LWT - Food Science and Technology 95 (2018) 9–16

Contents lists available at ScienceDirect

LWT - Food Science and Technology

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

Real-time monitoring of enzyme-assisted animal protein hydrolysis by NMR spectroscopy – An NMR reactomics concept

Ulrik K. Sundekilde, Lise Jarno, Nina Eggers, Hanne Christine Bertram*

Department of Food Science, Aarhus University, Kirstinebjergvej 10, 5792 Årslev, Denmark

ARTICLE INFO

Keywords:
Free amino acids formation
Meat metabolites
Alcalase
Enzymatic processing
Meat hydrolysis

ABSTRACT

This study presents an application of proton (^{1}H) NMR spectroscopy for monitoring of enzyme-assisted hydrolysis of muscle protein under both at-line and real-time conditions. Measurements were carried out on sample material > 1 g, and two different enzymes (alcalase and papain) were examined. The real-time monitoring of reactions was carried out directly in the NMR spectrometer, and the technique enabled the dynamic and quantitative detection of a total of 40 metabolites. The approach enabled to follow time evolution and kinetic modelling allowed extraction of kinetic parameters; a was calculated to 0.051 min^{-1} and 0.029 min^{-1} for alcalase and papain, respectively, revealing a higher hydrolysis velocity for alcalase than papain. The study demonstrates that NMR spectroscopy is an excellent analytical platform for real-time monitoring of the major metabolite composition changes occurring during an enzymatic hydrolysis.

Industrial relevance: Protein hydrolysis is a commonly applied technology in food industry and the presented NMR methodology for kinetic studies of metabolite generation during a hydrolysis process would be applicable for testing and optimization of different raw materials and processing conditions.

1. Introduction

Protein hydrolysis is a commonly applied technology in food industry. It is used widely in the manufacturing of protein ingredients where the technology aids in the production and valorization of ingredients with functional properties (emulsification, texture and foam stabilization, fat substitution, flavor enhancement etc.), ingredients with enhanced nutritional value or ingredients with bioactivity (Nongonierma & FitzGerald, 2017; Zhang & Mu, 2017). While the hydrolysis may be facilitated by various strategies including acid, heat, or application of hot-pressure, the hydrolysis reaction is frequently enzyme-assisted. Several studies have examined enzyme-assisted hydrolysis processes, and focus in these studies have been on characterizing the degree of hydrolysis, amino acid profile and molecular weight distribution of the final products (Chakka, Elias, Jini, Sakhare, & Bhaskar, 2015; Damgaard, Lametsch, & Otte., 2015; Dong et al., 2014; Pagan, Ibarz, Falguera, & Benitez., 2013; Piazza & Garcia, 2014; Song et al., 2016). The kinetics of enzyme-assisted hydrolysis of food proteins has also been studied (Jovanovic et al., 2016; Zhou et al., 2017). However, examinations of hydrolysis kinetics are complicated and tedious due to the fact that enzymes must be inactivated to preserve the sample unmodified. The sampling and enzyme inactivation is likely to introduce measurement errors. Consequently, dynamic studies of enzyme-assisted protein hydrolysis processes would be preferable; however, such studies are sparse.

Proton NMR spectroscopy is a valuable non-destructive technique that enables to monitor the complete profile of low-molecular metabolites present in a complex sample including free amino acids. Proton NMR spectroscopy is not selective for any specific metabolite classes and the metabolite profiling can therefore be considered unbiased and untargeted. The advantages of the concomitant analysis of multiple metabolites in a food matrix, which proton NMR spectroscopy offers, have been demonstrated in numerous studies, e.g. on muscle tissue (Sundekilde, Rasmussen, Young, & Bertram, 2017), processed milk (Jansson et al., 2014), and juice (Betoret et al., 2017) as some recent examples. Furthermore, the non-destructive character and the compliance with measurement on unmodified complex samples advocate that proton NMR spectroscopy could be a superior tool for the dynamic and direct monitoring of chemical processes and reactions in foods. Accordingly, Ebrahimi, Larsen, Jensen, Vogensen, and Engelsen (2016) demonstrated the potential use of proton NMR spectroscopy for the direct and dynamic monitoring of glucose fermentation by lactic acid bacteria. Based on the use of glucose solutions where lactobacillus cell cultures were added, the authors developed a methodology that allowed the kinetic analysis of 11 different metabolites, thereby establishing that proton NMR spectroscopy is a superior tool for real-time

