Application of high-resolution ultrasonic spectroscopy for detection of the plasmin activity toward β-casein

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ABSTRACT

High-resolution ultrasonic spectroscopy (HR-US) was applied for precise detection of plasmin activity towards β-casein in buffer at pH 7.8 and 37 °C. The evolution of ultrasonic velocity and ultrasonic attenuation measured at 15.5 MHz is related to the concentration of peptide bonds hydrolyzed and loss of β-casein aggregates, respectively. The ultrasonic assay presents sensitive and direct activity-based quantification of plasmin levels in milk. The variation in plasmin concentration between HR-US and ELISA method owed to the differing detection principles. The real-time ultrasonic profiles of hydrolysis were utilized to describe the kinetic aspect of plasmin activity. The non-linear activity curve was fitted with classic and inverse Michaelis–Menten type models. Within 1–8.6 mg·mL⁻¹ β-casein, the V max and K M obtained were (6.30 ± 2.21) × 10⁻⁵ mol·kg⁻¹·min⁻¹ and 10.33 ± 3.50 mg·mL⁻¹, respectively. The maximum peptide bond cleaved was 5–6 (2.7% degree of hydrolysis) achieved at 1 mg·mL⁻¹ β-casein.

1. Introduction

Control of the milk quality is rather important for further processing, e.g. ultra-high temperature (UHT)-treatment and pasteurization. Among the standard milk components that are routinely analyzed in the dairy laboratories are the concentration of proteins, fat and lactose (Dórea, Rosa, Weld & Armentano, 2018). Additionally, determination of the activity of endogenous proteases, particularly plasmin, is also important for processing and controlling the quality of dairy products (Rauh et al., 2014a).

Plasmin (EC 3.4. 21.7) is a highly specific serine protease that is responsible for cleavage of milk proteins, especially β-casein, with a pH optimum of 7.5 at 37 °C (Grufferty & Fox, 1988). Plasmin is a part of a complex system composed of various plasmin activators and inhibitors. It predominantly exists in zymogen form—plasminogen, in blood in which its activation by plasmin-activators (by tissue-type o.r urokinase-type plasminogen activator (Bastian & Brown, 1996)) plays an important role in the regulation of fibrinolysis; and in milk. Plasmin is either infiltrated into the milk before milking or activated in milk during milk storage (Ismail & Nielsen, 2010). While plasmin inhibitors in milk are heat-labile, plasmin is rather a thermostable protein and retain its proteolytic activity even after UHT treatment of milk (Richardson, 1983).

Increased activity of plasmin can be an advantage for cheese producers as it enhances the quality of both milk and milk products. On the other hand, high plasmin activity is not desirable for long term milk storage.

Considering above, the analysis of plasmin activity in milk is rather important. However, due to expensive tests by standard methods, the detection of plasmin is not feasible in the dairy laboratories. Currently, Enzyme-linked Immunosorbent Assay (ELISA) is only one of the commercially available test kits for plasmin detection (Collin, Compagnone, Ryba & Baer, 1988; Dupont, Bailly, Grosclaude & Collin, 1997). It is like other immunoassay technologies which quantify the amount of plasmin specifically and strongly bound to its antibody immobilized in a surface. Optical methods have been also incorporated into traditional enzymatic assay, but their applicability is limited to samples with optical activity (Rauh, Johansen et al., 2014b). Other non-optical alternatives include MALDI-TOF-MS, HPLC or capillary electrophoresis (see Dizon, Tatarko & Hianik (2020) for recent review). But these methods determine only the peptide fragments and not directly the plasmin activity; therefore, they are not suitable for kinetic studies of the peptide cleavage. In addition, the determination of plasmin activity in real milk samples is also complicated because plasmin is partially bound with casein micelles (Ismail & Nielsen, 2010). Furthermore, recently developed methods that provide enough sensitivity for plasmin
Chapter 2

