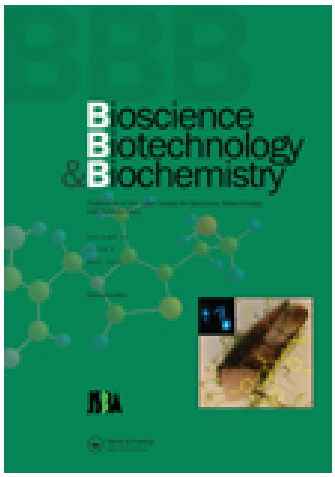

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Efficiency of 2.45 and 5.80 GHz microwave irradiation for a hydrolysis reaction by thermostable β-Glucosidase HT1

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Note

Efficiency of 2.45 and 5.80 GHz microwave irradiation for a hydrolysis reaction by thermostable β-Glucosidase HT1

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Microwave irradiation at different frequencies gave unique results for the hydrolyses of glycosyl bonds by β-Glucosidase HT1. With the observed relative complex permittivity data for the reaction buffer, 2.45 GHz microwave radiation affected both waters and ions, while 5.80 GHz only affected waters. We, here, propose that would be one of the unique "microwave nonthermal effects".

Key words: microwave; enzyme; glycosidase; glucosidase; hydrolysis

It has become common in recent years to use microwave for chemical reactions. Although microwave is used mainly as an effective heating tool, the authors reported some results that would be caused by microwave nonthermal effects.[1–5] Meanwhile, a few reports claimed that microwave gives only thermal effects caused by a rapid heating[6,7] and a controversy has arisen this year between Dr Kappe and Dr Dudley about whether microwave does, in fact, have a unique nonthermal effect.[8,9] We thought that investigating the efficiency of different microwave frequencies could reveal a solution to this dispute. Glucosidase, which hydrolyzes glycosyl bonds, is one of the most common enzymes in the medicinal and biomass research fields. β-Glucosidase HT1 is a commercially available glycosidase[10] and its optimum temperature is relatively high, at 60 °C in 50 mM sodium acetate buffer (pH 5.0). Here, we approached the microwave nonthermal effects with controlling the reaction temperature by conventional method (incubator) and by using 2.45 and 5.80 GHz microwave irradiations for this β-Glucosidase HT1 reaction.

4-Methoxyphenyl glucopyranoside has been chosen as the substrate for this research.[11] (Scheme 1) The reactions were performed at 40, 50, 60, and 70 °C in 20 mM sodium acetate buffer (pH 5.0) with 10 mM NaCl. Three types of heating methods were applied: electrical incubator EC-40R (AS ONE Corp.) for conventional heating, commercially available MWS-1000 (EYELA, Tokyo Rikakikai Co., Ltd.) for the 2.45 GHz microwave, and an in-house build up machine based on ATMW500B-5.8 G (Amil Co., Ltd.) for the 5.80 GHz microwave[12] (Fig. 1).

First, the reactions were heated conventionally. We found that 60 °C gave the best results, which corresponded with the supplier's information that the optimum temperature is 60 °C. The reaction proceeded even at lower temperatures smoothly, but enzyme inactivation occurred within 20 min at 70 °C and the reaction was stopped in only 69% yield.

Then, we performed the same reactions but with heating by 2.45 GHz microwave using MWS-1000 (EYELA), which could strictly control the reaction temperature. The power was set as 100 W. Practically, the irradiation power became initially 100 W but sooner was automatically attenuated 0–40 W (40, 50, and 60 °C) or 50–70 W (70 °C) to keep set temperature. A total of 50 °C gave the best performance for the reaction completed within 20 min, in contrast to the 30 min required by conventional heating. Even at 40 °C, the reaction was completed within 30 min, but it was not finished until 60 min in the incubator. When the reaction was performed at 60 °C, the enzyme became inactive and the reaction was stopped by 90% in 20 min. Thus, although an optimum temperature for the enzyme is reported at 60 °C, 60 °C by 2.45 GHz microwave inactivated the enzyme. From these results, we concluded that 2.45 GHz microwave affected more effectively than conventional heating, lowering the optimum temperature, and accelerating the reaction.

