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Application of high-resolution ultrasonic spectroscopy for real-time monitoring of trypsin activity in β-casein solution

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ABSTRACT

High-resolution ultrasonic spectroscopy (HR-US) was applied for real-time monitoring of β-casein hydrolysis by trypsin at various conditions for the first time. The technique is based on the precision measurement of hydration changes proportional to the number of peptide bond hydrolyzed. As HR-US exhibits ultrasonic transparency for most solution, the analysis did not require optical transparency like for 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay. Appropriate enzymatic models were fitted with degree of hydrolysis (d_h) profiles to provide kinetic and mechanistic description of proteolysis in terms of initial hydrolysis rate, r^0, and rate constant of hydrolysis, k_h, and enzyme inactivation, k_d. Maximal r^0 and d_h were obtained at 45 °C and pH 8. The exponential dependence of kinetic parameters allowed determination of the activation (E_A = 50.3 ± 7 kJ/mol) and deactivation (E_D = 62.23 ± 3 kJ/mol) energies of hydrolysis. The ultrasonic assay provided rapid detection of trypsin activity even at sub-nanomolar concentration.

1. Introduction

Proteolysis plays an important role in various fields of bioscience and biotechnology. Technologically, there are broad applications of proteolysis in food processing (Vorob'ev, Vogel, Güler & Mäntele, 2011). For instance, the proteolytic activities in milk affect the texture and flavor of dairy products (Datta & Deeth, 2002). Milk has an average protein concentration of 3.2% in cow's milk in which 80% of the proteins are caseins and 20% are whey proteins. β-casein is one of the major casein proteins (~35% of bovine caseins) of 209 amino acids per monomer with corresponding average molecular weight of 23.6 kDa. It is a non-compact globular protein which usually exists in the form of micelle associated with other caseins such as α_{s1}-caseins, α_{s2}-caseins and κ-caseins in milk. Since dominant milk protein, modification of β-casein alters the overall properties and qualities of milk yielding both positive and negative impacts. Specifically, proteolytic activities have also been linked with the release of caseinophosphopeptides (CPPs), the phosphorylated bioactive peptides from milk casein which can be used as supplements for fortifying foods, with a view to improving mineral bioavailability (Cruz-Huerta, García-Nebot, Miralles, Recio & Amigo, 2015; García-Nebot, Alegría, Barberá, Clemente & Romero, 2009). Therefore, detection and quantification of proteolytic activity in milk has important industrial impacts.

Trypsin is a highly specific serine protease which selectively cleaves peptide bonds on the carboxyl-terminal side of arginine (Arg-X) and lysine (Lys-X) (Huber & Bode, 1978). Its monomeric form consists of 223 amino acids with corresponding average molecular mass of 23.3 kDa. It is an important digestive enzyme which is produced in pancreas as an inactive precursor, trypsinogen. It is commonly used as a model protease because it is inexpensive and readily available (Sato & Kato, 2016). Traditional methods for trypsin detection involve multiple clinical tests including radioimmunoassay, gelatin-based film techniques, enzyme-linked immunosorbent assay (ELISA) and colorimetric assay. However, these methods are time-consuming and costly (Gao, Tang, Li & Su, 2012). To overcome the limitations of traditional discontinuous methods, a variety of novel techniques providing the real-time data of trypsin activity have been reported. Among them are fluorescence techniques based on fluorescence sensors which have the advantage of the lowest detection limit compared with the other methods (Sato & Kato, 2016; Gao, Tang, Li & Su, 2012; Zhang, Qin, Cui, Zhou & Du, 2016; Zhang et al., 2018; Song et al., 2019). However, the efficiency of these techniques is dependent on the optical transparency or transmission of medium and can be affected by light scattering in dispersions.

High-resolution ultrasonic spectroscopy (HR-US) provides a potential technology for real-time, non-invasive and precision monitoring of enzyme activity in a wide range of concentrations and substrates as well as medium. It does not require optical transparency or optical markers

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and has ultrasonic transparency for all complex solution (Buckin, 2018). The capability of HR-US to follow a variety of reactions catalyzed by enzymes, namely, proteinase K, cellobiose, and lactase (Buckin & Craig, 2005; Resa & Buckin, 2011; Sierra, Resa, Buckin & Elvira, 2012; Altas, Kudryashov & Buckin, 2016) has been previously demonstrated. In addition, providing the precise information on number of peptide bonds cut, the ultrasonic technology has also been utilized to assess the characteristics of biocatalysis such as Gibb's free energy of reactions and validation of enzyme kinetics models (Resa & Buckin, 2011).

This study aims to extend the application HR-US in real-time monitoring and detection of proteolytic activity of trypsin towards its native protein substrate, β-casein, in buffered solution. Recently advanced ultrasonic analysis for monitoring biocatalytic reactions, previously described by Buckin & Altas (2017), were employed to provide real-time precise information on progress of protein hydrolysis in terms of the evolution of number of peptide bond cut. The present ultrasonic method is rather simple and provide remarkably high precision compare to traditional discontinuous optical assay like 2,4,6-trinitrobenzenesulfonic acid (TNBSA) which require incorporation of optical marker. This method is time-consuming and laborious. Furthermore, the ultrasonically measured real-time hydrolysis profiles were then compared with appropriate enzymatic models to validate the kinetic and mechanistic aspect of trypsin activity towards β-casein at varying pH, temperature and enzyme concentration. Also, given the high sensitivity feature of the measurement, an ultrasonic assay method for trypsin activity detection at low nanomolar concentration (1–50 nM) was presented. According to our best knowledge such ultrasonic methodology has not been used so far for analysis of the cleavage of casein by trypsin. In consideration of the obtained results, it has been demonstrated that HR-US constitutes a potential alternative analytical tool for analysis of proteolysis under reaction conditions of research and industrial relevance.

2. Materials and methods

2.1. Materials

The ultrapure water (Millipore Super-Q-System equipped with Durapore® hydrophilic PVDF membrane filter (pore diameter: 0.22 μm) with the resistivity 18.7 MΩ.cm at 25 °C, was used for preparation of all aqueous solutions. 0.1 M potassium phosphate buffer (PB) of various pH values (6, 7.4 and 8) was prepared from dissolution of KH_2PO_4 (≥99.0%, P5655, Sigma-Aldrich, Germany) and K_2HPO_4 (ACS reagent, ≥98%, P3786, Sigma-Aldrich) powders in ultrapure water. Bovine trypsin (TPCK Treated, essentially salt-free, lyophilized powder, ≥10,000 BAEE units/mg protein, Mw ≈ 23800 g/mol, T1426, Sigma-Aldrich) stock solution was freshly prepared by dissolution of solid in 0.1 M potassium phosphate buffer and series of dilution with the buffer was performed to make up 11.1–222 nM initial concentration range of trypsin before addition to the reaction.