2. Materials and methods

2.1. Reagents

β-casein from bovine milk (Bioultra, Cat. No. C6905-250MG), plasminogen from bovine plasma (Cat. No. P9156-SUN) commercial plasmin from bovine plasma (Cat. No. 10602370001), were purchased from Sigma-Aldrich (Germany). Standard chemicals K$_2$HPO$_4$, KH$_2$PO$_4$, NaCl, KCl, NaOH, and HCl were of p.a. grade and purchased from Slavus (Slovakia). Phosphate buffer (PB) was prepared by mixing 0.1 M K$_2$HPO$_4$ and 0.1 M KH$_2$PO$_4$ until the desired pH 7.8 was reached. pH meter FiveEasy FE20 (Mettler-Toledo AG, Switzerland) was used for pH measurements. Ultrapure deionized water prepared by Pure Lab Classic UV (Elga Water Systems, UK) has been used for the preparation of all solutions. PB was stirred continuously for at least one hour, and the pH was further re-adjusted to 7.8. The PB was filtered using 0.22 µm pore size filters (Millipore, USA).

2.2. β-casein and plasmin sample preparation

Stock solutions of β-casein substrate were prepared in weight percentage (% w/w) by weighing of the powder followed by addition of buffer solution using MS 105 DU semi-micro balance (Mettler-Toledo AG, Switzerland). The samples of commercial plasmin solution were prepared in weight concentration by weighing the suspension followed by the addition of buffer using the same microbalance. The mass balance has a repeatability of ± 0.02 mg corresponding to the uncertainty of mass concentration below 0.0002%. The solutions were left to stir gently using magnetic stirrer overnight at room temperature until the solution was completely dissolved. Dissolution of protein in the buffer medium at pH 7.8 slightly changed the pH. The protein solutions were adjusted to desired pH 7.8 using minimal volume (incremental addition of 2 µL volumes) of concentrated HCl and NaOH.

2.3. Isolation of plasmin from experimentally prepared milk samples

The following milk samples were used: raw milk (Dairy-farm, Mosonmagyaróvár, Hungary), pasteurized milk (Dairy company “Óvártér” Mosonmagyaróvár) and UHT-treated milk (Commercially available “RISKA Zero”, Allöldi Tej Kft. Székesfehérvár, Hungary). In real milk samples the plasmin is partially free and partially associated with casein micelles. Therefore, for analysis of the activity of isolated plasmin by HR-US method, it was necessary to prepare casein-free samples. For this purpose, the milk has been centrifuged in a 15 mL centrifuge tube for 5 min at 3000 rpm using centrifuge EBA-21 (Hettich GmbH & Co. KG, Germany). After centrifugation, the fat layer was removed, and the rest of the milk sample has been filtered using paper filter Whatmann 1 (Cat. No. WHA1001325, Sigma-Aldrich). The pH of the sample was adjusted to 4.6. This pH corresponds to the isoelectric point of casein micelles. Neutral charge of casein micelles facilitates dissociation of plasmin from their surface. The milk proteins tend to precipitate at this pH. The sample was then filtrated using Whatmann grade 42 filter paper (Sigma-Aldrich) to remove the precipitates. The pH of the filtrated milk sample has been then increased to 6.6. The isolation of plasmin was performed as follows: 15 mL of filtered milk sample has been added into the special centrifuge tubes that contained filter for protein separation (Vivaspin 100 k, Vivaproducts, Inc., MA, USA) and centrifuged at 3500 g during 10 min. Approximately 0.5–1 mL (the final volume depended on the type and quality of used milk) of the sample filtrate solution containing plasmin was collected at the bottom of the
tube and this was used in the experiments without further treatment (see Benfeldt, Larsen, Rasmussen, Andreasen & Petersen (1995) for more details).

