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Biophysical Characterization of Temperature-Dependent Structural Modifications in β-Lactoglobulin during Tryptic Hydrolysis

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ABSTRACT

Determination of peptide fragments of proteins upon proteolysis process is crucial in many fields, including biotechnology, proteomics and food industry. Herein, our aim was to compare the structural changes of intact protein during enzymatic hydrolysis at various temperatures by using biophysical approaches. Proteolysis of β-Lactoglobulin (β-LG) was carried out with trypsin, and the changes were followed *in situ* during proteolysis at 37°C and 45°C by using Fourier transform infrared (FTIR) spectroscopy. Dynamical response of β-LG to enzymatic attack was identified on the molecular level. The IR signals of protein secondary structures decreased while carboxylate group signals arising from liberated products of proteolytic reaction increased in the 1605-1580 cm^{-1} range to the different extent. FTIR data revealed that the degree of protein changes and liberated products varies greatly based on the reaction temperature. This study demonstrates the potentiality of FTIR spectroscopy for *in situ* tracking of protein hydrolysis at various conditions.

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1. Introduction

Proteolysis (hydrolysis) is described as the breakdown of proteins into small polypeptide pieces or amino acids. Protein hydrolysis starts with the cleavage of peptide bonds by various enzymes (gastric, pancreatic etc.) and results in fragmentation of the polypeptide chains into small peptides forming a mixture of oligopeptides, micelles and even free amino acids. The hydrolysis of protein polypeptide chains must be taken under control as the sizes of

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the intermediate peptides or micelles change during hydrolysis process. Different treatment conditions (temperature, pH, concentration and conformation of the substrate etc.) affect the degree of protein structural changes and the content of released small peptides as the products of enzymatic reaction (Cheison et al., 2011; Iametti et al., 2002; Vorob'ev, 2022; Vorob'ev et al., 2023). However, there are still challenges to control target bioactive peptides and to avoid the generation of self-associated micelles or unwanted peptides.

Biophysical approaches such as vibrational spectroscopy has been widely used in many research areas such as biotechnology, biomedicine, biophysics, pharmacology, food and environmental research (Errico et al., 2023; Guler et al., 2019; Pirutin et al., 2023). Recently, Fourier transformed infrared (FTIR) spectroscopy has been frequently used to obtain detailed (bio)chemical composition and biophysical information about the biological specimens such as proteins, lipids, cells, tissues or body fluids (Derenne et al., 2014; G. Güler et al., 2016; Güler et al., 2024; Sen et al., 2015). Particularly, FTIR spectroscopy, as a kind of vibrational technique and a biophysical approach, is sensitive to vibrational modes of molecules, and thus, it provides abundant information on the proteins on the molecular level. In this regard, it has been successfully used to characterize the protein structures, conformational changes and dynamics, folding/unfolding, protein accessibility and enzyme reactions (Güler et al., 2011, 2016; Júnior et al., 2020; Korkmaz et al., 2015; Markoska et al., 2021; Vorob'ev et al., 2023; Yang et al., 2015, 2022).

The IR spectrum of a protein exhibits various vibrational bands arising from their peptide group and amino acid side chains. The peptide group generates nine distinct bands known as amide A, B, I, II, III, IV, V, VI and VII. However, only the amides I and II can be used for protein characterization. The amide I band (1700-1600 cm^{-1}) is the most sensitive region for determining protein secondary structure compositions. It is induced by C=O stretching (primarily), N-H bending, and C-N stretching vibrations of the amide functional groups. The amide II (1600-1500 cm^{-1}) band is mostly produced by the vibrations of N-H bending (40-60%) and C-N stretching modes (18-40%) and is conformation sensitive. The amide I vibration is used to identify protein secondary structure, whereas the amide II band can be used for both structural information and secondary structure prediction (Güler et al., 2016; Yang et al., 2015). Recently, FTIR has been used for the tracking of proteolytic activity, stability, and secondary structure estimation of the enzymes from the amide I band (Origone et al., 2024; Vorob'ev et al., 2023). Significantly, contribution of water absorption is a major challenge for protein characterization studies using FTIR spectroscopy. H_2O molecules in the buffer solvent are strongly absorbed in the amide I region owing to intense O-H bending vibration in the amide I band, and thus, its contribution overlaps with the IR signals of protein secondary structures. Thus, deuterated buffer (deuterium oxide, D_2O, heavy water) solvent has been frequently used to avoid the water absorption in the amide I region (Güler et al., 2011, 2016; Korkmaz et al., 2013).