The stock solution (2 mL) of bovine β-casein (BioUltra, ≥98% (PAGE), Mw ≈ 24000 g/mol, Sigma-Aldrich) was prepared in potassium phosphate buffer, at 1.5% (w/v) concentration. The protein solution was incubated at 4 °C without mixing for 12–16 h. It was then dialyzed against 3L of PB of the same pH for 72 h at 4 °C using dialysis tubing cellulose membrane with a molecular weight cutoff of 14 kDa (Sigma-Aldrich), according to the method described by O'Connell, Grinberg & de Kruif, 2003; Dezhampan, Esmaili & Khorshidi, 2017 with slight modifications. Dialysis was conducted without stirring and the buffer was not exchanged throughout the process. The protein concentration after dialysis was determined from the absorbance at 280 nm using Cary 60 UV/visible spectrometer (Agilent Technologies, Inc., USA). The β-casein concentration was adjusted to desired concentration, 0.5% (w/v), by further dilution with PB. using the molar extinction coefficient of the protein, 11000 $M^{-1}.cm^{-1}$. Finally, prior to the addition of casein to HR-US cell β-casein solution was filtered with 0.22 μm sterile PTFE syringe filter (Corning Inc., Germany). The recovery after filtration was evaluated by measuring UV absorbance at 280 nm of the pre-diluted protein solution before and after filtration was 95 ± 1.5%.

2.2. Determination of degree of hydrolysis by 2,4,6-trinitrobenzene sulfonic acid (TNBS) assay

The degree of hydrolysis (d_h) was determined by 2,4,6-trinitrobenzene sulfonic acid (TNBS) method following Adler-Nissen protocol (Adler-Nissen, 1979). Briefly, 10 μL trypsin was injected to 2.2 mL of β-casein solution (0.5% (w/v) in 0.1 M PB) incubated in water batch at 25 °C. Aliquots of reaction mixture (100 μL) were added to small test tubes and treated with 25 μL HCl (1 M) at different time points in order to stop the reaction. The acidified samples were stored at –25 °C. Duplicate aliquots of samples (10 μL, treated β-casein solutions/reaction mixture) were diluted into 140 μL of 1% sodium dodecyl sulfate (SDS) solution (≥98.5% (GC), BioReagent, L3771, Sigma Aldrich). No aliquots of reaction mixture were added in the blank. Meanwhile, standard solutions were prepared by a dilution series of concentrations, 20–250 mg/mL, of L-leucine (reagent grade, ≥98%, L8000, Sigma-Aldrich) in 1% SDS solution. As with the samples, the standards and blank were prepared in appropriate replicates. The blank, reaction sample and standard solutions were further diluted sequentially by adding 1 mL of sodium phosphate buffer (0.21 M, pH 8.2) and followed by 1 mL of 1% (w/v) TNBS (BioReagent, 5% (w/v) stock solution in H_2O, P2297, Sigma Aldrich, Germany). All solutions were mixed prior to incubation at 50 °C for 60 min in covered water bath. The TNBS reaction was stopped by the addition of 2 mL of 0.1 M HCl and was then allowed to cool at room temperature for 30 min. The absorbance readings of blanks and the reaction samples were performed using Cary 60 UV/visible spectrometer at 340 nm wavelength. (Agilent Technologies, Inc., USA), thermally controlled at 25 °C. Leucine calibration curve was constructed by plotting the absorbance readings at 340 nm wavelength with the corresponding L-leucine standard concentration. The slope of the linear calibration plot corresponds to the molar extinction coefficient, ε_{340} = 11507 ± 278 $M^{-1}.cm^{-1}$ (Spellman, McEvoy, O'Cuinn & Fitzgerald, 2003). The molar extinction coefficient was utilized to convert absorbance of trinitrophenyl (TNP)-amino complex into their respective molar concentration. Degree of hydrolysis, d_h, was calculated using the following equation:

$$d_h = \frac{N(t) - N^0}{c_b^0} = \frac{c_{bh}}{c_b^0} \quad (1)$$

where $N(t)$ and N^0 are the concentrations of free amino groups (expressed in $mol.kg^{-1}$) in β-casein at time t and zero, respectively, and they are equivalent to the molar concentrations of the (TNP)-amino complexes; c_b^0 is the concentration of peptide bonds in β-casein before hydrolysis; and c_{bh} is the concentration of peptide bonds hydrolyzed.

2.3. High resolution ultrasonic spectroscopy (HR-US) measurement of enzymatic hydrolysis

The change in ultrasonic parameters, velocity and attenuation, were measured using HR-US 102PT ultrasonic spectrometer (Sonas Technologies Ltd., Ireland), controlled by HR-US software v.4.50.27.35. HR-US consisted of two identical acoustic cavity resonators (2 mL cell volume each) and equipped with precision programmable temperature controller (Julabo FS18, with ± 0.01 °C accuracy and stability). Approximately 2 mL of each solution were degassed in a 10 mL disposable plastic syringe with attached pressurized cap and filtered with 0.2 μm sterile PTFE syringe filter prior to the addition to HR-US cell. One of the HR-US cell (measuring cell) was filled with analyzed protein sample using 1.1 mL precisely calibrated Hamilton syringe equipped with Chaney adapter (Hamilton Company, USA) and the other cell

(reference cell) was filled with the reference solution (PB buffer) of the same volume. The cells were enclosed with screw caps to avoid evaporation and heat jacket was applied above the cap to prevent condensation. After 10 min temperature equilibration, four frequency peaks parameters were analyzed and set into peak settings according to the manufacturer's manual. Ultrasonic measurements were performed at four frequencies: 2.8 MHz, 5 MHz, 8 MHz, and 15.4 MHz. To minimize the effect of temperature fluctuation, the measurement on ultrasonic parameters was performed under the differential measuring regime. The baseline (stability of β-casein) was measured for 30 min, and to activate the reaction, 5 μL of trypsin solution was added to the measuring cell through a rubber septum using precisely calibrated 5 μL Hamilton syringe equipped with Chaney adapter. The sample was immediately stirred using a bottom digitally controlled stirring system for 45 s at 1100 rpm. Each measurement was performed at least two times.

