Journal of Structural Biology 171 (2010) 88–94

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Second harmonic generation imaging – A new method for unraveling molecular information of starch

Zong-Yan Zhuo [a], Chien-Sheng Liao [a], Chen-Han Huang [a,c], Jiun-Yann Yu [a], Yu-Yi Tzeng [a], Wen Lo [a], Chen-Yuan Dong [a], Hsiang-Chen Chui [c], Yu-Chan Huang [b], Hsi-Mei Lai [b], Shi-Wei Chu [a,*]

[a] Department of Physics, National Taiwan University, Taipei 10617, Taiwan ROC
[b] Department of Agricultural Chemistry, National Taiwan University, Taipei 10617, Taiwan ROC
[c] Institute of Electro-Optical Science and Engineering, National Cheng-Kung University, Tainan 701, Taiwan ROC

ARTICLE INFO

Article history:
Received 29 December 2009
Received in revised form 23 February 2010
Accepted 25 February 2010
Available online 2 March 2010

Keywords:
Three-dimensional imaging
Polarization anisotropy
Nonlinear optics
Tensor analysis
Molecular orientation
Amylopectin

ABSTRACT

We present a new method, second harmonic generation (SHG) imaging for the study of starch structure. SHG imaging can provide the structural organization and molecular orientation information of bio-tissues without centrosymmetry. In recent years, SHG has proven its capability in the study of crystallized bio-molecules such as collagen and myosin. Starch, the most important food source and a promising future energy candidate, has, for a decade, been shown to exhibit strong SHG response. By comparing SHG intensity from different starch species, we first identified that the SHG-active molecule is amylopectin, which accounts for the crystallinity in starch granules. With the aid of SHG polarization anisotropy, we extracted the complete $\chi^{(2)}$ tensor of amylopectin, which reflects the underlying molecular details. Through $\chi^{(2)}$ tensor analysis, three-dimensional orientation and packing symmetry of amylopectin are determined. The helical angle of the double-helix in amylopectin is also deduced from the tensor, and the value corresponds well to previous X-ray studies, further verifying amylopectin as SHG source. It is noteworthy that the nm-sized structure of amylopectin inside a starch granule can be determined by this far-field optical method with 1-μm excitation wavelength. Since SHG is a relatively new tool for plant research, a detailed understanding of SHG in starch structure will be useful for future high-resolution imaging and quantitative analyses for food/energy applications.

1. Introduction

Laser scanning second harmonic generation (SHG) imaging has been proven to be a useful tool in biomedical tissue imaging. The first demonstration of SHG imaging was in the 1970s by Colin Sheppard and his colleagues (Gannaway and Sheppard, 1978) in nonlinear optical crystals. In the 1980s, Freund and his colleagues extended similar techniques to the study of crystallized collagen fibrils in mouse tail tendon (Freund et al., 1986), demonstrating its polarization anisotropy properties and deducing a full $\chi^{(2)}$ tensor. After the introduction of stable solid-state femtosecond oscillators and two-photon fluorescence imaging in the early 1990s (Denk et al., 1990), this imaging modality has been included in the *multi-photon* family and has been adopted for biological and material studies with many unique advantages. For example, the advantages of SHG imaging include intrinsic optical sectioning, non-invasive observation, deep tissue penetration, and endogenous contrast specificity. As a second-order nonlinear process, SHG intensity is proportional to the square of excitation intensity, thus providing intrinsic image sectioning without the need of a pinhole. The non-invasiveness originates from the virtual transition nature of SHG. Since only virtual transitions are involved with the SHG process, cell viability is significantly enhanced with reduced phototoxicity (Chu et al., 2003). In addition, since the wavelength dependency of SHG is relatively weak as compared to fluorescence signals, near-infrared lasers with wavelengths at the biologically transparent window are typically employed as excitation sources. As a result, not only can deeper penetration be achieved, but the SHG signal also falls within the visible range for easier detection (Yasui et al., 2009). Moreover, for most biomedical tissue studies, SHG contrasts come from endogenous proteins with non-centrosymmetric quasi-crystalline arrangements, requiring no exogenous staining or complicated sample preparation (Campagnola and Loew, 2003). Based on the high sensitivity and selectivity of SHG in non-centrosymmetric structures, SHG has been employed to probe the molecular structure of bio-tissues. Under the interaction of strong electric field and local crystallized molecules, the inherent nonlinear optical properties are characterized by a $\chi^{(2)}$ tensor, which reflects the underlying molecular details and their organization.

* Corresponding author. Address: 1, Sec. 4, Roosevelt Rd., Taipei 10617, Taiwan. Fax: +886 223639984.
E-mail address: swchu@phys.ntu.edu.tw (S.-W. Chu).

1047-8477/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.jsb.2010.02.020

Among biological tissues, collagen- and myosin-based animal tissues are probably the most-studied samples with SHG imaging (Chu et al., 2007; Lin et al., 2005; Plotnikov et al., 2006; Tiaho et al., 2007; Williams et al., 2005). Other endogenous proteins such as tubulin, silk fibroin, and glial fibrillary acidic protein have also been recently explored with SHG (Fu et al., 2007; Mohler et al., 2003; Rice et al., 2008). In plants, quasi-crystalline polysaccharides like cellulose and starch are known to exhibit strong SHG emission (Chu et al., 2002; Cox et al., 2005; Mizutani et al., 2000; Nadiarnykh et al., 2007; Thayil et al., 2008). However, detailed characterization of SHG in starch granules has not been performed.

