Morphometric analysis of paramylon particles produced by *Euglena gracilis* EOD-1 using FIB/SEM tomography

Makoto Anraku\(^a\)*, Daisuke Iohara\(^a\), Hajime Takada\(^b\), Takafumi Awane\(^b\), Jun Kawashima\(^c\), Madoka Takahashi\(^c\), Fumitoshi Hirayama\(^a\)

\(^a\) Faculty of Pharmaceutical Sciences, Sojo University, 4-22-1 Ikeda, Nishi-ku, Kumamoto 860-0082, Japan.

\(^b\) Kobelco Research Institute. Inc., 1-5-5, Takatsukadai, Nishi-ku, Kobe, Hyogo 651-2271, Japan.

\(^c\) Kobelco Eco-Solutions Co., Ltd., 1-1-4, Murotani, Nishi-ku, Kobe, Hyogo 651-2241, Japan.

*Correspondence e-mail: anraku@ph.sojo-u.ac.jp*
Summary

*Euglena gracilis* EOD-1, a microalgal strain, produces large quantities of paramylon, a class of polymers known as β-1, 3-glucans and has been reported to function as a dietary fiber and to improve the metabolic syndrome including obesity. However, despite its importance, the morphometric analysis of paramylon has not been conducted so far. In this study, we attempted to observe the detailed three-dimensional structure of paramylon by focused ion beam/scanning electron microscopy (FIB/SEM). Paramylon samples were fixed and three-dimensional image reconstruction and segmentation of the image stack were created using computer software (Amira v6.0.1, FEI). The results indicated that the inside of paramylon particles (diameter: 5μm, thickness: 3μm) was comprised of a dense structure with no evidence of the presence of large pores and gaps, although a small 100 nm crack was observed. The specific surface area of paramylon particles measured by the BET method, was not as large as activated charcoal, but similar to those of plant starches, indicating that the cholesterol-lowering effect of paramylon cannot be simply attributed to its adsorption ability. The FIB/SEM method was found to be useful for elucidating the internal structure of small solid particles.

Keywords

Paramylon; *Euglena gracilis* EOD-1; Focused ion beam/scanning electron microscopy; Ion milling; Electron microscopy; 3-D reconstruction
Introduction

*Euglena gracilis*, a microalgal strain, is known to produce paramylon, a type of polysaccharide. For example, the *E. gracilis* EOD-1 strain produces paramylon in high yields (70–80%) depending on the culture conditions.\(^1\) Purified paramylon is a polysaccharide composed of about 700 glucose units that are linked via β-1,3 glycosidic bonds and is assembled in the form of triple helices. Paramylon is produced in the form of crystalline granules.\(^2\) β-Glucans, including paramylon, have been reported to exhibit various biological activities such as immunomodulatory, anti-allergy, anti-obesity, antitumor and cytokine inducing effects.\(^3-7\) Paramylon also functions as a dietary fiber (an indigestible polysaccharide) and improves the metabolic syndrome by suppressing the intestinal absorption of cholesterol, thus reducing transition time in gastrointestinal tract and increasing fecal bulk. These bioactivities of paramylon have been attributed to the strong adsorption of cholesterol to the glucans in the intestinal tract, which results in the suppression of cholesterol absorption in the systemic circulation.\(^8\) However, the detailed mechanism responsible for this adsorption is not known. If adsorption were a main factor, the microscopic structure of the surface and inside of paramylon granules should be investigated in detail. However, despite its importance, the exact morphometric arrangement of purified paramylon granules is not fully elucidated.

Recent advances in analytical methods now permit the internal structures of solid and...
biological materials to be visualized in three dimensions. Among these methods are X-ray computed tomography, magnetic resonance imaging and confocal Raman imaging. Another approach to studies of the internal structures of materials is transmission electron microscopy (TEM), where serial sections of a material are anatomically produced, and the surfaces observed. Focused ion beam/scanning electron microscopy (FIB/SEM) is an alternative to the above TEM approach for visualizing the internal structures of materials. In this method, a FIB is used to remove the thin layers of materials and the exposed surface is then observed by the scanning electron beam.\textsuperscript{9,10} The milling/imaging data for a serially exposed surface is used for constructing and visualizing the internal structure of the sample. In this study, we used SEM equipped with a FIB (FIB/SEM), together with Brunauer–Emmett–Teller (BET) adsorption, to investigate the internal structure of paramylon produced by \textit{Euglena gracilis} EOD-1 and to obtain information regarding the issue of whether paramylon granules have sufficiently wide void spaces to permit biomaterials such as cholesterol to be adsorbed in the solid state.