The relative changes of sound velocity were determined from the changes of resonance frequencies, f and f_0, of the analyzed sample and the reference medium by the relationship:

$$a_i = \frac{u - u_0}{u_0 c_i} = \frac{f - f_0}{f_0 c_i}(1 + \gamma) \tag{2}$$

where a_i is the concentration increment of ultrasonic velocity; c_i is the concentration of the analyzed sample; u and u_0 are the ultrasonic velocities of the analyzed sample and of the reference solution, respectively; $\gamma \ll 1$ is a coefficient that can be neglected (Rybar et al., 2007). In addition, the intensity of the ultrasonic signal was small throughout (the pressure amplitude in the ultrasonic wave was less than 10^3 Pa), any effects of the sound wave on the structural properties of the protein were thus avoided.

To achieve better precision, stability measurements of both buffer and casein solution, consecutively, were performed prior to the activation of hydrolysis. The reproducibility of the measurements of the difference in velocity in the measuring and the reference cells was on the level of $\pm\ 2 \times 10^{-4}$ m.s^{-1}. For illustrative purposes, the evolution of the relative change of ultrasonic velocity, $\Delta u (= u(t) - u_0)$ from the time of enzyme addition has been measured continuously and was scaled to zero minutes by performing extrapolation of first few minutes (2 to 3 min) of the ultrasonic velocity data to time zero.

2.4. Hydrolysis kinetic modeling

The following exponential model was used to describe the kinetic curve of trypsin hydrolysis of β-casein at single protein substrate concentration (González-Tello, Camacho & Guadix, 1994,):

$$\frac{d(d_h)}{dt} = ae^{-bt} \tag{3}$$

The d_h denotes the degree of hydrolysis measured experimentally and its time derivative, $\frac{d(d_h)}{dt}$, can be calculated using numerical differentiation method. The parameter $a\left(\equiv\frac{k_h e_0}{c_{bh}}\right)$, where e_0 is the initial concentration of enzyme; k_h is rate constant of peptide bond hydrolysis expressed in min^{-1}; and $b\left(\equiv\frac{k_d}{k_h}\right)$ is the ratio between the rate constant of enzyme inactivation, k_d, assuming that the enzyme denaturation is second-order reaction, over the rate constant of hydrolysis, k_h. More convenient approach was fitting the real-time d_h profile with the logarithmic form of equation (4):

$$d_h(t) = \frac{1}{b}\ln(1 + abt) \tag{4}$$

The model fitting of equation (4) to determine parameter a and b was performed in OriginPro 2018 (OriginLab Corporation, MA, USA). Statistical analysis was performed using the same software.

The temperature dependence of the determined reaction rate constants, k_h and k_d, were modeled with classic Arrhenius equation (5). Following this, determination of activation and deactivation of energy, E_A and E_D, and their corresponding exponential factor A_h and A_d, respectively, was performed more conveniently by linear fitting of $-\ln(a)$ and $-\ln(ab)$, respectively, with inverse temperature, $\frac{1}{T}$, using linearized Arrhenius equation (the two respective equations in equation (6)) (Qi & He, 2006).

$$k_h = A_h e^{-\frac{E_A}{RT}};\ k_d = A_d e^{-\frac{E_D}{RT}} \tag{5}$$

$$-\ln(a) = \frac{E_A}{RT} - A_h;\ -\ln(ab) = \frac{E_D}{RT} - A_d \tag{6}$$

where $R = 8.314$ J.K^{-1}.mol^{-1} is the gas constant. Furthermore, the change in entropy, $\Delta S^{\ddagger}$, and in enthalpy, $\Delta H^{\ddagger}$, of activation were determined by linear fitting of $\ln(\frac{k_h}{T})$ with $\frac{1}{T}$ using the Eyring equation (7) derived from transition state theory (Erying, 1935):

$$\ln(\frac{k_h}{T}) = \ln(\frac{k_B}{h_P}) + \frac{\Delta S^{\ddagger}}{R} - \frac{\Delta H^{\ddagger}}{RT} \tag{7}$$

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \tag{8}$$

where k_B is the Boltzmann constant; h_P is the Planck's constant. All fittings and statistical analyses were carried in OriginPro 2018 (OriginLab Corporation, MA, USA). Following this, the change in Gibb's free energy, $\Delta G^{\ddagger}$ of activation was then calculated using equation (8).

2.5. Statistical analysis

All the ultrasonic and TNBS measurements of trypsin hydrolysis of β-casein were conducted at least in duplicates All the results were presented as the mean ± standard deviation (SD). The statistical analyses of the results were performed by means of ANOVA test with significance level of 0.05 using OriginPro 2018 (OriginLab Corporation, MA, USA).

3. Results and discussions

Fig. 1A shows the kinetics of the changes of ultrasonic velocity following addition of 0.41 μM trypsin into 0.5% (w/v) β-casein in 0.1 M PB, pH 7 at 25 °C, measured at four different frequencies: 2.8 MHz, 5 MHz, 8 MHz, and 15.4 MHz. The increase of the ultrasonic velocity is due to the hydrolysis of β-casein caused by increase of the hydration of the peptide fragments appeared after casein cleavage. It can be seen that the relative changes of ultrasonic velocity are lower at low MHz frequencies. The origin of this effect is the relaxation process associated with the proton transfer between the terminal α-amino group ($R–NH_3^+$ and $R–NH_2$) of protein hydrolysates and the phosphate group ($H_2PO_3^-$ and HPO_3^{2-}). The magnitude of frequency dependence observed are attributed to three interrelated factors: (1) the significant net volume effect of proton transfer reactions, i.e. the sum of volume of ionization of terminal α-amino group and phosphate group; (2) high equilibrium concentration of the participating species (utilization of 0.1 M PB); and (3) the relaxation time of the process which is comparable at 1–2 MHz. The third factor explains the emergence of relaxation contribution to ultrasonic velocity at lower frequency (≤2 MHz) but negligible at higher frequency (≥10 MHz) (Buckin & Altas, 2017). At our 2.8 MHz frequency measurements, the comparable time scale of oscillation of temperature and pressure in ultrasonic waves relative to that of relaxation process triggers perturbations of the equilibrium giving rise to relaxation contribution. In contrast, at 15.4 MHz, the time scale of oscillation of ultrasonic waves is significantly fast relative to the time scale of the proton transfer. In this case, the process is referred to be 'frozen' (Buckin & Altas, 2017). Our ultrasonic velocity profiles of hydrolysis measured at 15.4 MHz should not take account of the relaxation effects but directly attributed to changes in hydration proportional to increasing concentration of peptide bonds hydrolyzed.