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

Ultrasonic velocity and attenuation during hydrolysis were measured using HR-US 102PT ultrasonic spectrometer (Sonas Technologies Ltd., Ireland) equipped with precision temperature controller Lauda RK 8 CS (Germany) with ± 0.02 ◦C stability set to 37 ◦C. HR-US 102PT comprises two identical 1.5 mL quartz cells, which were used in a differential regime to minimize the effects of thermal fluctuations on the ultrasonic parameters. The cells were cleaned sequentially with liquid detergent, leaving overnight, and washed with MilliQ water and propa

ol. The cells were dried with a flow of air for 15 min. Prior to the measurements all samples were degassed through syringe method at room temperature with attached pressurized cap. One of the cells was filled with 1.1 mL of freshly prepared β-casein solution using pre-calibrated 1.1 mL Hamilton syringe fitted with precision volume Chan

ey adapter and the other cell was filled with buffer solution. The cells were left for 10 min for the temperature to equilibrate. Heat jacket was applied on the top of the surface of the cell at 50 ◦C to prevent condensation. In the measurement settings, the parameters of four frequency peaks found at 2.4 MHz, 5 MHz, 8 MHz and 15.5 MHz, respectively, were analysed and set for the measurements. The following reference values of ultrasonic velocity, and ultrasonic attenuation, used at 37 ◦C were 1523.65 m.s⁻¹ and 1.63 × 10⁻¹⁴ s²·m⁻¹, respectively. Upon initiation of the kinetic measurement and prior to the activation of hydrolysis, the stability of both buffer and protein solution under the ultrasonic measurement was monitored to achieve better precision. When the baseline curve reached stability, the hydrolysis was activated by adding 5 µL of freshly prepared solution of plasmin using pre-calibrated 5 µL Hamilton syringe to the measuring cell through a rubber septum. The sample was immediately stirred using a bottom digitally controlled stirring system for 1 min. at 1100 rpm. Ultrasonic measurements were repeated in duplicates to ensure the reproducibility of the results.

The measurement of the relative changes of sound velocity was determined from the changes of resonance frequencies, and f0 of the analysed sample and the reference medium, respectively, by the relation:

\[ a_i = \frac{u - u_0}{u_0 c_i} = \frac{f - f_0}{f_0 c (1 + \gamma)} \]  

(1)

where \( a_i \) is the concentration increment of ultrasonic velocity; \( c \) is the concentration of the analysed sample; \( u \) and \( u_0 \) are the ultrasonic velocities of the analysed sample and the reference, respectively; \( \gamma \) is a coefficient which is equal to \( \leq 1 \) and 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 10³ Pa), thus any effects of the sound wave on the structural properties of the protein were avoided. For illustrative purposes, the evolution of the relative change of ultrasonic velocity, \( \Delta u_i = u - u_0 \) 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.

2.5. Enzyme-linked immunosorbent assay (ELISA)

ELISA is a very sensitive immunochemical technique used for detection and quantification of specific analyte (antigen or antibody) in the given sample. It is also called a solid-phase enzyme immunoassay because it employs an enzyme-linked antigen or antibody as a marker for the detection of the analyte of interest. In the ELISA, an antigen must be immobilized on a solid surface and then complexed with an antibody that is linked to an enzyme. Detection is accomplished by assessing the conjugated enzyme activity via incubation with a substrate to produce a measurable product. In the experiments, a strip plate of quantitative sandwich ELISA kit for analyzing the presence of the plasmin in a milk was used. The theoretical kit detection range was 31.2–1000 ng.mL⁻¹ (0.367–11.76 nM). The estimated sensitivity of plasmin detection in biological samples was 5.0 ng.mL⁻¹. Purified bovine plasmin antibody was used to coat Microelisa strip plate wells. Following this, the plasmin and antiplasmin antibodies that have been labeled with horseradish peroxidase (HRP) were added into the wells. The reactants become antibody-antigen-antibody-enzyme complex. After washing the well completely, the 3,3′,5,5′-Tetramethylbenzidine (TMB) (Sigma-Aldrich) substrate solution was added. TMB becomes blue under HRP enzyme-catalyzed reaction. This reaction was terminated by addition of sulphuric acid solution (Garcia et al., 1990). In this assay, the enzyme-conjugated with an antibody reacts with a colorless substrate to generate a colored reaction product. The color change was measured spectrophotometrically at a wavelength of 450 nm. The concentration of plasmin in the samples was determined by comparing the optical density (O.D.) of the samples to the standard curve. Limit of detection for this analysis was 121.98 µM (10.37 ng.mL⁻¹). Absorbance was measured using BioTek microplate reader (Vermont, USA). Quantitative sandwich ELISA kit MBS030128 (MyBioSource, San Diego, USA) was used for determination of the level of the plasmin in raw milk, pasteurized milk and UHT milk.