Proteins are essential macromolecules for living organisms and play important roles, including transportation, metabolic activities, communication etc. in the body. β-lactoglobulin (β-LG) is the major nutritional whey constituents of milk (~3 g/L) and is considered as a major allergen in cows' milk. A transporter protein β-LG has naturally affinity for fatty acids and for some other hydrophobic ligands; hence, it has been investigated as a potential drug carrier (Broersen, 2020; Wei et al., 2018). β-LG is a noncovalent homodimer which comprises

mainly β-sheets. β-LG is a small globular protein of 162 residues with a molecular mass of 18,39 kDa (PDB ID: 1QG5) (Oliveira et al., 2001); thus, it has been mainly investigated in the studies of protein hydrolysis (Güler et al., 2011; Kristoffersen et al., 2019; Poulsen et al., 2016; Vorob'ev, 2022; Vorob'ev et al., 2011).

Trypsin is a type of endopeptidases and a kind of proteolytic enzymes from pancreas. Trypsin, a serine protease, generates free amino acids and small oligopeptides with C-terminal basic amino acids. Trypsin selectively hydrolyses the intact proteins at the carboxyl group of basic amino acids such as arginine and lysine (Toldrá & Mora, 2021). Trypsin is the most commonly used protease in many research areas, including food processes and proteomics. The optimum pH value is 7.8 at the temperature of 37°C for the tryptic hydrolysis of food proteins (Cheison et al., 2011; Olsen et al., 2004). It was also studied that trypsin is stable at 40°C but its activity decreases rapidly at 55°C (Galvão et al., 2001). Analysis of β-LG hydrolysates due to trypsin attack under acidic pH by using MALDI–TOF–MS/MS revealed that tryptic hydrolysis away from the optimum pH and temperature values produces different peptides and thus might provide a better controlling of hydrolysis process (Cheison et al., 2011). Our group has previously investigated the proteolysis mechanisms intensively. In those studies, the proteolysis process of various substrates (β-lactoglobulin, β-casein, bovine serum albumin etc.) with serine protease enzymes (such as trypsin, chymotrypsin) was studied by performing mathematical simulations (Golovanov et al., 2024; Vorob'ev, 2022) and by using atomic force microscopy (Vorob'ev et al., 2023), light scattering (Vorob'ev et al., 2013), fluorescence spectroscopy (Vorob'ev et al., 2013, 2015), Circular Dichroism (CD) spectroscopy and FTIR spectroscopy methods (Güler et al., 2011, 2016; Vorob'ev et al., 2023). Recently, our group has also carried out the tryptic hydrolysis of a non-globular protein β-casein. In that study, the nanoparticle rearrangement as well as the changes in protein secondary structures during hydrolysis were determined by combining AFM, FTIR spectroscopy and the methods of chemical kinetics (Vorob'ev et al., 2023). In aforementioned studies, spectroscopic data revealed that hydrolysis of proteins by proteolytic enzymes leads to the generation of C-terminals (COO^-) and N-terminals (NH_3^+), releasing of intermediate products and degradation of protein structures.

In the current study, our aim was to evaluate the influence of temperature variations on protein hydrolysis by using biophysical approaches, keeping the other environmental conditions constant. The protein changes were tracked *in situ* during tryptic hydrolysis at both 37°C and 45°C by means of FTIR spectroscopy methodologies. Herein, β-LG was used as a model protein due to its functional and structural features and widespread use in the food industry. The current work contributes to understanding the functional mechanism of proteolysis process, which might be crucial in biotechnology and proteomics and in determining food quality, storage performance, and food safety.

