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Enzymatic proteolysis of peptide bonds by a metallo-endoproteinase under precise temperature control with 5.8-GHz microwave radiation

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ABSTRACT

The present article examined the advantages of 5.8-GHz microwaves versus the more commonly used 2.45-GHz microwaves using a modular non-commercial apparatus on the proteolysis of the Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly peptide at the amino side of the aspartic acid (Asp) using the Asp-N metallo-endoproteinase enzyme. The microscale sample was subjected to microwaves' electric field (***E***-field) and magnetic field (***H***-field) radiation; good temperature control for samples at the μL scale was achieved using an apparatus that emitted microwaves at a precise frequency (5.800000 GHz). Temperature-dependent experiments with the Asp-N metallo-enzyme confirmed the activity of this enzyme to be greatest at 37 °C reached within 15 s on irradiation with the 5.8-GHz microwaves' *H*-field component with an input power of 1.7 W under air cooling conditions. Enzymatic activity decreased significantly above and below this temperature by a slight temperature change of 1 °C. Proteolysis yields of the peptide by the Asp-N enzyme at 30–42 °C under microwave ***E***-field and ***H***-field heating and under conventional heating revealed that enhancement of the proteolysis of the peptide at 37 °C by ***E***-field irradiation (42.6%) was 1.5 times greater than by conventional heating (27.5%), whereas under microwave ***H***-field irradiation (63.5%) it was 2.3 times greater than conventional heating. The relative dielectric loss factors (ε_r'') of the sample solution (peptide + enzyme) were also determined: 22.19 (5.8 GHz) and 12.78 (2.45 GHz) at 22 °C, which decreased with increasing temperature (faster for the 5.8-GHz microwaves) so that the initial heating efficiency of 5.8-GHz microwaves was nearly twofold greater than for 2.45-GHz microwaves. Results indicated that in addition to a thermal factor, microwave non-thermal factors also had a significant influence as the microwaves considerably enhanced the proteolytic process relative to traditional heating.

1. Introduction

Recent years have witnessed an increasing number of studies of enzyme reactions using microwave radiation ever since the use of this electromagnetic radiation in the field of food heating and cooking [1] that soon captured the attention of several researchers. For instance, microwave radiation has been used in the destruction of the growth inhibitor of the antitrypsin enzyme in soybeans by roasting above 105 °C for 2 min [2], and in the retrieval of antigens from formalin-fixed paraffin-embedded tissues at temperatures up to 100 °C in the presence of solutions containing metal cations [3]. The latter led to (i) omission of enzyme predigestion of tissues, (ii) to shorter incubation times of primary antibodies or to increased dilutions of primary antibodies, (iii) to achieving adequate staining in long-term formalin-fixed tissues, and (iv) to excellent staining of certain antibodies typically unreactive with formalin-fixed tissues. Adverse health effects of weak microwave radiation from cell phone use have focussed on DNA damage and cancer, though epidemiological studies have failed to support cell phone usage with causing cancer [4]. Nonetheless, low-intensity microwaves could induce a non-thermal heat-shock response that could enhance the expression of small stress-inducible heat-shock proteins. Apparently, improvement in the enzyme was ascribed to non-thermal microwave effects in such biological systems. Such effects were reported nearly two decades ago by subjecting thermophilic enzymes (e.g., 5-adenosylhomocysteine hydrolase) to 10.4-GHz microwaves that seemingly underwent induced protein structural rearrangements not related to temperature [5]. In this context, two recent review articles examined microwave-assisted enzymatic reactions [6,7] in which the influence of microwave

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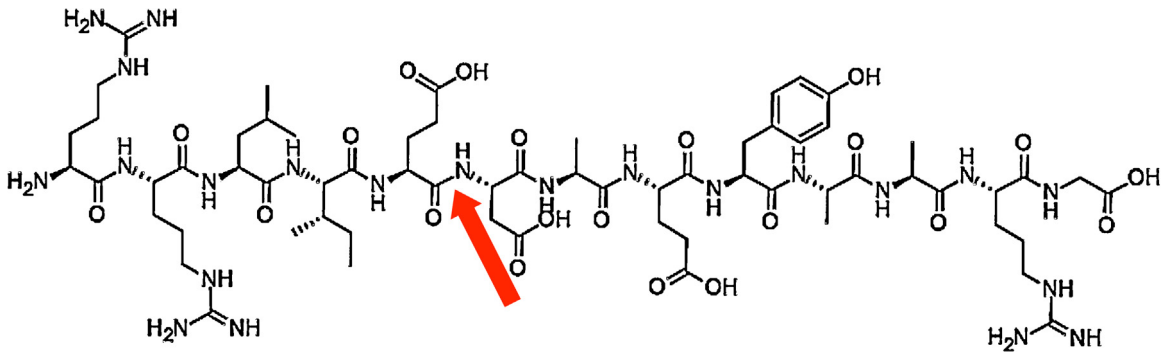

Scheme 1. Structure of the Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly peptide. This endoproteinase enzyme can specifically hydrolyze peptide bonds on the N-terminal side of aspartic and cysteic acid residues [19].

radiation on enzymatic properties and their application in enzyme chemistry were highlighted: (a) shortening of reaction times and (b) role played by specific microwave effects.