* Corresponding author.
E-mail address: hannec.bertram@food.au.dk (H.C. Bertram).

https://doi.org/10.1016/j.lwt.2018.04.055
Received 6 January 2018; Received in revised form 17 April 2018; Accepted 18 April 2018
Available online 23 April 2018

monitoring of dynamic processes involving the formation of multiple metabolites. Inspired by the work by Ebrahimi et al. (2016) and as a result of the need for efficient tools to study the detailed dynamics of multiple metabolites generated during protein hydrolysis, we conducted the present study with the aim to examine the potential use of proton NMR spectroscopy for monitoring enzyme-assisted protein hydrolysis of complex protein systems under both real-time conditions with monitoring of reactions directly in the NMR spectrometer and by semi-dynamic hydrolysis experiments conducted at-line. To the best of our knowledge, no previous reports on the use of proton NMR spectroscopy for characterization and monitoring of protein hydrolysis have been reported.

2. Materials and methods

2.1. Chemicals and regents

Deuterium oxide (D_2O) (99.9 D%) containing 3-(trimethylsilyl) propionic-2,2,3,3-d4 acid (TSP), Alcalase® 2.4 L from *Bacillus licheniformis* and Papain from *Caricapapaya* purchased from Sigma Aldrich (St. Louis, MO). All chemicals used were of analytical grade, and double-deionized water was used throughout.

2.2. ^{1}H NMR spectroscopy

Proton (^{1}H) NMR spectroscopy was performed on a Bruker Avance III 600 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with an autosampler with a capacity of 60 samples. For at-line hydrolysis experiments one-dimensional (1D) Nuclear Overhauser Effect Spectroscopy (NOESY) were acquired with 32768 time domain data points, 12.15 ppm spectral width and a total of 64 scans. As explained in subsequent text, two types of experiments were conducted: i) at-line experiments (section 2.3) and ii) real-time experiments (section 2.4). For both at-line and real-time hydrolysis experiments 1D Carr-Purcell-Meiboom-Gill spectra (CPMG) were acquired with 32768 time domain data points, 17.35 ppm spectral width, an echo time of 50 ms and a total of 64 scans. For both experiments the relaxation delay was 5 s. In the at-line hydrolysis experiments, the NMR acquisitions were carried out at 298 K, while in the real-time hydrolysis experiments, the NMR acquisitions were carried out at 328 K. The spectra were processed with zero-filling prior to Fourier transformation. All spectra were chemical shift referenced to the internal TSP signal at 0.0 ppm. Metabolite assignments were based on information previously published (Castejón, García-Segura, Escudero, Herrera, & Cambero, 2015; Garcia-Garcia, Lamichhane, Castejon, Cambero, & Bertram, 2018) and on the databases: HMDB (Human Metabolite Data Base http://www.hmdb.ca/) and Chenomx (NMR Suite version 7.6, Edmonton, Canada).

2.3. At-line hydrolysis experiments

Minced chicken meat purchased from a local butcher was used as a substrate for the hydrolysis experiments. The chicken meat had a fat content of approx. 5 g/100 g and the collagen/meat protein ratio was approx. 1:10. The samples were prepared by taking an amount of 250 mg of meat, which was put into an Eppendorf tube. A volume of 1000 μL D_2O (containing TSP as internal chemical shift reference) and enzyme substrate 1:1000 (w/w) was added to the Eppendorf tubes. Samples were heated to 55 °C while being shaken. The enzymatic reaction was allowed to proceed for following time intervals: 20, 40, 60, 70, 100, 120, 180, 240, 300 and 360 min. Hydrolysis was stopped by inactivation of the enzyme by heating the samples to 95 °C for 10 min. Subsequently sample material was cooled by placement in ice water until analysis. Two enzymes were tested, alcalase and papain. In addition, samples with no enzyme addition were included as a control samples.

