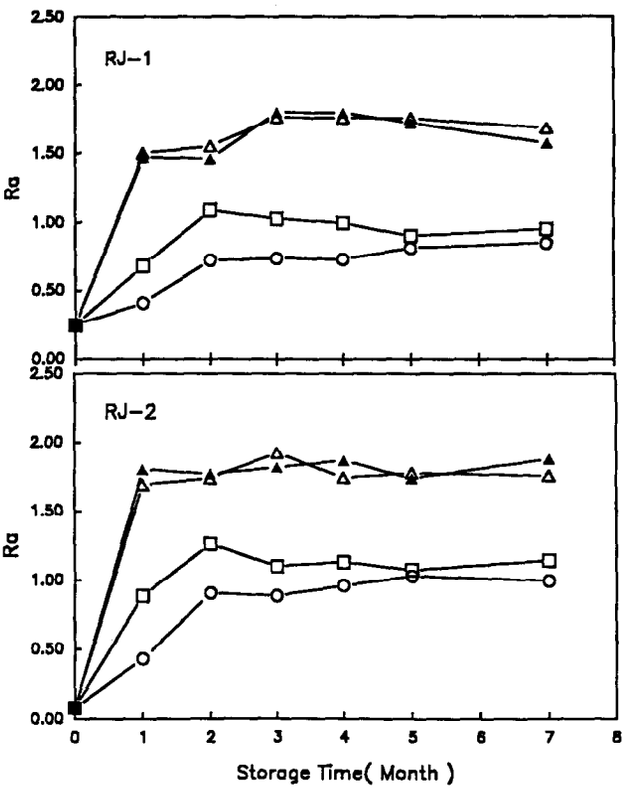

Fig. 4. The peak area ratios (R_a) of peak 1 and peak 2 in the gel filtration chromatograms of water-soluble proteins (WSP-2) of two royal jelly samples stored under different conditions. For an explanation of the symbols, see Fig. 1.