2. Material and Methods

2.1. Sample Preparation and Enzymatic Reaction

β-Lactoglobulin (Sigma-Aldrich, L3908) from bovine milk was used as a substrate and trypsin (Sigma-Aldrich, T1426) from bovine pancreas was used as an enzyme (serine protease) without any further treatment. The acid salt (KH_2PO_4) and base salts (K_2HPO_4) were used to adjust the pH value of buffer solutions. Thus, proper amounts of acid salt (KH_2PO_4) and base salts (K_2HPO_4) were mixed in deuterium oxide (D_2O, 99.9%, Sigma-Aldrich) and it

was used as the buffer solution at pD 7.9. Then, protein solutions were prepared by dissolving the lyophilized powder in 10 mM potassium phosphate (KPi) deuterated buffer at pD 7.9 (pD=0.4 + pH). Deuterated samples were equilibrated for 22 hours in D_2O buffer at +4ºC. The same conditions were also used to prepare the enzyme solutions. Prior to the enzymatic reaction, the substrate protein (β-LG) was adjusted to the initial concentration of 50 mg/ml, while the trypsin was adjusted to initial concentration of 0.25 mg/ml. Then, the enzyme and substrate solutions were mixed in the Eppendorf tubes at equal amounts of volume. The ratio of substrate to the enzyme was 200:1 (w/w). Additionally, blank solutions of β-LG (25 mg/ml) were prepared in the same manner in deuterated buffer without enzyme addition. All sample solutions were freshly prepared and analyzed to avoid the losing of enzyme activity and degradation of proteins.

2.2. FTIR Spectroscopy Measurements

PerkinElmer UATR Two FTIR spectrometer (USA) which is equipped with a MIR TGS (Mid-infrared Triglycine Sulfate) detector was used for recording of the spectra. A 3 µl sample solution was dropped on the CaF_2 (Calcium Fluoride) windows having a 14 µm pathlength. The outermost sides of the windows were coated with PTFE paste (Carl Roth GmbH, Karlsruhe) to prevent the leaking of the sample during measurement. The temperature was adjusted to 37°C or 45°C by using microcomputer-controlled cryogenic thermostatic bath (LabART, Turkey). The spectra were recorded in the range of 4000-1000 cm^{-1} with the resolution of 4 cm^{-1} and 8 scans for 120 minutes at certain time intervals. The "OPUS 7.0" software (Bruker, Germany) and software 'Kinetics' running under MATLAB were used to analyze the FTIR data, as described previously (Günnur Güler et al., 2018; Günnur Güler, Vorob'ev, et al., 2016). Accordingly, each absorbance spectrum was buffer-subtracted, baseline-corrected and area-normalized in the 1715-1385 cm^{-1} range. Afterwards, the FTIR difference spectrum was obtained by subtracting the first spectrum acquired at $t_0 = 1$ minute from each absorbance spectrum recorded at time t. The 2nd derivative of the IR absorbance spectra was applied with 9 smoothing points to resolve the superimposed bands that are not obvious in the absorbance spectra.

3. Results and Discussions

The FTIR spectra for blank β-LG in the absence of trypsin (so-called blank β-LG) and for hydrolyzed β-LG by trypsin (S:E ratio of 200:1) recorded for 120 min at 37°C (Figs. 1,2,5) and at 45°C (Figs. 3,4,6) revealed that the most substantial protein alterations were detected in the amide I (1700-1600 cm^{-1}) and amide II (1600-1500 cm^{-1}) regions, arise from the IR signals of protein secondary structural elements (α-helix, β-sheets, turns) and amino acid side chains. The color coded solid lines shown in all figures represent the spectra recorded at certain time intervals such that black color: 0-10 min, green color: 15-30 min, blue color: 35-85 min and red color: 90-120 min.

Hydrolysis at 37 °C

Fig. 1 represents the FTIR absorbance (Fig. 1A) and the second derivative spectra (Fig. 1B) of deuterated β-LG in the absence of trypsin recorded *in situ* at 37°C. The FTIR spectra exhibits characteristic peaks in the amide I band, such as absorbing at 1630 cm^{-1} which corresponds to the intra-molecular β-sheets (predominantly), 1622 cm^{-1}

(solution exposed β-sheets), 1647 cm^{-1} (α-helices), 1660-1658 cm^{-1} (tuns/loops), 1677 cm^{-1} (intra-molecular β-sheets) and at 1691 cm^{-1} (inter-molecular β-sheets). The spectral pattern shows only existing of slight spectral changes at the end of 120 min. This is due to the effect of deuteration since the protein is more accessible to the solvent at slightly-elevated temperature of 37°C. When hydrogen atoms are replaced by deuterium (i.e., N-H becomes N-D) in buffer-accessible amide modes of protein, the IR peaks shifts slightly towards lower wavenumbers (Güler, et al., 2016; Korkmaz et al., 2015). In deuterated buffer, the peaks detected at 1609 cm^{-1}, 1580 cm^{-1} and at 1563 cm^{-1} are respectively attributed to the side chains of Arg $\nu_{as}(CN_3H_5^+)$, Asp $\nu_{as}(COO^-)$, and Glu $\nu_{as}(COO^-)$ residues [for band assignment see refs. (Barth, 2000; Fabian & Mäntele, 2006; Güler et al., 2011)].