2.4. Real-time hydrolysis experiments

Samples for real-time hydrolysis experiments were prepared by placing 100 mg of minced chicken meat (identical to experiments in section 2.3) in an NMR tube and adding 550 μL of D_2O (containing TSP as internal chemical shift reference). Enzyme (0.5 μL) was added immediately (< 2 min) before initiating the NMR measurements. Proton NMR spectroscopic measurements were carried out consecutively over a 3.5 h-period to follow the hydrolysis in real time under 20 Hz rotation. Automatic shimming was conducted between acquisition of each spectra, and a total of 15 spectra were acquired.

2.5. Multivariate data analysis

The NMR spectra were subdivided into 0.01 ppm bins, reducing each spectrum into 950 separate variables in the regions 10.00–5.00 and 4.75–0.5 ppm. Principal component analysis (PCA) was performed in order to identify differences in the metabolite profiles as function of processing parameters (enzyme and time). The multivariate data analysis was performed using SIMCA-P + 14 (Umetrics AB, Umeå, Sweden). Selected metabolites were quantified using Chenomx NMR Suite 8.1.2 (Chenomx Inc, Edmonton, AB, Canada). Statistical significance was evaluated by Student's t-test using the Statistics Toolbox in MATLAB 2014a (MathWorks Inc., Natick, MA, USA).

2.6. Enzyme kinetics

For enzyme-catalysed reactions the kinetics is explained by modeling the hydrolysis as a zero order reaction and inactivation of the enzyme as a second order reaction (Zhou et al., 2016). According to the enzyme reaction intermediate complex theory, the process of enzymatic hydrolysis of a protein is expressed as follows:

$$E + S \overset{k1}{\rightleftarrows} ES \overset{k2}{\longrightarrow} E + P \tag{1}$$

where S is the concentration of substrate (g/L), E is the concentration of enzyme (g/L), P is the product (g/L), ES is the concentration of enzyme–substrate complex (g/L), k_1,k_2 are the reaction rate constants of enzyme adsorption (L/U min), and product formation (g/U min), respectively.

At a constant pH and temperature, the hydrolysis velocity can be determined by the irreversible stage (Marquez & Vazquez, 1999):

$$\mathrm{V} = S_0 \frac{\mathrm{d(DH)}}{\mathrm{dt}} = k3[ES] \tag{2}$$

where V is the hydrolysis velocity (g/L min), S_0 is the initial substrate concentration (g/L), DH is the degree of hydrolysis, and k_3 is the reaction rate constant for inactivation (L/U min).

According to the mechanism and kinetic model of enzymatic hydrolysis of a protein, the hydrolysis velocity also can be expressed by the following equation:

$$\mathrm{V} = \mathrm{aS_0e^{-b(DH)}} \tag{3}$$

In Eq. (3), $\mathrm{a} = \frac{\mathrm{k2E0}}{\mathrm{S0}}$ and $\mathrm{b} = \frac{\mathrm{k3KM}}{\mathrm{k2}}$, where a is the kinetic parameter (min^{-1}), b is a dimensionless kinetic parameter, and K_M is the Michaelis–Menten constant (g/L).

The relationship between the hydrolysis velocity and DH is expressed in Eq. (4) derived from Eqs. (2) and (3):

$$\frac{\mathrm{d(DH)}}{\mathrm{dt}} = \mathrm{ae^{-b(DH)}} \tag{4}$$

From Eq. (4), Eq. (5), which expresses the relationship between the degree of hydrolysis and the hydrolysis time can be obtained:

$$\mathrm{DH} = \frac{1}{\mathrm{b}} \ln(1+\mathrm{abt}) \tag{5}$$

The kinetic parameters a and b can be obtained by linear regression