Starch is the most important source of food for human, and the major energy reservoir for plants. It is also one of the most significant candidates for sustainable energy production in the future. The size and shape of starch granules vary with different species, origin locations, and climates. There are two major components in starch granules: amylose and amylopectin. Amylose is an essentially linear molecule composed of anhydroglucose units connected through (1 → 4)-α-linkages with a few (1 → 6)-α-linkages. Amylopectin, on the other hand, is a heavily branched structure built from about 95% (1 → 4)-α- and 5% (1 → 6)-α-linkages (Tester et al., 2004).

The structure of starch granules has been investigated with various techniques. A range of microscopic approaches, including optical, electron, and X-ray, have been applied to this research field (Chandrashekar et al., 1987; Gallant et al., 1997; Waigh et al., 1997). At the largest level of the granule structure, the semi-crystalline (soft) and crystalline (hard) shells are alternatively arranged with a thickness ranging from 120 to 400 nm, forming concentric layers observable under a light microscope. At a smaller structural level, the crystalline shell is further divided to crystalline and amorphous lamellae with 9 nm periodicity. The crystalline lamellae consist of 2D arrays of ordered double-helical side-chain clusters, and the amorphous lamellae are viewed as the double-helices junction zone. While the crystallinity is mainly maintained by the double-helix cluster of amylopectin, amylose is interspersed among amylopectin side-chains.

Due to native crystallinity, Maltese cross can be observed from most starch granules under a polarized light microscope. The positive birefringence of starch granules indicates the radial orientation of crystallinities (Gallant et al., 1997). Compared with a polarized microscope, SHG imaging provides additional three-dimensional resolution and deep-imaging capabilities. Both scanning and transmission electron microscopy have played a major role in determining the ultrastructures inside starch granules (Buleon et al., 1998). Although electron microscopy provides a much better spatial resolution as compared to optical imaging techniques, it is limited to surface imaging (or an extremely thin slice), and the sample preparation procedure is rather complicated, especially for fragile biological tissues. X-ray scattering, on the other hand, is useful in providing information about the degree of crystallization and molecular branching schemes (Waigh et al., 1997). Here, we will show that SHG imaging, with wavelengths much larger than X-rays, provides not only 3D molecular orientation but also information on molecular crystallization symmetry, as well as the helical pitch angles in the double-helical amylopectin within a subfemtoliter volume.

In this study, we first determine the SHG-active molecule in a starch granule by comparing SHG intensities from different species. With the aid of SHG polarization dependence (Tester et al., 2004), a complete $\chi^{(2)}$ tensor is extracted. Subsequently, the local 3D molecular orientation and molecular packing symmetry are deduced by relevant $\chi^{(2)}$ elements. More interestingly, the effective helical angle within an nm-sized double-helical amylopectin is found out through tensor analysis, though the excitation wavelength is orders of magnitude larger than the molecular structure.

2. Materials and methods

2.1. Starch granules

A number of different types of starch were used in this study. To determine the molecular origin of SHG, starches isolated from dehydrated Japonica type waxy rice (WR) and commercial Japonica rice flour (RF) were adopted. The former is composed of 0.5% amylose and 99.5% amylopectin, while the latter of 14% amylose and 86% amylopectin. The powders were prepared and purified based on the method in Juliano et al. (1981) and the amylose content of the rice starch was determined by a colorimetric method in the same reference. For imaging and polarization anisotropy studies, larger starch granules from fresh potato (*Solanum tuberosum* L.) were used. The granules were mounted on a slide glass without the addition of water to avoid Brownian motion during imaging.

Before the subsequent polarization analysis, it is vital to define spatial coordinate and relative angles between the optical field and the SHG-active molecule. A schematic drawing is given in Fig. 1. M is the SHG-active molecule and E is the laser polarization. The laboratory coordinate is XYZ, where laser propagates along Z, and scan across the sample on the XY plane. The molecular orientation angle between Y and the projection of M is denoted as θ_0 and θ is the orientation of the laser electric field E versus Y. Since M does not necessarily lie on the XY plane, the tilt angle δ is defined as the angle between M and XY plane.

2.2. SHG microscope setup

Our home-built laser scanning SHG microscope is shown in Fig. 2, composed of an ultrafast fiber laser, a mirror-based scanner, a microscope frame, and a synchronized detection system. With a near-infrared ultrafast laser source (Uranus 005, Polar Onyx, CA, USA), deep penetration into biological samples can be achieved. The central wavelength of the ultrafast laser is 1040 nm with an average power of 5 W. The pulse width is ~500 fs, and the repetition rate is 48 MHz. The typical average power after the microscopic objective is limited to 100 mW, and no photodamage was observed in the sample after continuous illumination.

The use of the mirror-based scanner has been reported recently (Yu et al., 2009). In brief, 2D raster scanning of the laser beam is accomplished via two galvanometric mirrors (MicroMax™ Series 671, Cambridge, MA, USA) and two sets of mirror-based telescopes. The major disadvantage of a conventional mirror-based telescope is the presence of geometrical aberrations, such as coma and astigmatism, from oblique incidence. This issue was solved by overlapping the sagittal axis of the first spherical mirror and the tangential

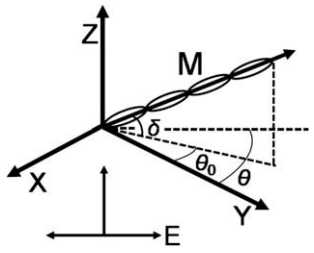

Fig. 1. Schematic drawing of the coordinate definition in the experiment. XYZ is a laboratory coordinate, with Z being the direction of laser beam propagation, and X and Y corresponding to the slow and fast scanning axes, respectively. M denotes the orientation of the SHG-active molecules, and E denotes the direction of laser polarization. θ is the angle between Y and E, and θ_0 is the molecular orientation angle between Y and the projection of M. δ is the tilt angle, corresponding to the angle between the M vector and XY plane.