Fig. 1B illustrates the kinetics of the changes of ultrasonic velocity

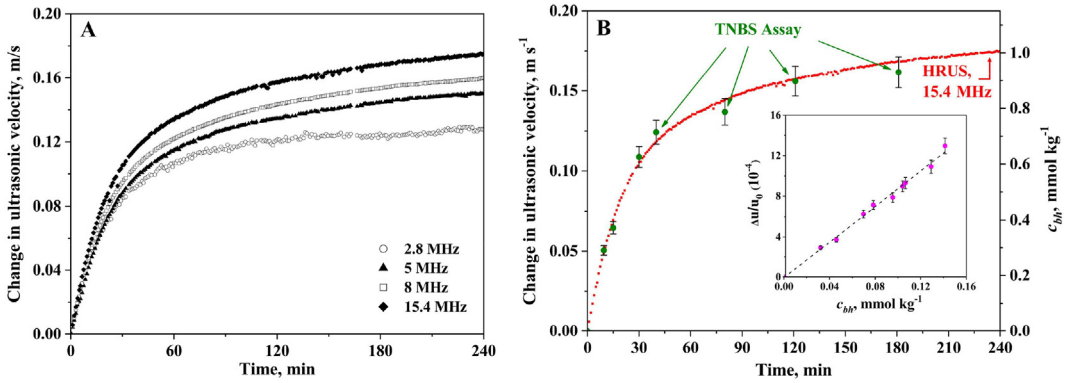

Fig. 1. (A) The kinetics of the changes of ultrasonic velocity following addition of 0.41 μM trypsin into 0.5% (w/v) β-casein in 0.1 M PB, pH 7 at 25 °C, measured at four frequencies: 2.8 MHz, 5 MHz, 8 MHz, and 15.4 MHz. (B) The kinetics of the changes of ultrasonic velocity measured at 15.4 MHz following hydrolysis of β-casein at presence of 0.41 μM trypsin. The black circles represent the results of TNBS assay at corresponding stage of hydrolysis. The error bars are at 0.05 confidence level. The inset is plot of the relative changes of ultrasonic velocity vs. concentration of peptide bonds, c_{bh}, determined by TNBS assay. The slope of the line yields the change in concentration increments of ultrasonic velocity of hydrolysis reaction, Δa_r. The data points represent the mean obtained from 2 separate measurements. The R^2 is 0.993 and the error bars are at 0.05 confidence level.

measured at 15.4 MHz following hydrolysis of β-casein at presence of 0.41 μM trypsin. The black circles represent the results of TNBS assay at corresponding stage of hydrolysis. The inset at Fig. 1B is plot of the relative changes of ultrasonic velocity vs. concentration of peptide bonds, c_{bh}, determined by TNBS assay. It can be seen perfect linear correlation ($R^2 = 0.993$) between ultrasonic velocity changes, expressed as $\frac{\Delta u(t)}{u_0}$ where $\Delta u(t) = u(t) - u^0$ ($u(t)$ and u^0 are ultrasonic velocity at time t and zero, respectively), and c_{bh}. The slope of this dependence ($=0.115 \pm 0.0075$ kg.mol^{-1}) corresponds to the change in concentration increment of ultrasonic velocity of hydrolysis, Δa_r. The y-intercept ($=1.344 \times 10^{-6} \pm 6 \times 10^{-6}$) was small and can be neglected. The error represents the calibration and replicate error at 0.05 confidence level.

The hydrolysis profile displays maximum initial rate of increase in c_{bh} within the first 60 min of the hydrolysis in the presence of high concentration of hydrolysable peptide bonds. This was followed by rate deceleration upon reaching reaction plateau at $d_h = 2.4\%$ (calculated as $\frac{c_{bh}(t)}{c_b^0}$) after 3 h and 20 min. The obtained d_h was equivalent to 5 peptide bonds cleaved in β-casein molecule out of 17 hydrolysable peptide bonds, based on the number Lys-X and Arg-X. No progress of hydrolysis was observed until 6 h. The deceleration of hydrolysis rate is attributed to the scarcity of hydrolysable peptide bonds, low affinity of trypsin to intermediate peptides released, and possibly enzyme inactivation during the hydrolysis. All of these factors are in accordance with the mechanistic description of the empirical kinetic model for hydrolysis of proteins by trypsin established by González-Tello, Camacho & Guadix (1994). Fitting the real-time hydrolysis profile with equation (4) provided two fitting parameters, $a = 1.47 \times 10^{-3} \pm 1 \times 10^{-4}$ min^{-1} and $b = 0.015 \pm 5.2 \times 10^{-4}$ of high precision. Consequently, k_h and k_d were equal to 3197.25 ± 255 min^{-1} and 47.96 ± 3.84 min^{-1}, respectively. The parameter k_h is inversely proportional to the concentration of enzyme. The standard deviation shown was at 0.05 confidence level. The physical interpretation for a was the initial slope of the conversion curve. This was the initial rate of hydrolysis reaction, $\frac{dd_h}{dt}$ (when d_h tends to 0) (Valencia et al., 2018). It is also appropriate to express the initial rate in terms of molar concentration, i.e. $r^0 = c_b^0 \frac{dd_h}{dt}$. On the other hand, parameter b is affected by the enzyme deactivation as well as the variance in enzyme affinity with the intermediate peptides. Both give rise to the characteristic curve (deceleration) at later stage of hydrolysis.