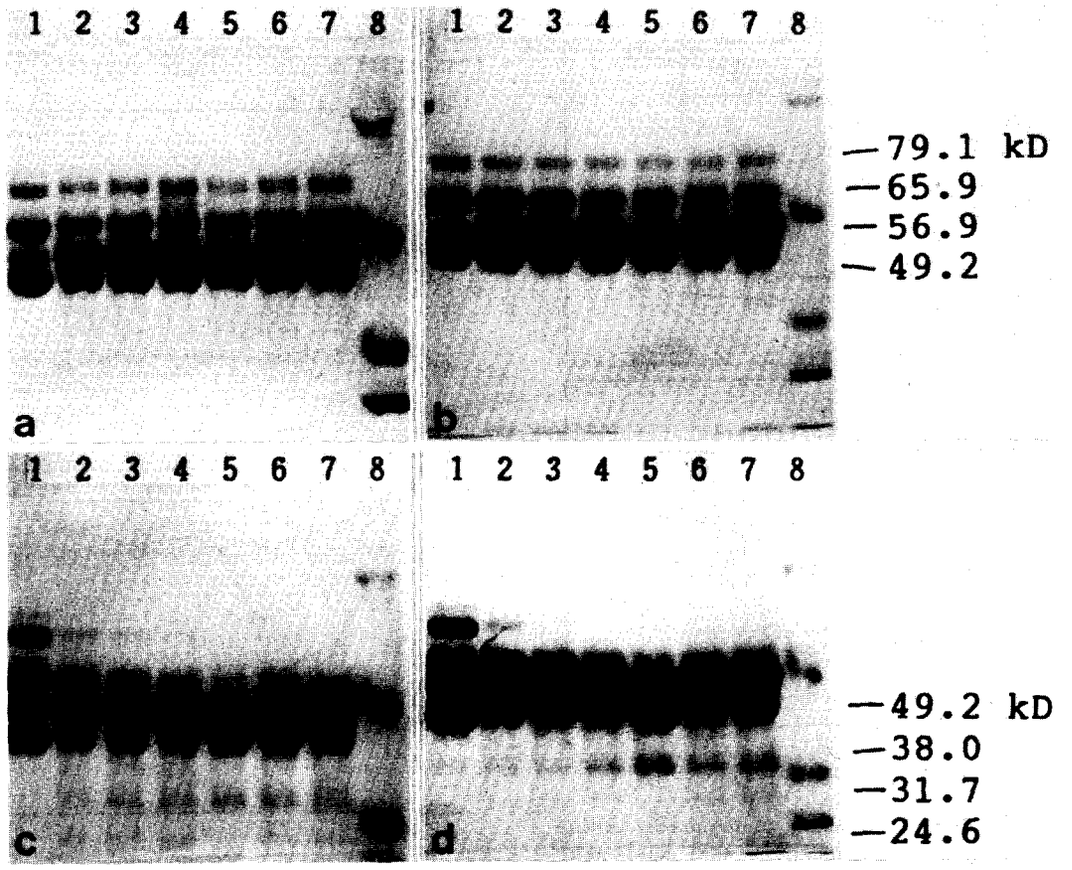

Fig. 5. SDS-PAGE patterns of water-soluble protein (WSP-2) of royal jelly (RJ-2) stored under different conditions. (a), –20°C; (b), 4°C; (c), room temperature and dark place; (d), room temperature and bright place. Lanes 1–7 refer to the storage period from 0 to 6 months, respectively; lane 8, marker proteins.

1986). This indicated that proteins in RJ hydrolysed soon after harvesting, especially at room temperature, and this was accompanied by the browning colour. On the other hand, the decrease in WSP-1 also implied that in increase of water-insoluble protein components occurred. In addition, a rapid increase in viscosity during the storage of RJ at room temperature was observed as above and also by Takenaka *et al.* (1986). It was thus suggested that some hydrolysates of WSP fractions in RJ aggregated or polymerised again during storage and led to the increase in both the amount of water-insoluble protein and viscosity.

Fractionation of WSP-2 in RJ

Two peaks, peaks 1 and 2, were obtained when WSP-2 was fractionated by the gel filtration chromatography (Fig. 3) as also shown by Tomoda *et al.*(1977). During the storage period, the peak area of the peak 1, which elutes first and has greater molecular weight increased, while that of peak 2 decreased. In addition, smaller fragments with lower molecular weights were probably produced as the shape of peak 2 tended to spread out gradually. If the ratio of areas of peak 1 to peak 2, R_a, was calculated, it was found that the R_a values increased significantly within 2 months; this tendency also depended upon the storage temperature (Fig. 4). Higher temperatures had greater effects on them, while the effect of light was little. This indicated that both polymerisation and hydrolysis reactions occurred simultaneously in RJ during storage. It was probable that the Maillard browning reaction resulted in polymerisation of components in RJ as is also shown in Fig. 2, and the decrease in area but spreading in shape of peak 2 was due to the hydrolysis of protein components in RJ by the proteolytic reaction. Protease has been previously detected in RJ (Chiu, 1992).

The changes in the components of WSP-2 in RJ were also shown by the results of SDS-PAGE. Four distinct and a faint bands were found for a fresh RJ, with molecular weights ranging from 50 000 to 88 000 (Fig. 5). Depending upon the extraction methods of WSP, Tomoda *et al.* (1977) reported at least five protein fractions in the WSP of a fresh RJ, based on solubility in a phosphate buffer containing 0·1 M NaCl. Takenaka and Echigo (1983) obtained five proteins from the WSP extracted by 0·1 M phosphate buffer, and precipitated with 60% ammonium sulphate. Instead, Patel *et al.* (1960) showed four bands in a water-extracted protein using paper electrophoresis. The changes of these bands were indiscernible in a frozen RJ during storage; however, the changes became significant as storage temperature increased. For example, the bands with molecular weight greater than 65 000 from the sample stored at room temperature disappeared gradually, resulting in the appearance of new bands with lower molecular weights (20 000–40 000). In other words, the results obtained from the SDS-PAGE probably only reflected changes of peak 2 in gel filtration chromatography; i.e. some components in the RJ disintegrated into smaller fragments during storage. The polymerised products (peak 1) were not detected or differentiated by electrophoresis under the experimental conditions used.

