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High myo-inositol concentration in the hemolymph of planthoppers

Norichika MORIWAKI,[1,†] Kazuhiro MATSUSHITA,[2] Masami NISHINA,[2] Kazuhiro MATSUDA[3] and Yoshiaki KONO[1,*]

[1] Laboratory of Applied Entomology and Zoology, Institute of Agriculture and Forestry, University of Tsukuba; Tsukuba, Ibaraki 305–8572, Japan
[2] Department of Immunity, Saitama Medical School; Moroyama, Irumagun, Saitama 305–0451, Japan
[3] Laboratory of Insect Science and Bioregulation, Graduate School of Agriculture, Tohoku University; Sendai, Miyagi 981–8555, Japan

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Abstract
Myo-inositol was found in the hemolymph of three species of rice planthoppers, *Nilaparvata lugens*, *Laodelphax striatellus* and *Sogatella furcifera*, along with the major common components by ^{1}H-NMR spectroscopy. Myo-inositol concentration was about three to five times higher than trehalose concentration. *N. lugens* nymphs as well as adults contained myo-inositol. Trace experiments with ^{13}C-NMR spectroscopy showed that myo-inositol was produced from glucose and accumulated in the hemolymph with a peak concentration appearing later than that of trehalose. The role of myo-inositol in rice planthoppers is discussed.

Key words: Rice planthoppers; homoptera; hemolymph; myo-inositol; NMR

INTRODUCTION

Since the first discovery of trehalose in insects by Wyatt and Kalf (1956), trehalose has been found in the hemolymph of most insect orders and is known as the main blood sugar in insects (Wyatt, 1967). Very high concentrations of trehalose are found in aphid hemolymph as compared to other insects (Ehrhardt, 1962; Hardie, 1987; Rhodes et al., 1997; Moriwaki et al., 2003). Recently, an unusual trisaccharide, isobemisiose [i.e., α-D-glucopyranosil-(1-6)-α-D-glucopyranosil-(1-1)-α-D-glucopyranose], was reported to occur in the hemolymph of whiteflies, *Bemisia argentifolii*, *Trialeurodes vaporariorum* and *Aleurodiscus dugesii* (Hendrix and Salvucci, 2001). This trisaccharide is likely to be a main storage carbohydrate in whiteflies, but is also present in aphids as a minor component (Hendrix and Salvucci, 2001). Homopterous insects, such as aphids, whiteflies and planthoppers, feed on plant phloem sap whose sugar components are comprised of excessive amounts of sucrose ranging from two to over 30% (w/v) (Ziegler, 1975; Zimmermann and Ziegler, 1975). Thus, it is thought that homopterous insects have developed the ability to synthesize various sugars in the hemolymph in order to adapt to their diets abundant in sugar. However, the regulation of hemolymph sugar levels in homopterous insects is poorly understood because of their small size. We tried to determine the sugar components in the hemolymph of rice planthoppers by ^{1}H-NMR, a more useful tool for characterizing the components of insect hemolymph as compared to chromatographic methods (Thompson, 1990; Kono et al., 1993, 1995; Phalaraksh et al., 1999; Lenz et al., 2001; Moriwaki et al., 2003). As a result, high concentrations of myo-inositol were found in these insects.

MATERIALS AND METHODS

Insects. Three species of rice planthoppers, *Nilaparvata lugens*, *Laodelphax striatellus* and *Sogatella furcifera* were used in this study. *N. lugens* and *L. striatellus* were maintained on rice seedlings under 16L–8D at 25±0.5°C at Takeda Chemical Industries, Ltd. *S. furcifera* was maintained on rice seedlings under 16L–8D at 25±2°C at the National Institute of Agrobiological Sciences. Macropterous adult females of each species (more than 25 d old

* To whom correspondence should be addressed at: E-mail: ykono@sakura.cc.tsukuba.ac.jp
† Present address: Toxicological division, Kashima Laboratory, Mitsubishi Chemical Safety Institute, Ltd.; Sunayama 14, Hasaki-machi, Ibaraki 314–0255, Japan

after hatching) were mainly used for the collection of hemolymph. In *N. lugens*, brachypterous females and macropterous males were also used.