The hydrolysis of protein is accompanied by a change in the intrinsic properties and the interactions with the environment affecting the hydration characteristics of the protein and its hydrolysates. Such changes lead to the change in compressibility and density of the mixture and affect the ultrasonic wave characteristics. In the reaction medium, the terminal α-amino and α-carboxylic groups liberated from hydrolysis subsequently interacts with water molecules and forms higher hydration shells compare to the original neutral amide bond. The water in the hydration shell forms more rigid structure around these atomic groups, thus, the hydration layer is less compressible than of the bulk water. The increase production of these atomic groups results with subsequent hydration which leads to an increase in ultrasonic velocity. The contribution caused by the change in intrinsic properties is relatively small compare to the hydration effects. Thus, the magnitude of change in velocity is mainly determined by the difference in the hydration characteristics and is proportional to the concentration of the bonds hydrolyzed (Altas, Kudryashov & Buckin, 2016; Buckin & Altas, 2017), by equation:

$$\frac{\Delta u(t)}{u_0} = \Delta a_r c_{bh}(t) \tag{9}$$

where Δa_r is the concentration increment of ultrasonic velocity of the reaction (relative change of ultrasonic velocity per unit of concentration of bonds hydrolyzed) defined as $\Delta a_r \left(\equiv \frac{1}{u_0}\frac{du}{dc}\right)$. As a result, rearranging equation (9) into equation (10), $c_{bh}(t)$ can be recalculated from the measured ultrasonic velocity.

$$c_{bh}(t) = \frac{u(t) - u^0}{u_0 \Delta a_r} \tag{10}$$

It is important to note that the precision of ultrasonic measurements of hydrolysis curve can be calculated in concentration of bonds hydrolyzed, according to the following equation:

$$P_{c_{bonds}} = \frac{P_u}{u_0 \Delta a_r} \tag{11}$$

where $P_{c_{bonds}}$ is the precision of ultrasonic measurements in concentration of bonds hydrolyzed and P_u is the precision of differential ultrasonic velocity. Thus, precision of our ultrasonic measurement was 2x10^{-4} m.s^{-1}, which corresponds to 1 μmol.kg^{-1} concentration of peptide bonds hydrolyzed.

The Δa_r serves as the proportionality constant between ultrasonic velocity and c_{bh} represent the difference between the concentration increment of the product, a_P and of the reactant, a_R, of the reaction, i.e. $a_P - a_R$, (Buckin & Altas, 2017). The physical meaning of this parameter is related to the hydration characteristics of intrinsic properties of the atomic groups of the reactants and of the products affected by hydrolysis, described by change in apparent volume and apparent adiabatic compressibility. Molecular interpretation of concentration

increments of ultrasonic velocity of hydrolysis reaction was previously discussed in detail elsewhere (Resa & Buckin, 2011; Altas, Kudryashov & Buckin, 2016). In non-concentrated mixtures, in an absence of specific interactions between the reactants and products, the value of Δa_r is expected to be constant during the reaction, especially if the physicochemical properties of the products are similar to the properties of the reactants.

The reliability of Δa_r depends on the ultrasonic calibration method utilized. Four methods were previously described by Buckin & Altas (2017). The most appropriate method in our case is the "ultrasonic calibration method 2″ where Δa_r is determined by the correlation of measurement of ultrasonic velocity during the hydrolysis reaction with the concentration of peptide bonds hydrolyzed from a discontinuous method (TNBS assay) at different reaction times. TNBS was utilized method as reliable assay for determination of degree of hydrolysis of proteins by proteases. The reaction is based on the nucleophilic aromatic substitution reaction of 2,4,6-trinitrobenzene sulfonic acid with primary amino groups (at *N*-terminal side and lysine side chain groups) which formed 2,4,6-trinitrophenyl (TNP)-amino complex. The complex has local optimum absorbance at 340 nm in which the corresponding concentration was determined using the molar extinction coefficient (ε_{340} = 11507 ± 278 $M^{-1}.cm^{-1}$) calculated from the slope of L-leucine standard curve. The overall calculated relative error of TNBS measurement of concentration of peptide hydrolyzed was not more than 8%.

The observed linear dependence between the ultrasonic velocity and $c_{bh}(t)$ agrees with the proposed kinetic mechanism of tryptic hydrolysis of casein substrates. Hydrolysis of casein is regarded as two-stage process in which during the first stage, called demasking, the initially enzyme-inaccessible peptide bonds are converted into demasked bonds. On the second stage, these demasked bonds are more accessible to the enzyme and subsequently are hydrolyzed in a faster rate. It has been reported that demasking process is the limiting stage of β-casein hydrolysis. The demasking rate constants for tryptic hydrolysis of β-casein are at least one order of magnitude lower than the maximum hydrolysis rate (Vorob'ev, 2009; Vorob'ev, Vogel & Mäntele, 2013). Therefore, polypeptides with intermediate degree of hydrolysis are nearly absent in the reaction mixture, thus the y-intercept of the linearity shall be zero.

Fig. 2A illustrates the effects of temperature on degree of β-casein (0.5% w/v) hydrolysis (d_h) by trypsin (20 nM) in 0.1 M PB, pH 7.4 solution. It can be seen that the d_h value increases with increasing temperature. The real-time degree of hydrolysis profile was recalculated from ultrasonic velocity using equation (10). The Δa_r value non-linearly depends on temperature, and it was calculated as $\Delta a_r = -0.000058\ T^2 + 0.03411\ T - 4.9122$ (R^2 = 0.99, unpublished results), where T is in Kelvin unit. The negative slope indicates lowered hydration effect with temperature due to the breakage of hydrogen-bonded network of the hydration resulting to more relaxed water. The degree of hydrolysis profile shows that both initial rate, r^0, and degree of hydrolysis, d_h, of enzyme activity increased with temperature at the range of 15 °C to 45 °C. The maximum degree of hydrolysis was 6.7% at 45 °C after 10 h and it was approximately 2.5 times higher than the degree of hydrolysis (=2.5%) at 15 °C. Our findings are in accordance with observation by Seabra and Gil (Seabra & Gil, 2007), who studied the effect of temperature on the catalytic properties of trypsin, in free and immobilized forms, towards synthetic substrate. The authors reported the optimal temperature around 45–55 °C of both forms. Above this temperature, trypsin activity tends to decrease due to the enzyme destabilization such as denaturation.

Table 1
The kinetic parameters of trypsin hydrolysis (20 nM) of β-casein (0.5% w/v) in 0.1 M PB at various pH and temperatures determined from exponential model fit, equation (4). The error values shown were at 0.05 confidence level.

Temperature,°C	pH	Initial rate, r^0, (10^{-5}), mol.kg^{-1}.min^{-1}	k_h, min^{-1}	k_d, min^{-1}
15	7.4	0.55 ± 0.014	273.62 ± 7	2.13
25	6	0.273 ± 0.006	136.73 ± 3	1.37
25	7.4	0.96 ± 0.026	482.05 ± 15	1.80
25	8	1.05 ± 0.032	523.07 ± 17	2.28
35	7.4	1.79 ± 0.104	889.14 ± 53	4.14
45	7.4	3.915 ± 0.43	1957 ± 222	8.75

r^0 was calculated as $c_b^0 \frac{dd_h}{dt}$, at $d_h = 0$; k_h was calculated as $\frac{ac_b^0}{e_0}$. The parameter a was obtained from fitting the real-time $d_h(t)$ profile with equation (4) ; k_d was calculated as bk_h. The parameter b was obtained from fitting the real-time $d_h(t)$ profile with equation (4). The standard deviation was below 1%. All determined values were statistically significant according to ANOVA test at the level of 0.05.