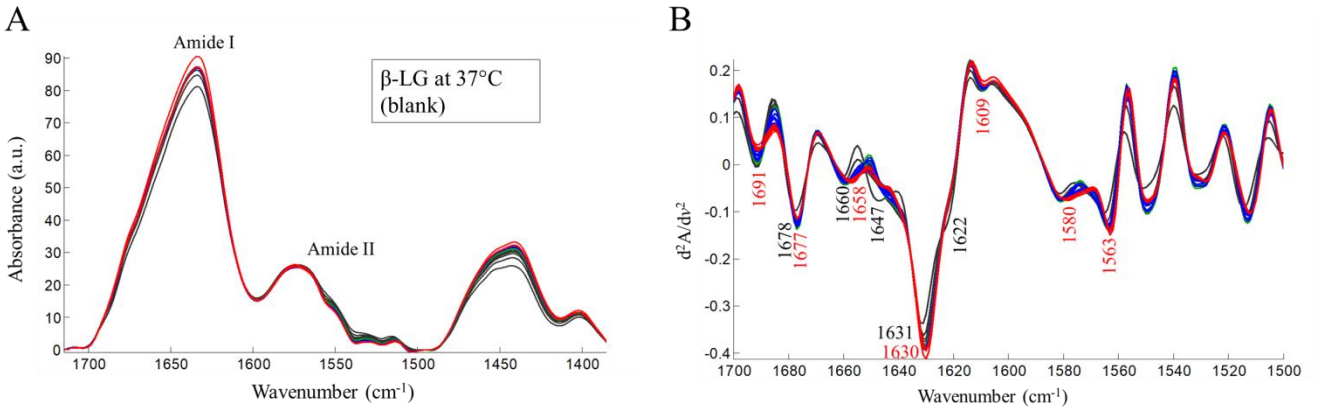

Figure 1. The FTIR absorbance spectra (A) and its second derivative spectra (B) for blank β-LG (25 mg/ml) solved in 10 mM KPi pD 7.9 buffer without enzyme addition, recorded for 120 min at 37°C. The absorbance scale represents rescaled absorbance values since all spectra were rescaled by normalization, as described in the M&M section.

Fig. 2 shows the FTIR spectra of tryptic hydrolyzed β-LG (S:E ratio of 200:1) in deuterated buffer recorded *in situ* at 37°C. It is clearer in the second derivative spectra (Fig. 2B) that the major signal at 1633 cm^{-1} (intramolecular β-sheets) decreases and shifts to 1636 cm^{-1} (disordered structures) (shown as 1633→1636 cm^{-1}) and the peak at 1647 cm^{-1} (α-helices) disappears during tryptic attack when compared to blank β-LG (Fig. 1). The other spectral shifts in the amide I and amide II bands during hydrolysis are detected as follows: 1692→1685 cm^{-1} (inter-molecular β-sheets), 1676→1675 cm^{-1} (intra-molecular β-sheets), 1661→1654 cm^{-1} (turn/loops), 1580→1577 cm^{-1} (Asp residues), and 1563→1561 cm^{-1} (Glu residues). It is also clearly observed that a new peak absorbing at 1664 cm^{-1} (turns) and the shoulder absorbing at 1629 cm^{-1} (solvent exposed β-sheets) arise gradually while the peak absorbing at 1608 cm^{-1} (Arg residues) disappears due to tryptic hydrolysis. Detection of the two accompanying peaks absorbing at 1685 and 1617 cm^{-1} are attributed to emerging of inter-molecular β-sheets (due to molecular contacts and/or aggregation) during tryptic hydrolysis at 37°C. The interesting point is that the IR signals absorbing around 1601-1587 cm^{-1} (shoulder) and around 1400 cm^{-1} increase (Fig. 2A and 2B), which corresponds to free carboxylates groups (antisymmetric and symmetric stretching vibrations of COO^- groups, respectively), which are liberated during tryptic hydrolysis. This altogether shows that the secondary structural elements of β-LG undergo strong changes: the content of regular β-sheet and α-helical structures decreases while both turn and disordered structures increase and the products (C-terminals COO^-) are generated as a consequence of enzymatic hydrolysis [for band assignment see refs. (Barth, 2000; Fabian & Mäntele, 2006; Güler et al., 2011)].

