JOURNAL OF PROTEOMICS 80 (2013) 160–170

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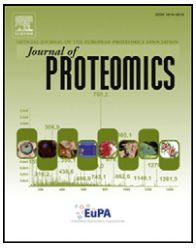

Evaluating the potential nonthermal microwave effects of microwave-assisted proteolytic reactions

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ARTICLE INFO

Article history:
Received 20 December 2012
Accepted 9 January 2013
Available online 24 January 2013

Keywords:
Microwave
Protein digestion
Enzyme
Nonthermal effect
Mass spectrometry

ABSTRACT

Microwave-assisted proteolytic digestion methods have evolved into a highly effective approach and serve as an alternative to conventional overnight digestion. This approach typically exploits the unique microwave properties to facilitate the digestion of proteins into their peptides within minutes. Conventional digestion is carried out at 37 °C while microwave-assisted digestion requires much higher and sometimes inconsistent temperatures. Thus, this study aims to investigate whether the faster reaction rate is due to the microwave quantum effect or the thermal effect. Quantitative mass spectrometry was used to conduct kinetic analysis of tryptic digestion for several proteins by microwave and conventional heating. The percentages of digestion products relative to internal standards showed no significant difference between microwave and conventional heating conditions at the same digestion temperature. The optimum temperature for tryptic digestion was determined to be 50 °C. Furthermore, this study compares the digestion completeness indicators of several proteins under microwave and conventional heating. Again, the values obtained from microwave and conventional heating were similar given identical temperatures. The overall results prove that a nonthermal effect does not exist in microwave-assisted tryptic digestion. Therefore, conventional heating at high temperatures (50 °C) can be also used to accelerate digestion reactions.

1. Introduction

Microwave heating is widely used as an efficient non-conventional energy source for causing chemical reactions [1,2]. In general, microwave reactions operate by agitating polar molecules that oscillate under the effect of the electromagnetic field. Recently, the advantages of microwave technology have also been exploited in fields including drug discovery [3], material sciences [4], proteomics [5,6], nanotechnology [7], and biochemistry [6,8,9]. Most related studies have shown that, compared to conventional heating techniques, microwave heating results in significantly reduced reaction times and enhanced product yields/purities [2]. There are two main hypotheses to explain the acceleration of chemical reactions (i.e., changes in reactivity and selectivity) under microwave irradiation. One holds that it is just a transfer of heat (i.e., a thermal effect) while the other is based on specific radiation effect (i.e., microwave or nonthermal effect). The role of nonthermal effects in organic synthesis is highly contentious [2,8–12], with some researchers asserting that microwaves are simply a convenient means of heating [1,13], while others have produced experimental evidence of a nonthermal effect [2,14]. This problem was addressed in a recent review by Hoz et al. [2], who examined the evidence for nonthermal effects of microwaves on chemical reactions. They concluded that the microwave irradiation effect on a chemical reaction is very complex and involves thermal and nonthermal effects. Other studies have examined the nonthermal effects of microwaves on protein conformation and function [15–17].

Proteomics attempts to comprehensively elucidate biological processes by systematically analyzing the proteins expressed in a cell or tissue. The speed, accuracy, selectivity, and sensitivity

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http://dx.doi.org/10.1016/j.jprot.2013.01.005

of mass spectrometry (MS) have made it particularly useful for analyzing the tryptic digestion of proteins [18–20]. On the other hand, conventional proteolytic digestion methods are often time consuming. Several approaches were developed for rapid proteolysis through thermal denaturation [21], chemical denaturation [22], ultrasound [23], and enzymes immobilized on various supports [24,25]. In recent years, microwave-assisted proteolytic digestion has developed into a highly useful technique and an interesting alternative for conventional overnight digestion [5,6]. This technique normally exploits the unique properties of microwaves to cleave proteins into their peptides within minutes. Furthermore, microwave-assisted proteolytic digestion procedures, such as tryptic [5,26–29] and acid-mediated proteolysis [30–35], allow for the rapid preparation of samples for bottom-up analysis. Pramanik et al. [5] investigated how microwave irradiation affected protein digestion with trypsin in 10 min. That study examined proteins including cytochrome c, ubiquitin (tightly folded and resistant to denaturation), lysozyme, and myoglobin. Juan et al. [26] extended this approach to digest several known proteins in gel with trypsin under 5 min of microwave irradiation. Moreover, the protocol for microwave-assisted digestion was further optimized to analyze clinical samples [28].