Next, we tested 5.80 GHz microwave effectiveness by using a microwave irradiation machine prepared in-house. The oscillated power from the magnetron was 50 W and attenuated by the cold water load to 10–15 W, then irradiated intermittently to keep fixed temperature manually. The reactions were not accelerated as in the experiment with 2.45 GHz microwave,

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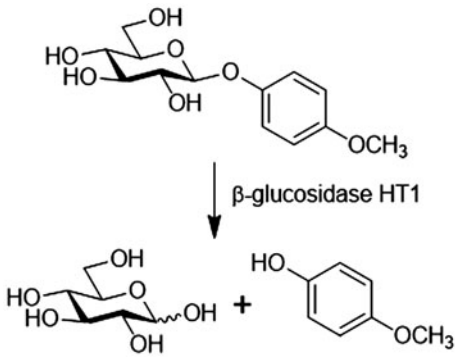

Scheme 1. Hydrolysis by β-Glucosidase HT1.

and the initial rates of the reactions were similar to that of conventional heating. In addition, the enzyme activities were lost by 20 and 10 min at 60 and 70 °C, respectively. From these results, it was apparent that 5.80 GHz microwave did not improve this reaction.

Although we got fruitful results using 2.45 GHz microwave irradiation which accelerated the glucosidase reaction, 5.80 GHz microwave did not cause any improvement. To determine how and on which molecules microwave worked, we examined the absorption property of the microwave for water and the buffer solution.[13] Relative complex permittivity at room temperature was measured from 200 MHz to 14 GHz by the reflection probe method. The result is shown as the Nyquist diagram in Fig. 2 for distilled water (left) and for 50 mM sodium acetate buffer (pH 5.0) (right), where the horizontal and the vertical axes corresponded to the real and the imaginary parts, respectively. Dielectric property within a complex plane was presented semicircular by Equation (1).

$$\left[\varepsilon_r'(\omega) - \{\varepsilon_r(0) + \varepsilon_r(\infty)\}/2\right]^2 + \{\varepsilon_r''(\omega)\}^2 = \left[\{\varepsilon_r(0) - \varepsilon_r(\infty)\}/2\right]^2 \quad (1)$$

The higher the conductivity, the further the actual diagram departs from the semicircle at the lower frequency and showed rectilinear.[14] In the case of distilled water (Fig. 2, left), the approximate curve was semicircular in all the measured bands, which suggests that pure water behaved only as a dielectric under irradiation microwaves. On the other hand, the result from the buffer solution experiments showed the rectilinear locus as an electric conductor from 200 MHz to 2.45 GHz and the semicircular locus as dielectric from 2.45 to 14 GHz (Fig. 2, right). When the impressed voltage of the electromagnetic wave was constant, the electromagnetic energy was lost and the irradiated material was heated proportionally with the imaginary part, which is the vertical axis of the figure. The dielectric was heated based on the vibration phase delay of the dipole to the vibration of the impressed electric

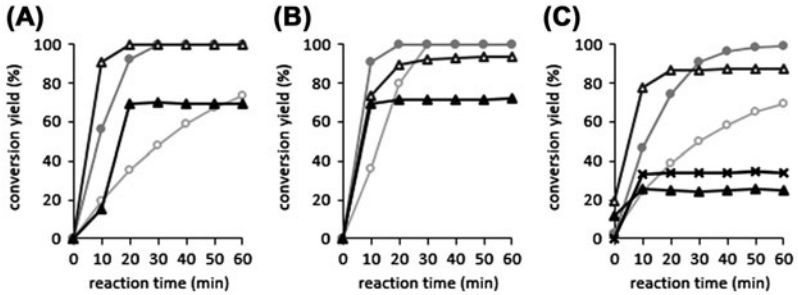

Fig. 1. Results of hydrolyses.

Notes: Reactions were heated by incubator (A), 2.45 GHz (B), and 5.80 GHz (C) microwaves at 40 °C (○), 50 °C (●), 60 °C (△), 70 °C (▲), and 80 °C (×). In the case of 2.45 GHz microwave experiment, the irradiation power was controlled automatically, so we set the staring time ($t = 0$ min) when the reaction was started, and was immediately irradiated the microwave to take the set temperature. Meanwhile in the case of 5.80 GHz microwave experiment, attenuation should be performed manually after the microwave irradiation was started, so we set the start time ($t = 0$ min) when the reaction reached the target temperature. Therefore, some reactions were already proceeded to some extent even at $t = 0$ min.

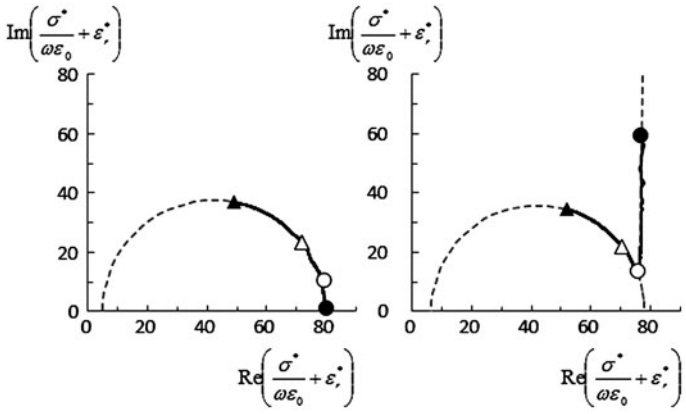

Fig. 2. Relative complex permittivity.