The results of the experimental determination of the degree of hydrolysis, d_h, of β-casein by trypsin (Fig. 2A) allowed us to calculate the initial rate of hydrolysis, r^0, as well as the kinetic parameters, k_h and k_d, of enzyme reaction. These values are presented in Table 1. The value r^0 increased exponentially at temperature range 15–45 °C as it can be seen from Fig. S1 (see Supplementary material), thus exhibiting classical Arrhenius behavior. The kinetic parameters, k_h and k_d, were initially determined from parameters a and b at each temperatures using equation (5). To estimate the thermodynamic parameters of trypsin hydrolysis of β-casein at studied range of temperatures, parameter a and b were fitted with linearized Arrhenius model as a function of inverse temperature, $\frac{1}{T}$; equation (6), as shown in Fig. 2B. Since b at 15 °C highly deviated from linear Arrhenius model, only 25–45 °C range were used to estimate E_D. The slope determined the activation and deactivation energy of hydrolysis, E_A = 50.3 ± 7 kJ.mol^{-1} andE_D = 62.23 ± 3 kJ. mol^{-1}, respectively. The y-intercepts

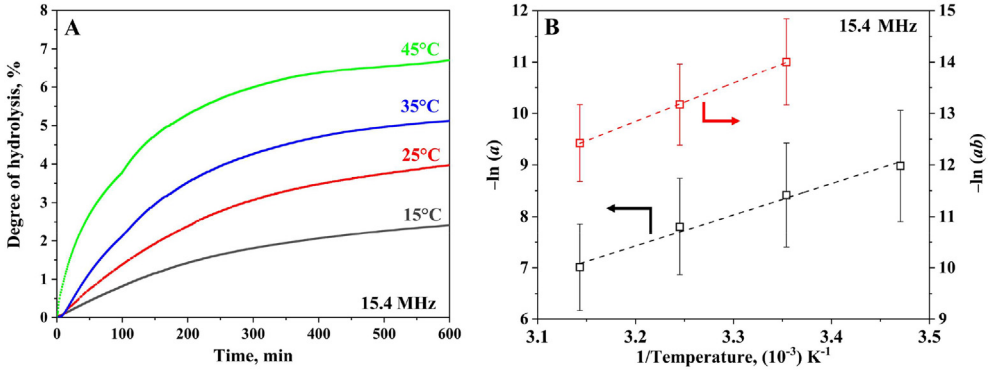

Fig. 2. (A) Real-time degree of hydrolysis, d_h, profiles of β-casein (0.5% w/v) catalyzed by trypsin (20 nM) in 0.1 M PB, pH 7.4 at different incubation temperature-15 °C (gray line), 25 °C (red line), 35 °C (blue line) and 45 °C (green line). The degree of hydrolysis was recalculated from ultrasonic velocity (at 15.4 MHz) from equation (10) using the appropriate change in concentration increment of ultrasonic velocity of reaction, Δa_r, at each temperature. (B) The linearized Arrhenius plot, equation (6), of – ln(a) (black open square, primary y-axis) and – ln(ab) (red open square, secondary y-axis) with respect to reciprocal of temperature, $1/T$. The error bars correspond to 8–13% relative error at 0.05 confidence level and the R^2 is in the range 0.97–0.99. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

corresponds to the frequency factor A_h = 11.9 ± 3.0 and A_d = 11.11 ± 1.5, respectively. The higher value of E_D indicates the higher energy barrier required towards thermal deactivation of trypsin during the hydrolysis. Obtained experimental values are in range of values for tryptic hydrolysis of BSA estimated by the same approach (Qi & He, 2006). Maximova & Trylska, (2015) determined E_A = 35.6 kJ.mol^{-1} of trypsin hydrolysis of β-casein (1:1000 enzyme to substrate molar ratio) in 0.05 M PB (pH 7.5) by discontinuous multiple injection assay using isothermal calorimetry (ITC) at temperature range 20–37 °C. The authors performed the hydrolysis at higher enzyme to substrate molar ratio by ten-fold which resulted to E_A approximately twice lower than our experimental value.

Following this, the changes in entropy, $\Delta S^{\ddagger}$, in enthalpy, $\Delta H^{\ddagger}$, and in Gibb's free energy, $\Delta G^{\ddagger}$ were calculated to be $\Delta S^{\ddagger}$ = –35.2 ± 12 J.K^{-1}.mol^{-1}, $\Delta H^{\ddagger}$ = 47.0 ± 0.81 kJ.mol^{-1} and $\Delta G^{\ddagger}$ = 57.74 ± 4 kJ.mol^{-1}, respectively, using equation (7) and equation (8). The coefficient of determination was R^2 = 0.987. The positive $\Delta H^{\ddagger}$ value confirms endothermic nature of enzymolysis reaction. The negative value of $\Delta S^{\ddagger}$ indicates more orderly distribution of enzymes and reasonable high thermostability of trypsin (Hernández-Martínez, Gutiérrez-Sánchez, Bergmann, & Prado-Barragána et al., 2011; Dabbour, He, Mintah, Tang & Ma, 2018).

The pH dependence of catalytic activity of trypsin on 0.5% (w/v) β-casein in 0.1 M PB at 25 °C was also investigated (Fig. 3). The degree of hydrolysis profile was recalculated from ultrasonic velocity using equation (10) and Δa_r equals to 0.113 ± 0.0015 kg.mol^{-1}, 0.1 ± 0.0015 kg.mol^{-1} and 0.091 ± 0.0015 kg.mol^{-1}, at pH 6, 7.4 and 8, respectively. The pH dependence of Δa_r attributes to the change of hydration caused by the ionization of relevant weak acids (particularly terminal α-amino group, phosphate, histidine and cysteine) determined by their apparent acid dissociation constant, pK_A^{app}. Since all of our hydrolysis reaction were performed at pH within the effective buffering region (pK_A^{app} of the phosphate buffer is 6.87 ± 0.04), there was a minimal pH change during the hydrolysis, particularly at low