2.6. Statistical analysis

All the ultrasonic and ELISA measurements of plasmin 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

3.1. Ultrasonic velocity and attenuation profile

Evolution of ultrasonic parameters, ultrasonic velocity and ultrasonic attenuation, during the plasmin hydrolysis of β-casein, was monitored in real-time using the HR-US spectrometer. The cleavage of peptide bonds catalyzed by proteases in the presence of water released two atomic groups – carboxylate group, –COO⁻, and amino group, –NH₃⁺:

\[ R₁ – C(O)NH – R₂ + H₂O → R₁ – C(O)O⁻ + NH₃⁺ – R₂ \]  

(2)

In addition to Eq. (2) the proteolysis is also accompanied by a change in the intrinsic properties of proteins (i.e. loss of aggregate structure), and a change in peptide interactions with the medium, particularly solvation effects (hydration in aqueous solutions). The terminal α-amino and α-carboxylic groups liberated from hydrolysis subsequently interacts with water molecules and forms higher hydration shells than the original neutral amide bond. Hydration shell constitutes a rigid less ‘compressible’ network of water molecules surrounding these atomic group than in the bulk phase. The hydration effect is the main attribute to the decrease in compressibility during hydrolysis which is precisely measured as an increase in ultrasonic velocity presented in Fig. 1A. The magnitude of change in velocity presented is mainly determined by the difference in the hydration characteristics and is proportional to the concentration of the bonds hydrolyzed (Buckin & Altas, 2017), by Eq. (3):

\[ \frac{u - u_0}{u_0} = \Delta a_{Ch_0} \]  

(3)
where $\Delta \alpha$ is the change in concentration increment of ultrasonic velocity during the hydrolysis, and it is the sum of partial contributions $\Delta \alpha_P$ and $\Delta \alpha_\beta$. The value $\Delta \alpha$ is the change in concentration increment of ultrasonic velocity of hydrolysis of one peptide bond in a protein. Whereas, the $\Delta \alpha$ is the change of hydration due to the ionization of titratable atomic groups (end terminal groups, polar side chain groups and buffers) present in the hydrolysis mixture. The $\Delta \alpha$ is usually determined experimentally using ultrasonic calibration methods and theoretical calculations previously outlined by Buckin & Altas (2017). In the ultrasonic calibration method, the $\Delta \alpha$ corresponds to the slope of the change of ultrasonic velocity of hydrolysis and concentration of peptide bond hydrolysed measured by TNBS method (see Adler-Nissen (1979) for the TNBS assay method for determination of the degree of hydrolysis). In this study, since plasmin and trypsin almost possess the same specific cleavage sites in $\beta$-casein and the $\Delta \alpha$ is independent on any peptide bond hydrolysis, $\Delta \alpha_P = 0.127 \pm 0.003$ kg.mol$^{-1}$ (corrected to 37°C (Melikishvili, Dizon & Hianik, 2021)) of trypsin hydrolysis of $\beta$-casein, with the inclusion of the ionization correction, $\Delta \alpha = -0.052 \pm 0.003$ kg.mol$^{-1}$ was used to predict $\Delta \alpha$, 0.075 $\pm 0.003$ kg.mol$^{-1}$, for the hydrolysis of $\beta$-casein by plasmin in 0.1 M phosphate buffer pH 7.8 at 37°C. The value of $\Delta \alpha$ is expected to be constant during the reaction under the case of (1) non-concentrated mixtures, where there is an absence of specific interactions between the reactants and products especially if the physico-chemical properties of the products are similar to the properties of the reactants; and (2) buffered reaction, where there are no significant pH changes during the hydrolysis as the changes in ionization of relevant atomic groups significantly affects hydration.