Simple sugars in RJ

The major sugars found in the RJ were glucose, followed by fructose and sucrose. Depending upon the age and beekeeping foods, glucose constituted 50–70% of the total sugars (Tourn *et al.*, 1980; Lercker, 1986). Both glucose and fructose increased rapidly within 2 months and then remained constant or decreased slightly, while sucrose decreased as storage time increased (Fig. 6). In addition, the hydrolysis of sucrose was enhanced at higher temperatures; however, the light had little effect on it. These results indicated that the initial increase of glucose and fructose probably came from the hydrolysis of sucrose, and some of these reducing sugars might react with the hydrolysates of protein components eventually due to the Maillard browning reaction as described previously.

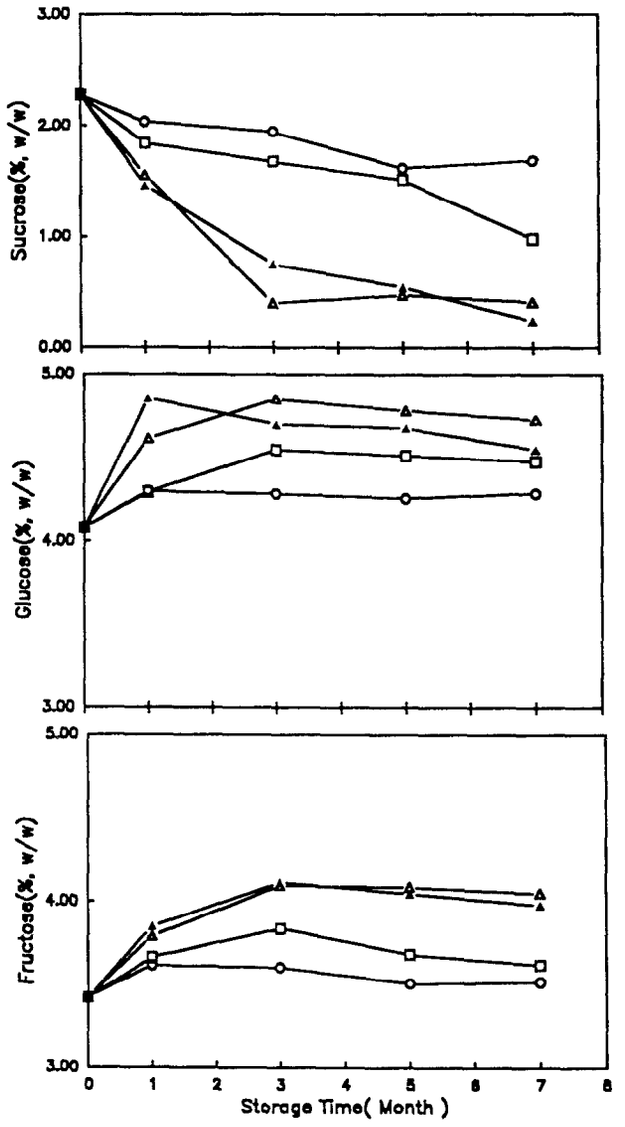

Fig. 6. Changes in sugar contents of royal jelly (RJ-2) stored under different conditions. For an explanation of the symbols, see Fig. 1.

These results clearly indicated that the browning reaction of RJ during storage was due to the Maillard browning reaction. This type of reaction was sensitive to the ambient temperature. Higher storage temperatures stimulated the reaction rates; however, less or little stimulating effect was found in the presence of light. The actual contribution of light to the browning reaction of RJ stored at room temperature was probably masked by the effect of the higher temperature used in this study. In addition, the changing patterns of parameters such as brown intensity, R_n, and R_a during storage were similar between two RJ samples. The results also showed that measurements of colour, viscosity, and WSP fractions basically reflected the quality changes of raw RJ during storage. It was therefore possible to correlate quality loss of RJ products with the changes of these parameters, and allow us to detect the quality loss during storage or freshness of raw RJ products by using these methods.

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