In search of real images of insect cuticular lipids: Synchrotron radiation FTIR ATR microspectroscopy study on insect body surfaces

The exoskeleton of insects is covered with lipid compounds called “cuticular lipids.” Until the early 1960s, these lipids had been studied from the viewpoint of protective coats. However, the development of microchemical analyses revolutionized cuticular-lipid research [1]. We now understand that cuticular lipids are involved in various activities of insects; for instance, some components contribute to chemical communication.

Although microchemical analyses have revealed the physiological roles of cuticular lipids, the state of the lipids on the body surface is not well understood. This situation is due to the difficulty in accessing the cuticular lipid layer, which is assumed to have thickness of 0.01 to 1 μm order on average. Therefore, cuticular lipids are still vaguely regarded as a thin film that cover the body surface uniformly.

To fill this void, we take note of the characteristics of Fourier-transform infrared (FTIR) spectroscopy, which can provide a wide range of structural information of lipid compounds, although it has rarely been employed in cuticular lipid research except by Gibbs [2]. We previously combined FTIR spectroscopy and attenuated total reflection (ATR) sampling for *in situ* structure analyses and demonstrated the potential of this technique [3]. What stands out is that the ATR method provides the IR spectrum of the cuticular lipid layer while avoiding the strong absorption of the underlying thick chitin layer. As the next step, we aim to clarify how lipids are distributed over the insect body surface, which is essential for considering their diverse functionalities.

FTIR ATR microspectroscopy is suitable for our purpose as it enables a two-dimensional (2D) IR absorption intensity map to be drawn focusing on a specific IR band. However, the ATR measurement requires a considerably larger number of spectral scans than the standard transmission measurement because the efficiency of infrared light utilization is much lower and the change in absorbance is much smaller. In addition, we must measure and integrate spectra at many measurement points to construct 2D images. To obtain reliable information, we must confirm the reproducibility and investigate the effects of differences in species and sex; it is essential to measure high-quality spectra as efficiently as possible. We have performed FTIR ATR microspectroscopy measurements using the high-luminance infrared synchrotron radiation at SPring-8 **BL43IR** as a light source to meet this requirement [4].

Figure 1 shows an example of 2D measurements on a forewing of a male two-spotted cricket (Fig. 1(a)), where the IR bands of cuticular lipids show significant position-dependent intensity changes; two pronounced IR absorptions, a CH_2 symmetric stretch $\nu_s(CH_2)$ at about 2850 cm^{-1} and a CH_2 antisymmetric stretch $\nu_a(CH_2)$ at about 2920 cm^{-1}, show an intensity variation of one order of magnitude among 15 sampling points (Fig. 1(b)). As if to keep up with this, the amide group bands of the underlying cuticular layer, such as the N–H stretch band at around 3280 cm^{-1}, the amide I band (mainly due to C=O stretch) at around

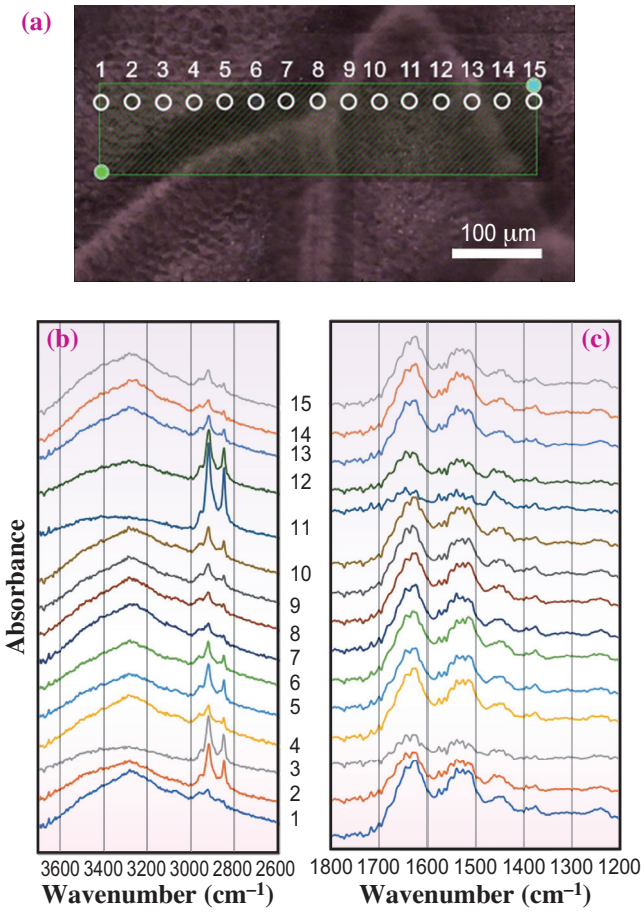

Fig. 1. Visible image and microscope FTIR-ATR spectra of a forewing. **(a)** Image of the measured sample. The rectangle delineated by the green dotted line represents the mapping measurement area with 90 sampling points (6 rows × 15 columns). Shown are 15 sampling points in the second row from the top. **(b)** Spectra in the 3700 to 2600 cm^{-1} region at the sampling points in the second row. **(c)** Spectra in the 1800 to 1200 cm^{-1} region measured at the same sampling points.