**Materials and Methods**

**Materials**

The \textit{Euglena gracilis} EOD-1 strain produces paramylon in high yields of 70–80%, depending on the culture conditions.\textsuperscript{6,11} Purified paramylon extracted from \textit{Euglena gracilis}
EOD-1 was obtained from Kobelco Eco-Solutions Co., Ltd. (Kobe, Japan), which was produced according to the methods reported by Aoe\(^6\) et al. and Takahashi et al.\(^\text{11}\). Other chemicals and solvents used were of analytical grade, and deionized double-distilled water was used throughout the study.

**SEM observation**

The paramylon granules were fixed on the stage and sputter-coated with osmium in about 2 nm under vacuum. These granules were observed by a field emission scanning electron microscope with (FE-SEM SU-70, Hitachi High-Technologies) operating at 2 kV.

**FIB/SEM imaging**

The FIB/SEM imaging experiment was conducted according to the method of Murata et al.\(^\text{12}\) with minor modification. Briefly, the surface of paramylon granules was fixed on the stage and their surface were observed by backscatter electron imaging using a conventional field emission SEM with FIB (FIB/SEM, Focused Ion & Electron Beam System nanoDUE'T NB5000, Hitachi High-Technologies GLOBAL). The particles were coated with thin layers of evaporated carbon\(^\text{13}\) to avoid charge-up of samples from the FIB milling. A series of serial images of surfaces that were exposed by repeated cycles of the milling was acquired using the Mill & Monitor operating software (Hitachi High-Technologies). FIB milling of samples
was undertaken with a gallium ion beam at an accelerating voltage of 20 kV and at a milling pitch 50 nm/step and 107 cycles. SEM imaging was done using a 1 keV electron beam. The resulting image stack was processed by Amira software (FEI Visualization Science Group, Burlington, MA).

BET measurement

The BET experiment involved the use of a Quantachrome Auto sorb-IQ-MP gas sorption analyzer. Paramylon granules (0.5 g) were degassed and dried in an oven for 3h at 60 °C and the nitrogen adsorption was measured at temperature 77 K. The specific surface area was estimated with BET equation applied for absorption isotherms which were obtained in the range of relative pressures 0.04-0.28. 14)

Results and Discussion

SEM images of the purified paramylon from Euglena gracilis EOD-1 are shown in Fig. 1. A micro-sized particle structure with an elliptical shape and a high dispersible ratio were maintained without coagulation. We next investigated the internal structure of dried purified paramylon particles. Fig. 2 shows reconstructed, three-dimensional images of paramylon particles from Euglena gracilis EOD-1 (Fig. 2a). The segmentation of the whole paramylon particle revealed that the paramylon particles are spread out densely and packed closely in a
protective membrane (Fig. 2b). The paramylon (diameter: 5μm, thickness: 3μm) particles had a dense structure without gaps, although a small crack area with a length of 100 nm was observed (Fig. 2c). These FIB/SEM observations indicate that the paramylon granules from strains contained no large void spaces on the surface and inside of the particles.

In order to confirm the above results, we measured the specific surface areas of paramylon particles obtained from *Euglena gracilis* EOD-1. As shown in Fig. 3, the plots of the BET equation was linear, giving a specific surface area of 2.438 m$^2$/g for the paramylons from Euglena EOD-1. These surface areas are significantly smaller than that (900-1600 m$^2$/g) of activated charcoal, and similar to those (about 5 m$^2$/g) of corn and potato starches, indicating that the adsorption ability of the paramylons was not as large as that of activated charcoal.

Aoe et al. recently investigated the effect of paramylon at dosages of 2.5 and 5% on obesity in mice and reported its potential preventive effect on obesity. They also reported that paramylon was resistant to biological degradation in the intestinal tract, and that its original shape was maintained, even in feces. As described in this introduction, bioactivities of polymer including paramylon have been attributed to the adsorption of biological component such as cholesterol in the intestinal tract. In fact, the form of triple helices of purified paramylon might be related the adsorption of biological component. However, the present FIB-SEM findings and the surface area measurements indicated that paramylon granules do
not contain sufficiently wide void spaces to permit large amounts of biological components to be adsorbed, at least in the solid state. Therefore, the cholesterol-lowering effect of paramylon particles cannot be simply ascribed to their adsorption ability, and that other physicochemical and biological factors may be considered to elucidate fully the bioactivity of paramylon particles. Further, the results indicate that FIB/SEM can be useful for observing not only the surface but also the internal structure of pharmaceutical dosage forms such as granules and tablets.