Collection of hemolymph and sample preparation. The forelegs of rice planthoppers were pulled out with forceps and the exuding hemolymph collected with a 1 μl microcapillary tube (EM minicaps, Hirschmann® laborgerate). Hemolymph obtained was immediately diluted with 500 μl heavy water (D_2O) containing 0.1 ppm validoxylamine A (VAA) and with a small amount of phenylthiourea as previously described (Moriwaki et al., 2003). A total of 2–10 μl of hemolymph was collected from 20 to 100 individuals of each species. Homogenate samples of first and fourth stadium nymphs (1–3 day-old and 8–10 day-old after hatching, respectively), and adults of *N. lugens* were also prepared. All samples (hemolymph and homogenates) were centrifuged at 11,000×*g* for 20 min at 4°C. The supernatant (450 μl) was transferred to a tube with 50 μl of 1 mM sodium 3-trimethylsilyl 2, 2, 3, 3, tetradeuteropropionate (TSP) as an internal standard and stored at −20°C until NMR analysis.

Administration of ^{13}C-glucose to *N. lugens*. Microinjection technique was adopted to administer labeled glucose into the planthoppers according to the methods described by Yoshiyama et al. (2000). A glass needle was made from a 5 μl microcapillary tube (Calibrated Micropipets, Drummond) with a Model PN-3 horizontal pipette puller (Narishige) and an EG-400 microgrinder (Narishige). The needle was connected to a syringe with a rubber tube and mounted on a micromanipulator, LEICA MZ-7.5 (LEICA). Macropterous female adults of *N. lugens* were aligned on double sided Scotch® tape on a glass slide after CO_2 anesthesia, and were injected with about 0.1 μl of $^{13}C_1$-D-glucose (99 atom%, Cambridge Isotope Laboratories) at 333.3 μg/μl into the coxa of the middle legs through a glass needle by air pressure. *N. lugens* were placed in a small chamber with no food at 25°C, 100% RH until hemolymph collection. The hemolymph was collected from 30 individuals 1 h, 2 h and 4 h after injection as described above.

NMR analysis of the hemolymph. Hemolymph samples were subjected to ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) analysis with a JEOL JNM-EX 400 FT-NMR spectrometer. ^{1}H-NMR spectra were obtained by the following parameters; 6.0 μs (45°) of pulse width, acquisition time of 2.048 s, delay of 4.952 s, repetition time of 7.0 s and 128 pulses with 32,768 data points covering a spectral width of 8,000 Hz at 23.0°C. The water signal was suppressed by pre-saturation (homogated decoupling). ^{13}C-NMR parameters were a 4.6 μs (45°) pulse width, acquisition time of 0.544 s, delay of 1.456 s, repetition time of 7.0 s and 600 to 30,150 pulses with 32,768 data points covering a spectral width of 10,500 Hz at 23.0°C. Assignment of the chemical signals on ^{1}H-NMR and ^{13}C-NMR spectra was carried out by adding each standard chemical (2 mg) to the sample and checking for overlap of the signals according to Kono et al. (1993). The concentrations of the hemolymph components were estimated by calibration curves of standard chemicals (mM) against the ratio of signal strengths of each compound to that of TSP by ^{1}H-NMR, as previously described (Moriwaki et al., 2003).

RESULTS AND DISCUSSION

Major hemolymph components in macropterous female adults of *N. lugens* detected by ^{1}H-NMR are shown in Fig. 1. Components common in insect hemolymph such as trehalose, amino acids, organic acids and other compounds were detected. Concentrations (mean±SD mM, N=3) of the main components were as follows: trehalose (6.1±2.5), valine (3.3±0.6), alanine (4.7±0.9), proline (4.1±0.5), acetic acid (0.7±0.5), dimethylamine (1.1±0.3) and phosphorylcholine (1.4±0.5). Signals at 0.86 ppm and 1.25 ppm show the presence of lipids at low concentrations. Uncommon compounds, myo-inositol and putrescine were identified by assignment using authentic chemicals with ^{1}H-NMR. Myo-inositol (33.2±2.4 mM) concentration in the hemolymph of *N. lugens* was very high, while trehalose concentration was low.

Myo-inositol was also detected in the hemolymph of two other planthoppers, adult female of *L. striatellus* (32.7±1.4 mM) and *S. furcifera* (33.4 mM) (Fig. 1B and 1C, Table 1). Furthermore, myo-inositol was present in the hemolymph of males (31.6±6.1 mM), brachypterous females (34.2 mM) (Table 1), first stadium nymphs (3.7±0.3 μg/mg insect) and fourth stadium nymphs (4.5±0.9 μg/mg insect) of *N. lugens* at the same levels as in macropterous adult females.