Proteins (or polypeptides) constitutes various acids (i.e. side chains and terminal atomic group). The ionization (protonation/deprotonation) of these weak acids is accompanied with a change in hydration i.e. proton transfer, in which equilibrium process could be perturbed by the ultrasonic wave, and give rise to excess in ultrasonic attenuation caused by ultrasonic energy dissipation into the system (Buckin & Altas, 2017). Concomitantly, the contribution to ultrasonic velocity arises from the change of the compressibility and volume of the mixture as the distribution between the two different states (protonated and deprotonated states) shifts ($\beta$). The ultrasonic profiles of hydrolysis of 8.64 mg mL$^{-1}$ $\beta$-casein catalysed by 5 nM plasmin in 0.1 M PB pH 7.8 at 37°C measured at 2.4 MHz, 5 MHz, 8 MHz and 15.5 MHz are presented in Fig. 1. The amplitude of the change of relative ultrasonic parameters showed greater frequency dependence at frequencies below 8 MHz and became independent at higher frequency. It can be seen from the figure that the relaxation effect gives rise to a decrease in amplitude of change of ultrasonic velocity (Fig. 1A), and high excess of change of ultrasonic attenuation (Fig. 1B) which is maximal around 2.4 MHz region. This observation is in accordance with the theoretical framework of the relaxation process in both homogenous and heterogeneous solution (Nachman et al., 1990). It also outlines the relationship between the compressibility and ultrasonic attenuation. Furthermore, the origin of frequency dependence below 8 MHz is the fast kinetics of the relaxation process, i.e. proton transfer, between the terminal $\alpha$-amino group (R = $NH_2$ with apparent pKa of 7.68 $\pm$ 0.03) of the protein hydrolysates and the phosphate group ($HPO_4^{2-}$, with apparent pKa of 6.88 $\pm$ 0.02). A similar phenomenon was also reported previously by Melikishvili, Dizon & Hianik (2021) in the ultrasonic profile of hydrolysis $\beta$-casein by trypsin in 0.1 M phosphate buffer pH 7. In homogenous and non-concentrated protein solution, ultrasonic relaxation phenomenon in hydrolysis system may only be significant when: (1) the ultrasonic frequency applied is close to the relaxation frequency (0.5–3 MHz) (Strom-Jensen & Dunn, 1984; Cho, Leung, Mok & Choy, 1985); (2) there is a high equilibrium concentration of protonation and deprotonation between weak acids; and (3) there is a significant volume change of proton transfer between the two participating atomic groups (Jürgens & Baumann, 1985). The first mentioned factor is explained by the comparable time frame between the relaxation time of proton transfer and the low ultrasonic frequency. Under this case, this shall allow the ultrasonic wave perturbation and energy transfer. At higher ultrasonic frequency range, the relaxation contribution becomes negligible because the characteristic time of proton transfer is relatively very slow; and therefore, the fast oscillation of ultrasonic wave perceived the process to be ‘frozen’ (Buckin & Altas, 2017). On the other hand, in the presence of phosphate ions, the solution exhibited unusually high ultrasonic absorbance due to significant volume change between the proton transfer reaction of amino groups of peptides and phosphate ions. If the phosphate is to be replaced by another buffer such as Tris, the relaxation effect becomes negligible due to smaller volume change of proton transfer between Tris ions and the terminal amino group of the peptides (Jürgens & Baumann, 1985). Furthermore, the emergence of the relaxation contribution is also known to be only substantial at pH close to the pKa of participating atomic groups due to high equilibrium concentration. This explains the high ultrasonic attenuation of native protein solution in water at high pH (high concentration of hydroxyl ions) and low pH (high concentration of hydrogen ions), particularly close to the pKa of acidic and basic side chains (Cho, Leung, Mok & Choy, 1985). On the other hand, the presence of phosphate buffer resonates this effect with the terminal $\alpha$-amino at pH 6.5–7.5. Therefore,
the substantial equilibrium concentration of protonated/deprotonated states, together with high volume effect of proton transfer between these two atomic groups, give rise to the increase of relative ultrasonic attenuation at pH 7.8, proportional to the concentration of terminal amino groups. On the other case, side-chains and terminal carboxyl group in which pKa is relatively far from the pH of hydrolysis shall present little or negligible contribution due to low equilibrium concentration of protonated/deprotonated states as well as low volume change of the reaction. Overall, the experimental results obtained are in accordance with the above-mentioned explanations and theory where the relaxation effect is maximal at 2.4 MHz. The emergence of relaxation effect is highly selective to an ultrasonic frequency close to relaxation frequency, and high concentration equilibrium of species under highly absorbing solution.