1630 cm^{-1}, and the amide II band (N–H bend) at around 1530 cm^{-1}, also change markedly in intensity. However, the changes are in the opposite direction; as the intensities of lipid bands increase, that of the amide band decreases, and vice versa (Fig. 1(c)). The tendency of the hydrocarbon and amide bands to show mutually opposite intensity changes is evident in the two 2D images obtained from $\nu_s(CH_2)$ and amide I bands (Fig. 2). The ATR spectral analysis using the two-layer model (Fig. 3), which includes the characteristics of the evanescent wave, reveals that the relative intensity varies several tens of times in the range of 0.01 to 1 μm thickness of the lipid layer. These spectral features are observed in the two-spotted cricket, regardless of sex or body site, and in the American cockroach, which strongly suggests that this uneven distribution of cuticular lipids occurs in a wide range of insect species.

The $\nu_s(CH_2)$ and $\nu_a(CH_2)$ bands tend to shift to lower frequencies as their intensities increase, which implies that the marked uneven distribution of cuticular lipids has been induced by solid-liquid phase separation and the coarsening of solid-phase domains [5]. Each cuticular lipid component is considered to play a different physiological role. Therefore, the separation of lipid components during lipid-layer thickening should significantly affect the body surface function. The results of the present experiments using infrared synchrotron radiation at SPring-8 strongly suggest that the topological features of cuticular lipids should be taken into account in the study of physiological functions.

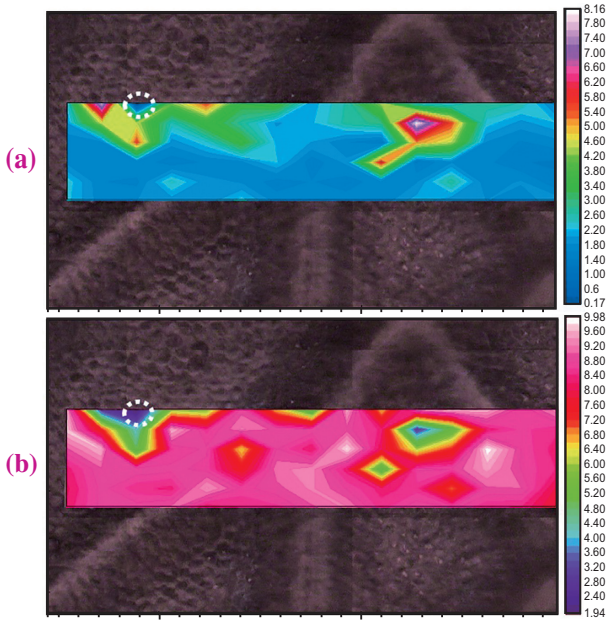

Fig. 2. FTIR images synthesized from the spectra at 90 sampling points (Fig. 1(a)). **(a)** FTIR images obtained by integrating the area under the 3020–2880 cm^{-1} region, where the $\nu_a(CH_2)$ band predominantly contributes. **(b)** FTIR images obtained by integrating the area under the 1727–1588 cm^{-1} region, where the amide I band predominantly contributes. The region inside the white dotted circle shows weak absorption in both spectra, which is due to poor contact between the internal reflection element (IRE) and the sample surface.

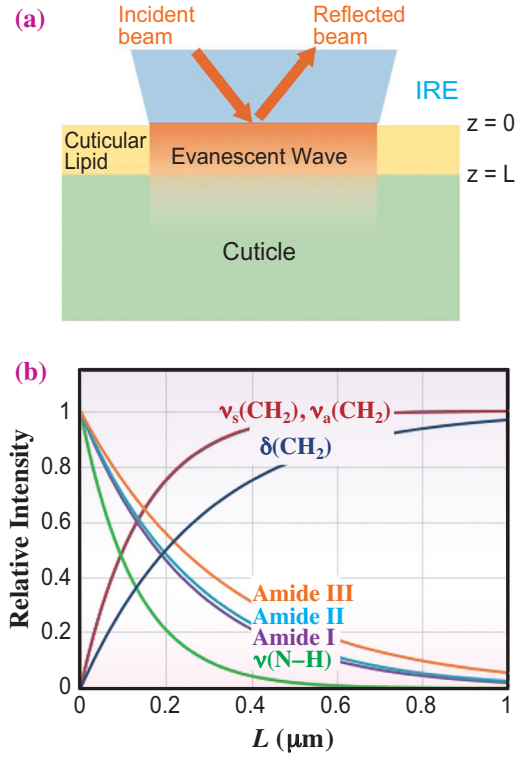

Fig. 3. Effect of the lipid-layer thickness on the IR band intensity. **(a)** The two-layer model used for evaluation consists of an upper lipid layer with thickness *L* and a lower cuticle. The ATR method employs the electric field called evanescent waves, generated by IR total reflection at the interface between the prism (IRE) and the sample surface. **(b)** Estimated relative intensity changes of each band against its maximum intensity.

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