Active usage of microwave radiation in organic syntheses [8] and in the syntheses of nanomaterials [9] has been ongoing since the latter part of the 1980s. Usage of microwaves in enzymatic catalysis by Young et al. [10] examined specific microwave effects with high levels of microwave radiation under conditions wherein minimal catalytic activity arose from thermal heating of the bulk solvent. Their work indicated that hyper-thermophilic enzymes could be activated at temperatures far below their optimum, thereby offering the prospect of using hyper-thermophilic enzymes at ambient temperatures to catalyze reactions with thermally labile substrates and products, together with the possibility of using microwaves to regulate biocatalytic rates at low temperatures for enzymes from less thermophilic sources.

Enzymatic catalysis with immobilized enzymes in dry media and microwave activation in solvent-free conditions at temperatures near 100 °C enhances both reactivity and selectivity of supported lipases in such enzymatic reactions as esterification and transesterification reactions [11]. For instance, the transesterification of methylacetoacetate in various *n*-alcohol solvents with the Novozym 435 enzyme exhibited significant microwave-induced rate enhancement in all the alcohols relative to conventional heating [12].

The polymerase chain reaction technique (PCR) is a common biological molecular tool used to amplify DNA; it features thermal cycling between two or three distinct temperatures to achieve denaturing of DNA, primer annealing, and DNA extension. A recent report on rapid PCR amplification demonstrated the successful use of microwaves for direct substrate heating that resulted in rapid thermal cycling, thereby enabling DNA amplification on a microfluidic device [13]. Many reported studies, however, used commercial microwave heating devices with which strict sample temperature control at the milliliter scale of samples proved difficult [14]. Consequently, reproducibility and thermal management of radiation intensity of the microwaves at the microliter scale would prove even more difficult. Indeed, commercial devices/equipment available prior to 2007 lacked efficient stirring and critical internal temperature measurement of microwave-heated reactions as inefficient agitation can cause temperature gradients within the reaction mixture as a result of field inhomogeneity in the microwave cavity [14], and not least the use of external infrared temperature sensors can result in significant inaccuracies in temperature measurement as emphasized in the first PCR amplification experiment by Fermér et al. [15] who noted that the PCR sample must be heated with a high temperature gradient in a very reproducible and uniform fashion. These authors also pointed out that domestic microwave ovens are designed to heat large samples and so are less reliable for small loads because of magnetron overheating by non-absorbed microwave power. Moreover, because the domestic multi-mode technique provides a field pattern with areas of high and low field strengths, the temperature can vary drastically between different positions of the sample, which could lead to formation of localized hot spots and cold spots, with the former possibly denaturing irreversibly the protein structure of the polymerase and destroy its enzymatic function. Clearly, the use of domestic microwave ovens for small scale PCR amplification is to be avoided [15]. Therefore, delineation of microwave effects in enzymatic reactions will require precise and constancy of sample temperature and microwave exposure to different frequencies.

The present study proposes some solutions to the above problems using an apparatus that can deliver microwaves at the precise frequency of 5.800000 GHz using a single-mode applicator. Our earlier studies have described the possible advantages of 5.8-GHz microwaves in organic syntheses [16] and in nanoparticle syntheses [17], especially as the shorter wavelengths of the 5.8-GHz microwaves can heat nonpolar solvents [18]. We used a Teflon guide for the thermometer in existing PCR tubes so as to achieve high reproducibility and precise thermal control in our examination of the proteolysis of the Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly peptide (Scheme 1) in the presence of the Asp-N enzyme as a model enzymatic reaction to ascertain the advantages of the proposed protocol. This endoproteinase enzyme is specific for cleavage of protein or polypeptide bonds N-terminal to aspartate or cysteic acid residues [19].

2. Experimental

2.1. Materials and reaction methodology

The peptide Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly was obtained from Takara Bio Inc. (Pfu Methionine Aminopeptidase) and used as the model substrate for the proteolysis reaction with the Asp-N enzyme, a metallo-endoproteinase enzyme also obtained from Takara Bio Inc. (Lot No. ACXP056).

The peptide (0.10 nmol μL^{-1}; 15 μL) was introduced into the PCR polypropylene tube (diameter: 5.0 mm; internal diameter: 4.0 mm; height 20.0 mm) containing 7-μL of an aqueous Na_3PO_4 buffer solution (250 mM, pH 8.0), following which the endoproteinase Asp-N enzyme (0.05 μg μL^{-1}; 8 μL) was added to the PCR tube. According to the data sheet supplied by the vendor, the optimal reaction temperature of the Asp-N enzyme is 37 °C. Termination of the enzymatic reaction was achieved by adding an EDTA solution (1.0 M; 2 μL) to the reaction mixture.