The small decrease in relative change of ultrasonic attenuation at 8 MHz and 15.5 MHz indicated the absence of relaxation process but rather was attributed by the decrease in the amplitude of thermal and shear wave scattering associated with the reduction of particle size of \( \beta \)-casein during the hydrolysis. Such scattering has resulted from the ultrasonic wave-particle interaction which is dependent on the frequency, particle radius and volume fraction (Bryant, & McClements, 1999). In general, the wave scattering phenomenon is substantial if the particle radius is comparable with the wavelength. In our case, the size of the \( \beta \)-casein micelle (10–20 nm, (O’Connell, Grinberg & de Kruijf, 2003)) is much smaller than the wavelengths of ultrasonic waves, within the frequency range of HR-US, thus the ultrasonic wave scattering contribution shall be negligibly small. However, a portion of ultrasonic energy is converted and scattered in other forms of energy measured as thermal and shear wave scattering because their wavelengths, ranging from 0.05 to 1 \( \mu \)m, are comparable with the \( \beta \)-casein micelle size. In addition, these secondary ultrasonic waves were also affected by the thermophysical properties of the continuous and dispersed phase (Bryant, & McClements, 1999). These thermophysical properties are also important on determining the effect of particle size on ultrasonic velocity as a function of frequency and volume fraction. Particularly, the effect is originated from the contribution of heat exchange (thermal scattering or heat dissipation) on the interface between the proteins and the medium to compressibility under oscillating temperature and pressure. In general, the scattering at our ultrasonic frequency range shall appear minimal in a non-concentration homogenous solution of \( \beta \)-casein. Therefore, by estimation based on the level of excess attenuation obtained at 15.5 MHz as well as the level of concentration and homogeneity of the reaction mixture, the contribution of the loss of \( \beta \)-casein micelle during hydrolysis to ultrasonic velocity shall be approximately small and can be neglected. Finally, the preferred frequency range for the direct ultrasonic velocity measurement of the number of peptide bonds hydrolyzed, excluding relaxation and scattering effects, is 8–20 MHz.