Notes: They measured by the reflection probe method at room temperature at 200 MHz (●), 2.45 GHz (○), 5.80 GHz (△), and 14.0 GHz (▲) for water (left) and for 50 mM sodium acetate buffer (pH 5.0). Bold lines are observed results and broken lines are the approximate lines.

field, and the calorific power was the largest in the specific frequency. In contrast, the electric conductor was heated based on the Joule's heat by monopole migration to the impressed electric field, and the higher frequency showed lower heating. Therefore, *the 5.80 GHz microwave was able to heat dipole water molecules by dielectric heating but did not heat monopole ions* such as sodium cations and acetate anions because the frequency was too rapid and the Coulomb force of the impressed electric field was ignored. On the other hand, the relative complex permittivity at 2.45 GHz showed ranges in both dielectric and conductive. Hence, the heating was not only dielectric but also Joule's heating of ions; *the 2.45 GHz microwave could affect both dipole water and monopole ions* in the buffer solution.

For hydrolysis of 4-Methoxyphenyl glucopyranoside by β-Glucosidase HT1, 2.45 GHz microwave heating gave advantages, the reaction became faster, and the optimum temperature decreased to 50 °C, but 5.80 GHz microwave heating gave no significant advantage. We consider that this is why 2.45 and 5.80 GHz microwave affected the reaction at the molecular level in different ways. A possible explanation is that buffering ions formed ion-bonds, for example, to carboxyl groups on Glu or Asp, which are key functions for hydrolysis reaction, or coordinated to the enzyme as the buffer stabilized it. When an enzyme acted as the catalyst of a reaction, the following steps occurred: out the coordinated water and bonded ions from a reaction pocket, occupying the substrate in the pocket, reaction and release of the resulting product. As we discussed concerning the heating mechanism, *5.80 GHz microwave affected only the water molecules in buffer solution, but 2.45 GHz worked on both water molecules and buffering ions*. Therefore, even if microwaves were not used as a heating tool, 2.45 GHz microwave irradiation would give some benefit for the first step of the reaction, that is, taking ions out to cleave the ion bond, decrease entropy penalty, etc. On the other hand, 5.80 GHz microwave would work only on water molecules that would not produce a special advantage for the reaction like the 2.45 GHz irradiation. Meanwhile, both 2.45 and 5.80 GHz microwaves enhanced inactivation of the enzyme. We would not propose any clear consideration for dependency of microwave frequency at this point.

Acknowledgments

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[11] Typical procedure of the reaction: the reaction was carried out in 1.5 mL scale of 1 mM of 4-Methoxyphenyl glucopyranoside and 100 mU/mL Glucosidase HT1 in 50 mM sodium acetate buffer (pH 5.0). The reaction was heated by either incubator EC-40R (AS ONE), MWS-1000 (EYELA) for using 2.45 GHz microwave or our own making machine for using 5.80 GHz. The reaction was sampled (5 μL) every 10 min and stopped the reaction by mix of 15 μL of 8 M guanidine hydrochloride solution. The reactions were monitored by HPLC [column: Inertsil ODS-3, ϕ 4.6 mm × 250 mm; eluate: acetonitrile with 0.1% TFA, gradient increase from 15 to 35% over 15 min; column oven temperature: 30 °C; flow rate: 1 mL/min, detector: 220 nm] and the reaction ratio was calculated by signal intensities of the starting material, 4-methoxyphenyl glucopyranoside (Rt 7.5 min) and resulting product, 4-methoxyphenol (Rt 14.5 min).

[12] The 5.80 GHz microwave irradiation equipment was applied the system of ATMW500B-5.8 G (Amil Co., Ltd.). Microwave was oscillated by the magnetron, attenuated by the attenuator, matched by EH tuner to minimizing reflective power and then irradiated to the target. Dummy load was set on the waveguide terminal to absorb transmitted wave and the target was heated by the progressive wave. The quartz tube (i.d. 9 mm and o.d. 10 mm), practically NMR tube, was used for the reaction vessel. The vessel was set parallelly along the direction of the vibration of electric field at the center of the E surface of 20 × 40 mm waveguide. Temperature was measured directly by AMOTH FL-2000 fiber optic thermometer (ANRITSU METER Co., Ltd.).

[13] Relative complex permittivity was measured by open-probe measurement system (KEYCOM Corp.) using ZVB14 vector network analyzer (Rohde & Schwarz) calibrated by open (air), short (copper foil) and load (Kanto Chemical Co., Inc. 11307-3B distilled water).

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