Conclusion

FIB/SEM was used to observe the interior of purified paramylon granules. These results, together with the BET adsorption data, suggest that paramylon particles have a dense internal structure and contain no gaps. The specific surface area of paramylon particles was not as large as activated charcoal, and similar to those of starch granules, indicating that the cholesterol-lowering effect of paramylon cannot be simply attributed to its adsorption ability, and that other physicochemical and biological factors also need to be considered. FIB/SEM is useful to observe not only the surfaces but also the internal structure of pharmaceutical dosage forms such as granules and tablets.
Conflict of Interest

The authors declare no conflict of interest.
References
1) Ishibashi KI., Nishioka M., Onaka N., Takahashi M., Yamanaka D., Adachi Y., Ohno N., *Nutrients*, **11**, E1144 (2019).
2) Watanabe T., Shimada R., Matsuyama A., Yuasa M., Sawamura H., Yoshida E., et al. *Food Funct.*, **4**, 1685–1690 (2013).
3) Nakashima A., Suzuki K., Asayama Y., Konno M., Saito K., Yamazaki N., Takimoto H., *Biochem. Biophys. Res. Commun.*, **494**, 379-383 (2017).
4) Levine R., Horst G., Tonda R., Lumpkins B., *Mathis G., Poult. Sci.*, **97**, 3494-3500 (2018).
5) Bianchi V.A., Castro J.M., Rocchetta I., Conforti V., Pascual M., Luquet C.M. *Immunol.*, **51**, 17-25 (2016).
6) Aoe S., Yamanaka C., Koketsu K., Nishioka M., Onaka N., Nishida N., Takahashi M., *Nutrients*, **11**, E1674 (2019).
7) Sugiyama A., Hata S., Suzuki K., Yoshida E., Nakano R., Mitra S., Arashida R., Asayama Y., Yabuta Y., Takeuchi T., *J. Vet. Med. Sci.*, **72**, 755–763 (2010).
8) Nakashima A., Sugimoto R., Suzuki K., Shirakata Y., Hashiguchi T., Yoshida C., Nakano Y., *Food Sci Nutr.*, **7**, 139-147 (2018).
9) Bushby AJ., et al., *Nat Protoc.*, **6**, 845–858 (2011).
10) Knott G, Marchman H, Wall D, Lich B., *J Neurosci*. **28**, 2959–2964 (2008).
11) Takahashi M., Kawashima J., Nishida N., Onaka N. “Beta-Glucan Derived from Euglena (Paramylon)” CMC Publishing Co., Ltd.; Tokyo, Japan: 2018. pp. 174–182.
12) Murata K., Hirata A., Ohta K., Enaida H., Nakamura K., *Sci Rep.*, **19**, 6329 (2019).
13) Ohta K, et al., *Micron.*, **43**:612–620 (2012).
14) Rouquerol, J.; Llewellyn, P.; Rouquerol, F., *Stud. Surf. Sci. Catal.*, **160**, 49– 56 (2007).
15) Pavlovic S., Brandao P.R.G., *Minerals Engineering*, **16**, 1117-1122 (2003).
Fig. 1. FE-SEM Micrographs of Paramylon Particles from *Euglena gracilis* EOD-1
Scale bars are 10 μm (a) and 5 μm (b), respectively.
Fig. 2. FIB-SEM Cross-sectional and Three-dimensional Images of a Paramylon Particle from *Euglena gracilis* EOD-1

a) Three-dimensional image showing 108 serially exposed surface images of paramylon at the posterior pole, 3.9×2.0×2.5 in μm. b, c) Cross-sectional image (Slice No. 25 (b), No. 43 (c)) of paramylon. d) Three-dimensional image of paramylon segmented by color (green: crack, yellow: light elements area, red: holes appeared by the FIB treatment).
Fig. 3. BET Plot of Nitrogen Adsorption by Paramylon Granules
Correlation coefficient = 0.99940, Calculated surface area= 2.438 m²/g