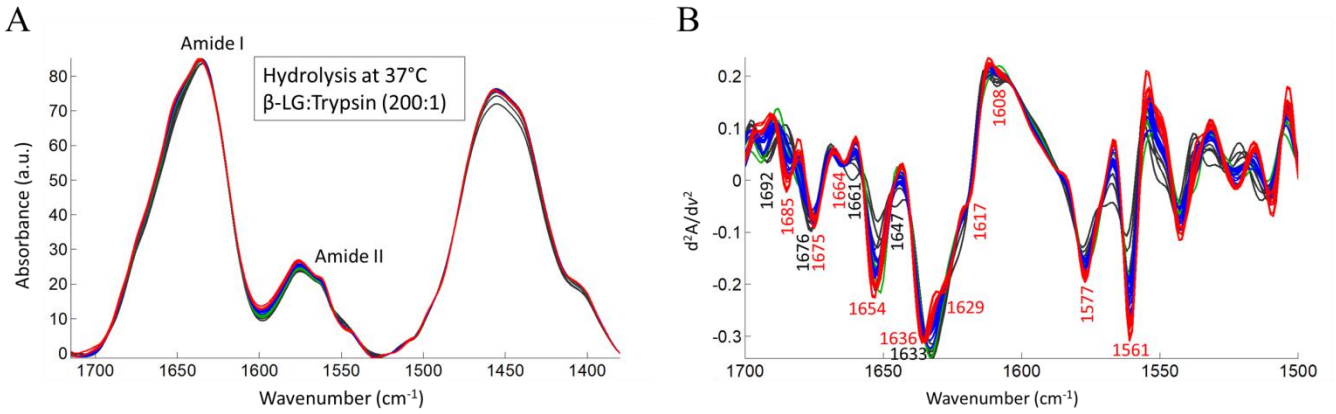

Figure 2. The FTIR absorbance spectra (A) and its second derivative spectra (B) for tryptic hydrolyzed β-LG (S:E ratio of 200:1) solved in 10 mM KPi pD 7.9 buffer, recorded for 120 min at 37°C. The absorbance scale represents rescaled absorbance values since all spectra were rescaled by normalization, as described in the M&M section.

Hydrolysis at 45°C

Fig. 3 illustrates the FTIR absorbance and its second derivative spectra for deuterated blank β-LG in the absence of trypsin recorded *in situ* at 45°C. When compared to blank β-LG at 37°C (Fig. 1), the spectra comprises of similar IR signals in the amide I band such as bands absorbing at 1631 cm^{-1} and 1679 cm^{-1} (intra-molecular β-sheets, predominantly), 1622 cm^{-1} (solution exposed β-sheets), 1647 cm^{-1} (α-helices), 1660 cm^{-1} (turns/loops), 1668 cm^{-1} (turns) and at 1690 cm^{-1} (inter-molecular β-sheets). Only slight differences are detected in the intensity and positions of IR signals of secondary protein structures due to the effect of further deuteration at slightly-elevated temperature of 45°C.