3.2. Effect of plasmin concentration

The effect of enzyme concentration on its activity is one of the key factors in the developments of assay methods for enzyme detection and activity characterization as well as enzyme-based formulations. In this study, the range of concentration of plasmin utilized covers the levels of plasmin naturally present in raw milks as well as the amounts used in dairy-related processes. Fig. 2A presents the real-time ultrasonic profiles of the hydrolysis of \( \beta \)-casein catalysed by different concentrations of plasmin (0.2–25 nM). The data presented was the average taken from two measurements performed. Provided the high precision HR-US measurement of peptide bonds hydrolysed (Melikishvili, Dizon & Hianik, 2021), and the level of ultrasonic velocity (0.03 m.s\(^{-1}\) at 1st hour) signal detected at 0.2 nM plasmin hydrolysis, detection of plasmin activity towards \( \beta \)-casein as low as 0.05 nM (equivalent to 4.25 ng.mL\(^{-1}\)) concentration is assumed to be possible. This offers evidence of remarkably high sensitivity of HR-US method assay in volume that is comparable to those based on surface acoustic assay. However, longer incubation time may be needed to accumulate the desired signal and to increase the limit of detection. Nonetheless, the limit of detection was not reported in this study as no further hydrolysis reaction was measured at lower concentrations.

It is evident that plasmin concentration enhanced both the initial rate and amplitude of change of ultrasonic velocity. For our more detailed kinetic analysis, ultrasonic data were re-calculated into more useful bioinformation such as the concentration of peptide bonds hydrolyzed, \( c_{bh} \), and degree of hydrolysis, \( d_h \), i.e. ratio between \( c_{bh} \) and the total concentration of peptide bonds, \( c_{PT} \), by ultrasonic calibration method as described above. Given the substrate concentration of 0.5% w/w of \( \beta \)-casein, the \( c_{PT} \) is calculated to be 0.044 ± 0.001 mol.L\(^{-1}\). Fig. 2B illustrates the re-calculated \( d_h \) profiles overlapped with the ultrasonic velocity data. The \( d_h \) varied within 0.69–1.77% (±0.08%), taken at 1st hour of hydrolysis, from highest to lowest concentration of

![Fig. 2](image-url)
plasmin, respectively. The range of $d_{\text{h}}$ was equivalent to 1–4 peptide bond cleaved by plasmin; taking the total number of peptide bonds (208) in the $\beta$-casein monomer. The number of peptide bonds cleaved was in accordance with the number of main cleavage sites in $\beta$-casein – Lys$_{292}$-Lys$_{292}$, Lys$_{910}$-His$_{910}$, and Lys$_{107}$-Glu$_{106}$, mainly observed in milk; as well as Lys$_{113}$-Tyr$_{114}$ and Lys$_{913}$-Asp$_{184}$ which has also been observed in hydrolysis in vitro (buffer) solution (Fox, 2003).

The effect of enzyme concentration on its activity is commonly assessed by determining the initial rate of hydrolysis, expressed in terms of rate of change in ultrasonic velocity, from the linear part or steepest portion of reaction curve (within the first 15–20 min of the hydrolysis) at each plasmin concentration. Fig. 2B presents the calibration plot of the relative change in ultrasonic velocity derivative, $\frac{\Delta v}{\Delta t}$, as a function of plasmin concentration. The experimental points were fitted with a simple sigmoidal function,

$$\frac{dv}{dt} = v_{\text{max}} - c_{\text{pl}} - c_{\text{L}}$$

(4)

where $v_{\text{max}}$ is the maximum rate of change; and $c_{\text{pl}}$ and $c_{\text{L}}$ are the initial plasmin concentration and the half-maximal effective concentration of plasmin, respectively. By performing non-linear regression analysis, following fitting parameters yielded $v_{\text{max}} = 0.0035 \pm 3.48 \times 10^{-4}$ m.$\text{s}^{-1}$.min$^{-1}$ and $c_{\text{L}} = 1.06 \pm 0.39$ nM. The initial reaction rate curve demonstrated that specific plasmin activity in the bulk is dependent on its concentration with the analyzed range. Eq. (4) represents similar function used in inverse Michaelis-Menten model of enzyme activity where the concentration of the enzyme and the substrate can be interchanged under substantial magnitude of $E_{0}/S_{0}$ (enzyme to substrate ratio) ratio (Kargi, 2009). A similar approach by Romanzski et al. (2018) was used to describe the plasmin activity at different plasmin concentration towards $\beta$-casein immobilized on the surface measured using electromagnetic piezoelectric acoustic sensor (EMPAS). The authors reported the non-linear dependence of plasmin activity with the enzyme concentration that was fitted with Eq. (4) yielding $c_{\text{L}} = 3.292$ nM. The lower value of $c_{\text{L}}$ obtained in our study can be explained by the higher plasmin activity at optimal pH (7.8) and temperature (37 °C) (Kelly, O’Flaherty & Fox, 2006).