Functionalized magnetic nanoparticles (NPs) were also used as efficient affinity probes and heat (microwave) absorbers for microwave-assisted proteolytic digestion methods [29,36–38]. Chen et al. developed an efficient method using magnetite beads to digest proteins in a domestic microwave device within 1 min. Wu et al. successfully applied quantum dots (QDs) as enrichment and accelerating probes for microwave enzymatic digestion of proteins [39–41]. The same group [42] proposed a washing and separation-free method via the use of TiO_2 NPs as an accelerating and affinity probe for the microwave-assisted tryptic digestion of proteins. They reported that TiO_2 NPs (which provide good heat absorption) produced higher sequence coverage (100%) than CdS QDs (95%) for cytochrome c.

Our previous study demonstrated that, under the influence of rapid microwave heating, enzymatic reactions can proceed in a solvent such as chloroform, which, under conventional digestion conditions, renders the enzyme inactive [43]. Another work investigated how the digestion process was affected by various solvents and reaction times, different enzyme to protein molar ratios, and varying microwave temperatures [44]. Microwave-assisted proteolytic digestion often yielded miss-cleaved peptides, resulting from incomplete hydrolysis reactions between enzymes and substrates. To investigate whether microwave irradiation catalyzes proteolytic digestions only by heat or by a combination of thermal and nonthermal energies, this study compares the effects of microwave irradiation and conventional heating on protein digestion given identical reaction temperatures. Mass spectrometry is used to qualitatively and quantitatively determine the relative percentages of peptide digests vs internal standards for several proteins in various solvents, temperatures, different protein/enzyme (E/S) molar ratios and various enzymes, with and without microwave irradiation are determined. Comparisons are also made of the digestion efficiencies, number of miss-cleaved peptides (NMCP), incomplete digestion percentage (IDP), and sequence coverage of several proteins with and without microwave irradiation.

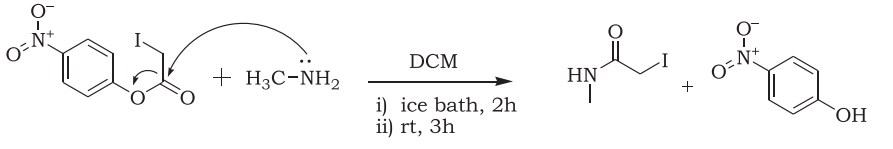
2. Experimental

2.1. Materials and chemicals

All reagents were of analytical grade and used as received. Ultrapure Milli-Q water was produced in the laboratory with a Millipore system (Bedford, MA, USA). HPLC grade acetonitrile (ACN), dichloromethane, and methanol were obtained from J.T. Baker (Phillipsburg, NJ, USA). Urea was from Wako (Osaka, Japan). Trifluoroacetic acid (TFA), 4-nitrophenyl iodoacetate, and glycoprotein hormone α (32–46) amide were purchased from Fluka (Buchs, SG, Switzerland). Immobilized trypsin (TPCK) was acquired from Pierce (Thermo Scientific, USA). Sequencing grade trypsin was obtained from Promega (Madison, WI, USA). α-Cyano-4-hydroxycinnamic acid (HCCA), ammonium bicarbonate, dithiothreitol (DTT), iodoacetamide (IAA), Tris buffer, lysozyme (chicken egg white), albumin (bovine serum), transferrin (human), β-lactoglobulin (bovine milk), cytochrome c (bovine heart), α-casein (bovine milk), myoglobin (horse heart) and trypsin (bovine pancreas) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methylamine (40% w/w aqueous solution) was obtained from Alfa Aesar (Ward Hill, MA, USA).

2.2. Synthesis of N-methyliodoacetamide (MIAA)

MIAA was synthesized according to the reported procedure [45] with minor modifications. A volume of 43 μL aqueous methylamine (40%, 11.63 M, 2 equiv.) was added dropwise to an ice-bath cooled solution of 4-nitrophenyl iodoacetate (76.7 mg, 0.25 mmol, 1 equiv.) in anhydrous dichloromethane (5 mL) over 20 min. 4-Nitrophenol was precipitated during the reaction (Scheme 1). The reaction mixtures were stirred for an additional 2 h at 0 °C and 3 h at room temperature. The resulting crude products were filtered through a paper filter to remove 4-nitrophenol. The filtrate was dried with 1 g of anhydrous Na_2SO_4 and concentrated in a rotary evaporator at 40 °C with a water vacuum pump. Chromatography (SiO_2,



Scheme 1 – Synthesis of N-methyliodoacetamide.