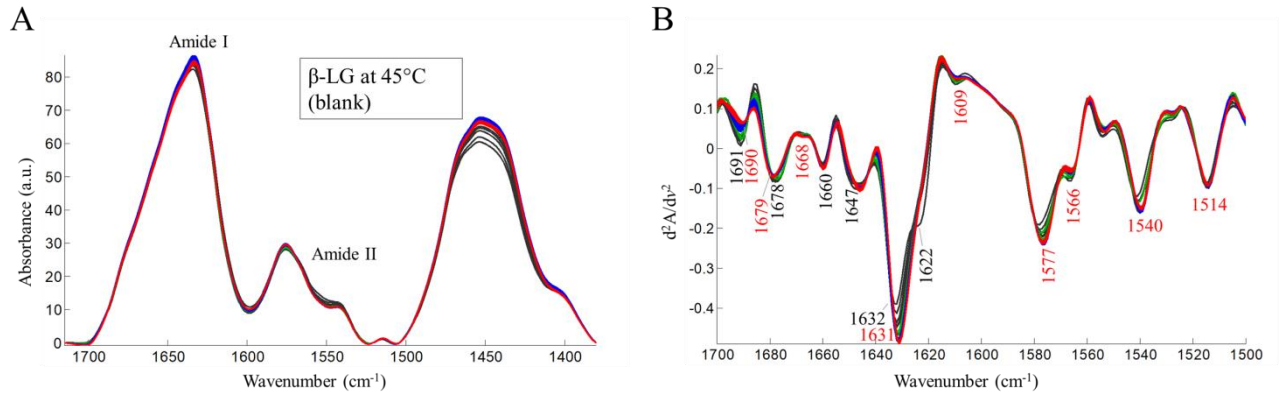

Figure 3. The FTIR absorbance spectra (A) and the second derivative spectra (B) for the blank β-LG (25 mg/ml) in 10 mM KPi pD 7.9 buffer at 45°C recorded for 120 minutes. The absorbance scale represents rescaled absorbance values since all spectra were rescaled by normalization, as described in the M&M section.

Fig. 4 demonstrates hydrolyzed β-LG by trypsin (S:E ratio of 200:1) in D_2O buffer recorded for 120 min at 45 °C. In comparison to blank β-LG recorded at 45°C (Fig. 3), the maxima of the amide I band at 1677 and 1633 cm^{-1} decreases with downshifting towards 1632 cm^{-1} (intra-molecular β-sheets) and the peak absorbing at 1622 cm^{-1} (solution exposed β-sheets) disappears during enzymatic reaction (Fig. 4B). The spectral changes are also detected as follows: 1691→1688 cm^{-1} (inter-molecular β-sheets), 1660→1658 cm^{-1} (turns/loops), 1577→1579 cm^{-1} (Asp residues), 1566→1564 cm^{-1} (Glu residues) and 1514→1515 cm^{-1} (Tyr residues).

Additionally, the intensities of the IR peaks absorbing at 1667 (turns), 1658 cm^{-1} (turn/loops) and at 1646 cm^{-1} (short α-helices/disordered structures) increase while the intensities at 1550-1520 cm^{-1} (α-helices/β-sheets) decrease. Moreover, a shoulder absorbing between 1605 and 1586 cm^{-1} as well as around 1400 cm^{-1} increases slightly (carboxylate groups of the C-terminals as products) (Fig. 4A). These altogether suggest that the amount of regular secondary structures of β-LG decreases while turns, short helices and disordered structures increase and intermediate products are formed upon hydrolysis.