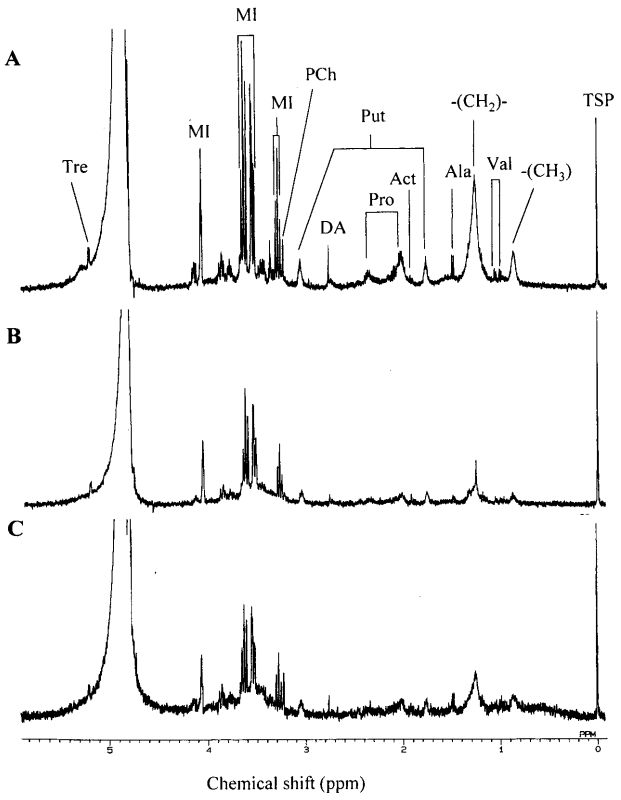

Fig. 1. ^{1}H-NMR spectrum of hemolymph from macropterous female adults of *Nilaparvata lugens* (A), *Laodelphax striatellus* (B) and *Sogatella furcifera* (C). Hemolymph volume is (A) 10 μl, (B) 1.9 μl, (C) 2.1 μl, respectively. TSP, sodium 3-trimethylsilyl 2, 2, 3, 3, tetradeuteropropionate (standard); Fa, fatty acid; Val, valine; Ala, alanine; Put, putrescine; Act, acetic acid; Pro, proline; DA, dimethylamine; PCh, phosphorylcholine; MI, myo-inositol; Tre, trehalose.

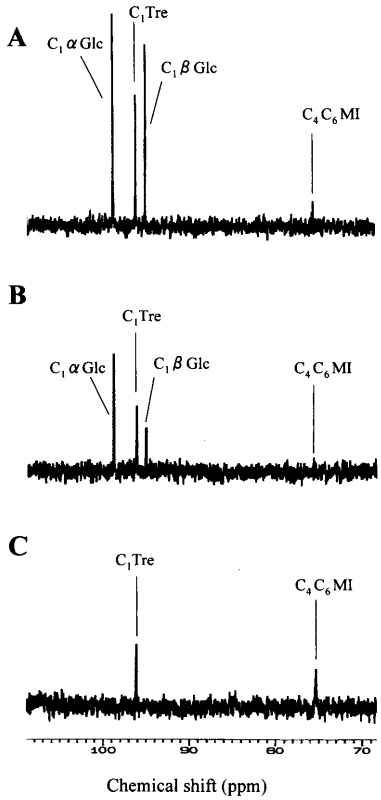

Fig. 2. ^{13}C-NMR spectrum of the hemolymph (A), 1 h; (B), 2 h and (C), 4 h after injection of 1 mg [$^{13}C_1$]-glucos, in macropterous female adults of *N. lugens*. Hemolymph volume is (A), 3 μl; (B), 3 μl; (C), 5 μl, respectively. Accumulation times of ^{13}C-NMR pulsation is (A), 2,528; (B), 600 and (C), 615, respectively. TSP, (see Fig. 1); αGlc, α-glucose; βGlc, β-glucose; Tre, trehalose; MI, myo-inositol.

Table 1. Comparison of trehalose and myo-inositol concentrations in rice planthoppers[a]

Insect species	Stage[b]	Trehalose (mM)	Myo-inositol (mM)
Nilaparvata lugens	A, ♀M	6.1±2.5	33.2±2.4
	A, ♂M	8.3±1.3	31.6±6.1
—	A, ♀B	5.8[c]	34.2[c]
Laodelphax striatellus	A, ♀M	7.3±0.2	32.7±1.4
Sogatella furcifera	A, ♀M	7.5[c]	33.4[c]

[a] Values expressed as mean±SD (N=3).
[b] A: adult, M: macroptera, B: brachyptera.
[c] N=1.