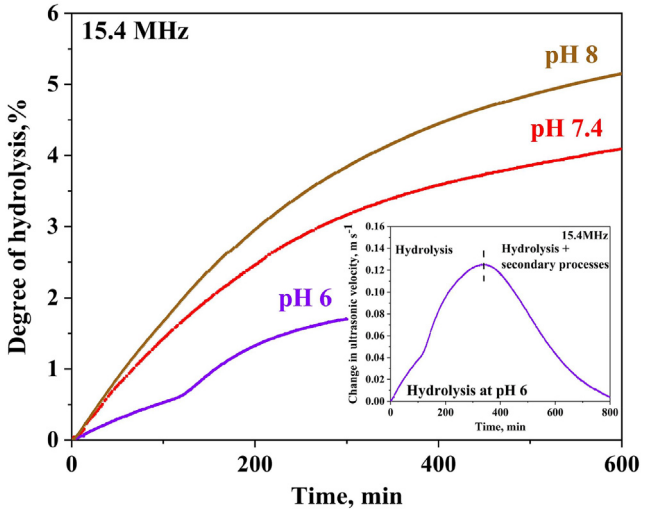

Fig. 3. Real-time degree of hydrolysis profiles of β-casein (0.5% w/v) catalyzed by trypsin (20 nM) performed at pH: 6 (violet line), 7.4 (red line) and 8 (brown line), in 0.1 M PB, 25 °C. The degree of hydrolysis was recalculated from ultrasonic velocity (at 15.4 MHz) from equation (10) using the appropriate change in concentration increment of ultrasonic velocity of reaction, Δa_r, at each pH. In the degree of hydrolysis curve at pH 6, only the time interval where there was an increase of ultrasonic velocity was presented. The insert presents the whole time spectrum of the changes in the ultrasonic velocity of hydrolysis at pH 6. In the later stage of the profile, the decrease in ultrasonic velocity due to the dominant contribution of secondary processes was excluded in the calculation of degree of hydrolysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

degree of hydrolysis. Following this, the estimated variation in Δa_r did not exceed 0.003 kg.mol^{-1} (max of 3%). This is also equivalent to negligible variation in c_{bh}, e.g. ≈ 5 μmol.kg^{-1} at 6th hour of hydrolysis. Therefore, Δa_r was concluded to be constant throughout the hydrolysis. The ionization contributions to Δa_r is the additive sum of the individual change in concentration of increment of ionization of all relevant weak acids which are partially ionized at the working pH.

Fig. 3 shows significantly higher activity of enzyme at pH 7.4–8 than the hydrolysis at pH 6. Similar effect of pH was observed on degree of hydrolysis at pH 8, 7.4 and 6, i.e. 5.1% (at 10th h of hydrolysis), 4.8% (at 10th h of hydrolysis) and 1.73% (after 5.5 h of hydrolysis), respectively. The initial rate of hydrolysis, determined as kinetic parameter a from the exponential model fitting, showed non-linear dependence within the range of experimental pH (Table 1). It is known that the optimal pH of trypsin activity towards protein is around 8. On the other hand, at pH 6, two curvatures are observed in the profile. The initial rate of the first curvature, 0–120th min of hydrolysis, was 2.73 × 10^{-6} ± 3 × 10^{-8} mol.kg^{-1}.min^{-1} and the initial rate of the second curvature, > 120th min (0.6% degree of hydrolysis), was twice higher, 6.24 × 10^{-6} ± 1.7 × 10^{-7} mol.kg^{-1}.min^{-1}. The initial slow hydrolysis of β-casein could be explained by the proteins initial aggregated state and low solubility at pH 6. The succeeding increase in rate may be attributed to transient loss of aggregate and improved solubility. It has been reported that tryptic digestion (up to 7.4% degree of hydrolysis) tends to improve the solubility particularly at pH close to the isoelectric point of the β-casein and its hydrolysates (Chobert, Harb, Dalgalarrondo & Nicolas, 1989). Obtained result suggests that the transition to second curvature occurred after the 1st peptide bond cut (> 0.5% degree of hydrolysis. Vorob'ev et al. reported that first cut takes place between Arg-25 and Ile-26 as the rate constant is the highest among the other specific sites. However, at pH 6, the slow peptide bond cut at this site was expected due to the effect of pH on the kinetic constant of hydrolysis as well as higher degree of masking on this peptide bond (Vorob'ev et al., 2013). Following this, the specific sites within the rest of the fragment (Ile-26 to Val-209) become more accessible to hydrolysis, and subsequently the altered protein is more prone to aggregation (Vorob'ev et al., 2013).

It is evident that pH has significant effects on both intrinsic enzyme activities, i.e. far from the optimal pH, and on the β-casein structure. The pH controls the ionization pattern of the catalytic residues, which undergoes acid-base catalysis, as well as all the electrostatic interactions which defines the integrity of the tertiary structure of enzyme. The pH also significantly affects the interaction properties of β-casein during hydrolysis. The peculiar hydrolysis profile observed at pH 6 displayed an initial increase in ultrasonic velocity (up to 1.7% degree of hydrolysis) was observed and followed by subsequent decrease (Fig. 3 insert). The decrease in velocity is most likely associated with dominant contribution of secondary processes such as formation of large aggregates during trypsinolysis of β-casein. As mentioned before, aggregation arises in the initial stage of proteolysis in parallel with demasking process (Vorob'ev et al., 2013) more significantly at pH closer to the protein's isoelectric point (pI = pH ~ 4.6 for casein). As the pH approach the pI, the caseins negative charges decrease gradually, steric and electrostatic stabilization are diminished and casein micelles induced self-assembly (Corredig, Nair, Li, Eshpari & Zhao, 2019). Another driving force of aggregation is the entropically-driven interactions between hydrophobic regions exposed during the hydrolysis. The presence of large aggregate structures has negative contribution to ultrasonic velocity due to the void volumes and soft, "less compressible" hydrophobic core. In general, there is a contribution from the particle size dependent ultrasonic wave scattering to ultrasonic velocity and attenuation (not shown), particularly at 15.4 MHz where wavelength is more comparable to the size of the aggregate structures. Therefore, presence of large aggregates gives rise to significant error in calculating degree of hydrolysis of β-casein using ultrasonic parameters.