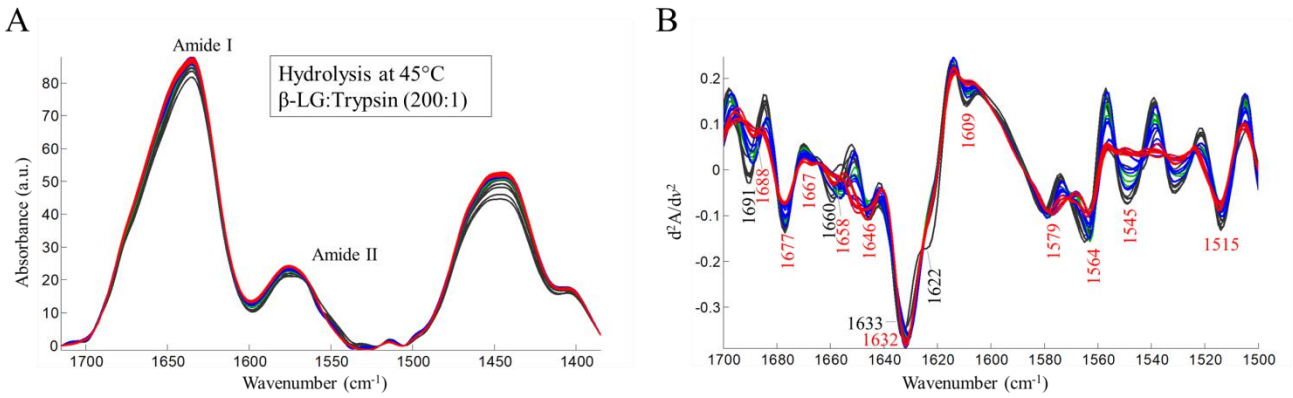

Figure 4. The FTIR absorbance spectra (A) and the second derivative spectra (B) for the digested β-LG (50mg/ml) at S:E ratio of 200:1 in 10 mM KPi pD 7.9 buffer at 45°C recorded for 120 minutes. The absorbance scale represents rescaled absorbance values since all spectra were rescaled by normalization, as described in the M&M section.

Comparison of tryptic hydrolysis at 37 °C and 45 °C

The FTIR difference spectrum has been frequently used to explore the small spectral alterations of proteins. Fig.5 demonstrates the FTIR difference spectra for each blank β-LG and digested β-LG in deuterated buffer at 37°C and Fig.6 shows the FTIR difference spectra of blank β-LG and of digested β-LG at 45°C. Herein, the first spectrum recorded at t=1 min of hydrolysis reaction was subtracted from the last spectrum recorded at t=120 min, refers to (final time)-*minus*-(initial time), during *in situ* measurements. Thus, the positive signals represent the increased part of β-LG while the negative peaks refer to the decreased part of β-LG due to tryptic hydrolysis.

Based on the FTIR difference spectrum recorded at 37°C (Fig. 5), the negative bands at 1630 cm^{-1} and 1692 cm^{-1} (β-sheets) as well as at 1624 cm^{-1} (solvent exposed β-sheets) are detected, meaning that β-LG loses its regular secondary structural components, β-sheets. However, hydrophilic turns/loops/short helices (positive peaks at 1670 and 1655 cm^{-1}), disordered structures (positive peak at 1639 cm^{-1}) and inter-molecular β-sheets (positive peaks at 1685 and 1615 cm^{-1}) increase due to enzymatic hydrolysis at 37°C. Additionally, trypsin attack causes an increase in the IR signals absorbing around 1601-1587 cm^{-1} (a positive broad peak centered at 1593 cm^{-1}) and around 1400 cm^{-1} attributed to the free carboxylate groups as proteolysis products. However, such spectral alterations are not observed for blank β-LG sample incubated at 37°C without trypsin.

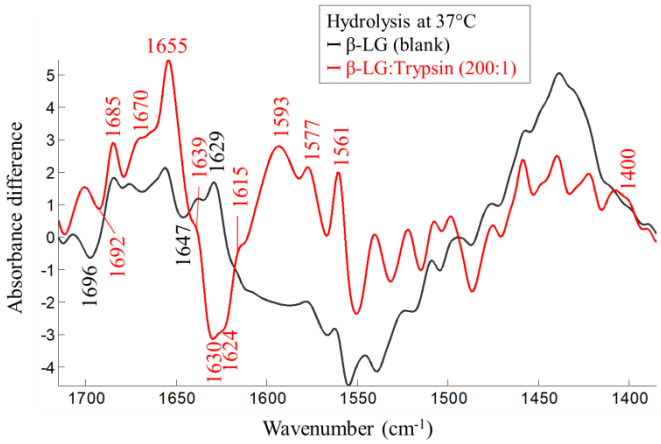

Figure 5. The FTIR difference spectrum {(spectrum recorded at t=120 min)-*minus*-(spectrum recorded at t=1 min)} for blank β-LG (25 mg/ml) and for tryptic hydrolyzed β-LG solved in 10 mM KPi pD 7.9 buffer at 37°C.

According to FTIR difference spectrum recorded at 45°C (Fig. 6), the two negative bands at 1693 and 1622 cm^{-1} (solvent exposed β-sheets) and broad positive peaks at 1688-1640 cm^{-1} (1668 cm^{-1}: turns, 1659 cm^{-1}: turns/loops, and 1642 cm^{-1}: short α-helices/disordered structures) are detected due to enzymatic hydrolysis at 45°C. Moreover, trypsin attack leads to an increment in the broad IR bands centered at 1592 cm^{-1} (positive peak) and around 1400 cm^{-1} attributed to the free carboxylate groups as proteolysis products. Such spectral alterations are not detected for blank β-LG equilibrated at 45°C for 120 min without trypsin.