Trace experiments in which $^{13}C_1$-glucose was injected into adult females of *N. lugens* and the products detected by ^{13}C-NMR spectroscopy showed that $^{13}C_1$-enriched trehalose (labeled at C_1) and myo-inositol (labeled at C_4, C_6) appeared in the hemolymph within 1 h after injection (Fig. 2). Myo-inositol deposition increased in the hemolymph up to 4 h, while the production of trehalose reached a maximum within 1 h. Four hours after injection, $^{13}C_1$-glucose disappeared from the hemolymph and the labeled myo-inositol concentration was comparable to the labeled trehalose concentration (Fig. 2). These results indicate that myo-inositol is a main sugar in the hemolymph of rice planthoppers and is synthesized in the planthopper body.

In a previous paper, we indicated that trehalose is a major component in the hemolymph of homopterous insects; aphid, leafhopper and cicada (Moriwaki et al., 2003). Trehalose synthesized in rice planthoppers may also be utilized as an energy source like in most insects. However, the presence of abundant myo-inositol in the hemolymph is rare among insects. The importance of myo-inositol for

rice planthoppers remains unknown, but several roles can be speculated.

The first possible role may be that of osmotic regulation. The solute concentration in phloem sap of host plants fed on by homopterous insects is about three times higher than that of the body fluids of the insects (Downing, 1978; Fisher et al., 1984; Wilkinson et al., 1997). If insects of low hemolymph osmotic pressure feed on a diet of high sugar content like phloem sap, they lose body water by osmosis (Kennedy and Stroyan, 1959). It is thought that homopterous insects have adapted to this diet by converting high concentrations of sucrose in the phloem sap of host plants into various oligosaccharides that are excreted as honeydew (Fisher et al., 1984; Rhodes et al., 1997; Salvucci et al., 1997; Wilkinson et al., 1997; Ashford et al., 2000; Hendrix and Salvucci, 2001). Aphids seem to maintain a high osmotic pressure by a high content of trehalose in the hemolymph (Moriwaki et al., 2003). In *B. argentifolli*, accumulation of sorbitol occurs in response to hyperosmolarity of the artificial diet (Wolfe et al., 1998). Accumulation of sugar alcohols (i.e. polyols), such as glycerol, sorbitol, myo-inositol and mannitol, occurs as a response to water stress in some organisms (Yancey et al., 1982). Thus, it is possible that rice planthoppers have developed the ability to accumulate myo-inositol in their hemolymph in response to the high osmotic pressure of phloem sap.

The second possible role is protection against cold or heat stress. In many overwintering insects, polyols are accumulated as cryo-protectants and are a main energy source during the winter (Sømme, 1982; Storey and Storey, 1991). Myo-inositol has been reported to accumulate during diapause in the alpine beetles, *Patrobus septentriones*, *Calathus melanocephalus* (Bakken, 1985), lady beetles, *Epilachna vigintioctomaculana* (Hoshikawa, 1987), *Ceratomegilla undecimnotata* (Koštal et al., 1996), *Harmonia axyridis* (Watanabe, 2002), and chrysomelid, *Aulacophora nigripennis* (Watanabe and Tanaka, 1997). In contrast, heat treatment of *B. argentifolli* causes the accumulation of sorbitol as a thermo-protectant (Wolfe et al., 1998; Salvucci et al., 1999). *N. lugens* and *S. furcifera* are known to migrate long-distances from China to Japan on the low-level jet stream during the wet season (Seino et al., 1987). During this time, myo-inositol in the hemolymph may play a role for not only flight energy, but also as a thermo- or cryo-protectant for changes in physiological conditions.

The third role is likely to meet the nutritional requirements of symbionts in planthoppers. Planthoppers harbor yeast like symbionts (YLS) in the mycetocytes of the fat body. The main role of YLS is thought to be a source of essential amino acids, vitamins and sterols, and to recycle the nitrogen of the hosts (Noda et al., 1979; Koyama, 1995; Sasaki et al., 1996; Hongoh and Ishikawa, 1997). Molecular phylogenetic analyses have shown that the YLS of three species of rice planthoppers belong to the fungi class Pyrenomycetes in the subphylum Ascomycotina (Noda et al., 1995). In fungi, myo-inositol is an important component of the cell wall and is synthesized by conversion of D-glucose-6-P to inositol-1-P. Validamycine A, known as a control agent against sheath blight disease of rice plants, inhibits myo-inositol production in *Rhizoctonia solani*, but a recovery of infectivity of this fungi has been observed by addition of myo-inositol to the culture media (Wakae and Matsuura, 1975). This suggests that myo-inositol is converted to glucose and is utilized as energy in fungi. It is unclear whether either planthoppers or YLS produce myo-inositol, but it is possible that requirements for myo-inositol are related to YLS.

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