Finally, the hydrolysis of 0.5% (w/v) β-casein in 0.1 M PB at 25 °C

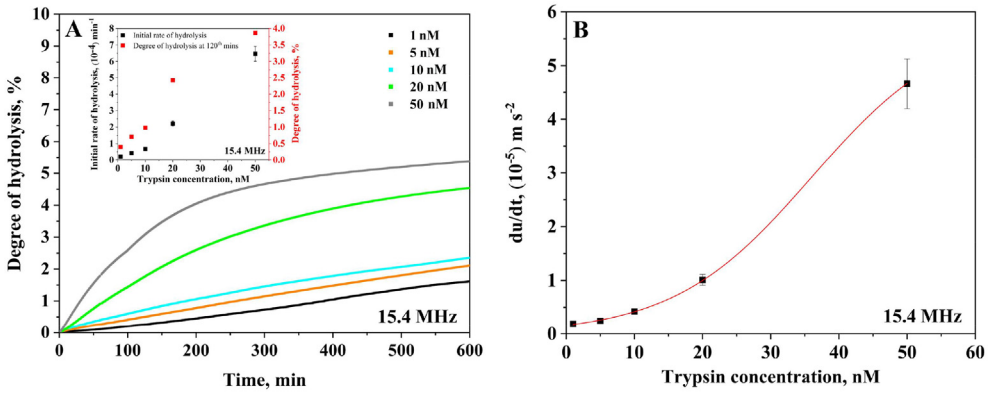

Fig. 4. (A) The effect of enzyme concentration, 1 nM (black line), 5 nM (orange line), 10 nM (light blue line), 20 nM (light green line) and 50 nM (gray line), on the real-time degree of hydrolysis profiles, d_h, of β-casein (0.5% w/v) in 0.1 M PB, pH 7.4, 25 °C. The degree of hydrolysis was recalculated from ultrasonic velocity (measured at 15.4 MHz) from equation (10) using the appropriate change in concentration increment of ultrasonic velocity of reaction, Δa_r, equal to 0.102 kg mol^{-1}. The Δa_r was invariant of enzyme concentration. The insert represents the effect of trypsin concentration on the initial rate (primary y-axis) and degree of hydrolysis taken at 120th minute (secondary y-axis). (B) The calibration plot of the initial rates of increase of ultrasonic velocity, (du/dt), (data at 15.4 MHz) during the hydrolysis of β-casein as a function of the trypsin concentrations at 25 °C in 0.1 M PB, pH 7.4. The data were fitted with logistic function Eq. (12). The data points represent the mean obtained from 3 separate measurements. The error bars shown are at 0.05 confidence level. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

at various trypsin concentrations, ranging from 1 nM to 50 nM has been monitored in real time. The effect of concentration of an enzyme on its activity is one of key factors in the development of enzyme-based formulations. Provided the precision of HR-US technique, it allowed to measure as low as 1 nM trypsin and be possibly able to measure even at sub-nanomolar concentration of trypsin. Fig. 4A illustrates the significant increase in initial rate and degree of hydrolysis with trypsin concentration. The degree of hydrolysis profiles was recalculated from ultrasonic velocity using $\Delta a_r = 0.103$ kg.mol^{-1}. It is interesting to note that, below 10 nM trypsin, the hydrolysis profile displayed a steadily linear curve, and non-linear curve profile was obtained above 10 nM trypsin. Plotting the initial rate of hydrolysis as a function of trypsin concentration, 1–50 nM, revealed the non-linear dependence between the two parameters (Fig. 4A insert). The insert plot also illustrates the effect of concentration of trypsin on the degree of hydrolysis, plotted at 120th minute. It should be noted that unlike the enzymatic hydrolysis of casein at the hydrophobic surfaces of biosensors described in the recent work of Romanszki, Tatarko, Jiao, Keresztes, Hianik & Thompson (2018), trypsin hydrolysis, within the experimental range of concentration, of β-casein in the bulk clearly does not follow inverse Michaelis-Menten kinetics, in which the concentration of the enzyme and the substrate are interchanged. Fig. 4B represent the plot of initial rate of change of ultrasonic velocity, *du/dt* as a function of trypsin concentration, c_{Try} , fitted with logistic function:

$$\frac{du}{dt} = \frac{r^*}{1 + e^{-p(c_{Try} - c_{1/2})}} \tag{12}$$

where r^*, p and $c_{1/2}$ are the fitting parameters corresponds to the upper limit of initial rate ($=6.659 \times 10^{-4} \pm 1.26 \times 10^{-5}$ m.s^{-2}), steepness of the curve ($=1.406 \times 10^{5} \pm 1.1 \times 10^{4}$ nM^{-1}) and concentration at the inflection point ($=24.98 \pm 0.08$ nM), respectively. The error values presented is at 0.05 confidence level.

Overall, the obtained initial reaction rate demonstrated apparently nonlinear character with respect to enzyme concentration indicating that the specific activity of the trypsin depends on its concentration within the analyzed concentration range.

4. Conclusion

The developed ultrasonic methodology for real-time characterization of the number of cuts of peptide bonds produced during hydrolysis of β-casein by trypsin has been demonstrated and applied to assess the trypsin acitivity under different reaction conditions. Compared to TNBS assay, the described ultrasonic method is simpler as it did not require any chemical derivatization to incorporate optical markers. Besides, it provided remarkably high precision of degree of hydrolysis (within the level of 1 μmol.kg^{-1} concentration of peptide bond hydrolysed). The precision ultrasonic profiles of hydrolysis is related to the difference in the hydration characteristics of the atomic groups of reactants and of products affected by the reaction.

Fitting of real-time hydrolysis profiles with exponential model provided both kinetic data and mechanistic description consistent with the intrinsic catalytic property of trypsin towards proteins. The temperature dependence kinetics obeys the classical Arrhenius model of catalysis within the experimental temperature window, 15–45 °C and this allowed determination of thermodynamic variables of hydrolysis. The sensitivity of the measurement to molecular arrangement allowed the detection of the formation of compressible polypeptide aggregates concomitant with the hydrolysis observed at pH 6. Its occurrence limited the quantification of degree of hydrolysis. The dependence of initial rate of hydrolysis on enzyme concentration were empirically fitted with logistic function and inverse Michaelis-Menten approach was not possible in this case. The present calibration plot constructed can be used to fast and real-time detection of trypsin activity even within sub-nanomolar enzyme concentration in volume.

For future work, the results presented in this study can be used as a reference model for ultrasonic analysis of proteolysis of β-casein in more complex media such as milk under wide range of hydrolysis conditions of research and industrial interest.

CRediT authorship contribution statement

Sopio Melikishvili: Investigation, Validation, Formal analysis. **Mark Dizon:** Conceptualization, Investigation, Validation, Writing - review & editing. **Tibor Hianik:** Methodology, Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.127759.

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