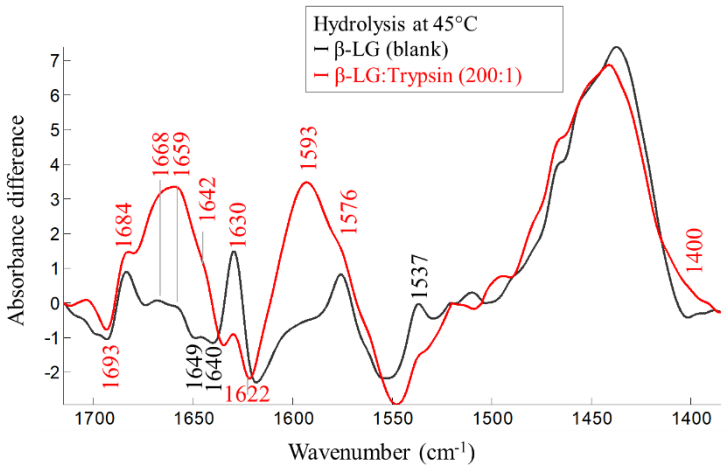

Figure 6. The FTIR difference spectrum {(spectrum recorded at t=120 min)-*minus*-(spectrum recorded at t=1 min)} for blank β-LG (25 mg/ml) and tryptic hydrolyzed β-LG solved in 10 mM KPi pD 7.9 buffer at 45°C.

To sum up, although the similar spectral changes are detected upon protein hydrolysis, the strength of protein degradation and arrangements of secondary structural elements differ with respect to incubation temperature. Tryptic hydrolysis of β-LG at 45°C (Figs. 4,6) results in higher hydrolysis of peptide bonds when compared to hydrolysis process performed at 37°C (Figs. 2,5), while the considerable decrease in intra-molecular β-sheets was found for 120 min of proteolysis at 37°C. The considered example shows that characterizing the proteolysis of β-LG by trypsin is much more difficult than searching for optimal conditions for the hydrolysis of low-molecular trypsin-specific substrates. The maximum overall rate of protein hydrolysis by trypsin was found at pH 7.8 and 37°C (Cheison et al., 2011; Olsen et al., 2004) and trypsin is stable up to 40°C (Galvão et al., 2001). It is possible that the search for proteolysis conditions that ensure maximum degradation of certain structural elements of the

protein may lead to other values. This work clearly shows the potential use of FTIR methods to track the temperature-dependent structural modifications in β-lactoglobulin during tryptic hydrolysis *in situ*.

4. Conclusions

In the current study, an IR spectroscopic analysis was employed to track the structural changes in the β-LG secondary structural elements (β-sheets, α-helices, turns) caused by tryptic attack at various temperatures. The measurements were performed *in situ* at the temperature values of 37 °C and 45 °C for blank β-LG (without enzyme treatment) and for trypsin hydrolyzed β-LG (S:E ratio of 200:1) solved in D_2O buffer. Overall, regular secondary structures of the β-LG (β-sheets) decrease while solvated and irregular structures (solvent exposed β-sheets, turns, short helices, disordered structures etc.) increase upon tryptic hydrolysis. Additionally, broad IR signals absorbing in the range of 1605-1580 cm^{-1} (centered at 1593 cm^{-1}) and at 1400 cm^{-1} were observed, which refer to the vibrations of free carboxylate (COO^-) groups corresponding to the reaction products. Consequently, β-LG lost its secondary structural elements due to the tryptic attack and the products (C-terminal groups, small peptides) were formed as a result of the enzymatic reaction. However, the FTIR data revealed no significant changes in the structure of blank β-LG (nature intact protein) for 2h-incubation at both 37°C and 45°C, except for negligible effects of deuteration. This study demonstrates that the degree of protein hydrolysis and arrangements of secondary structural elements due to tryptic attack are pretty different and are based on the incubation temperature. By making a manipulation for the hydrolysis environment (such as change in temperature), scientists can control the fragmentation of the intact protein and the release of small peptides as well as can avoid liberation of unwanted peptides. Thus, this work serves as guide for the development of novel methodologies to be useful in protein hydrolysis.

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Declaration of Competing Interest and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in OPS Journal belongs to the authors.

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