### 3.3. Determination of isolated plasmin concentration

The comparison of the kinetic activity of three isolated plasmin to 5 mg.mL$^{-1}$ $\beta$-casein in 0.1 M PB pH 7.8 at 37 °C is presented in Fig. 3. The concentration of each plasmin isolated from three different sources of milk – raw, pasteurized and UHT-treated samples, was quantified using the standard activity curve constructed above (Fig. 2B). The initial rate of hydrolysis of $\beta$-casein by isolated plasmin was determined from the linear initial part of the hydrolysis curve. The initial slope, $v_{\text{0}}$, was used to re-calculate the concentration of plasmin,

$$c_{\text{pl}} = \frac{v_{\text{0}}}{v_{\text{max}} - c_{\text{L}}}$$

(re-arranged from Eq. (4)). Since the injection of isolated plasmin into the $\beta$-casein solution further diluted the enzyme by 10 times, dilution correction was applied in the re-calculation of plasmin concentration. The plasmin concentrations from the three milk samples were listed in Table 1.

The results obtained for non-treated raw milk were consistent with the concentration of plasmin present in milk, ranging within 1.65–8.53 nM (Dacres, Wang, Anderson & Trowell, 2019; Richardson, 1983) measured using activity-based assay employing fluorogenic substrate. The plasmin concentrations in pasteurized and UHT samples were both at the same level which was three times lower than in raw milk. It was suggested that pasteurization shall not affect the plasmin activity due to its expected relatively high heat stability under the process (Ismail & Nielsen, 2010). Instead, plasmin isolated from pasteurized milk might have been survived under the heating process, and it may also display higher activity than the one isolated from raw milk due to the thermal inactivation of plasminogen inhibitor under pasteurization (Richardson, 1983). This was not observed in this study since the plasmin concentration from pasteurized milk used might have been possibly reduced by the skimming treatment. The high plasmin concentration in raw milk indicates that the skimming treatment does not affect the samples (Rauh, Johansen et al., 2014b). Overall, the variation of concentration of active plasmin in raw milk and heat-treated milk samples is mainly attributed to the effect of the sample treatment directly to the plasmin, or plasmin activators or inhibitors.

#### 3.3.1. Comparison with ELISA assay

Our HR-US results were compared with the enzyme-linked immunosorbent assay (ELISA) of determining plasmin concentration. The purpose of the study was to differentiate the activity-based assay by HR-US and the immune-affinity based assay by ELISA. It has been known that ELISA presents one of the most sensitive plasmin assays with a detection limit of 0.03 nM (5 ng.mL$^{-1}$) plasmin (Dupont, Bailly, Grosclaude & Collin, 1997). The concentrations of plasmin isolated from three milk samples derived from ELISA assay are presented in Table 1. It was observed that the differences in concentration between the isolated plasmin from milk samples quantified by ELISA were insignificant. On the other hand, the ultrasonic assay quantified the highest plasmin concentration observed in raw milk, while at pasteurized and UHT milk the concentrations were substantially lower even with those determined by ELISA. The variations in these results were owed to the difference in measurements principles and methodology. ELISA method directly quantifies the amount of plasmin but might not be able to differentiate...
between active (only those plasmin molecules that are involved in the cleavage of the β-casein) and inactive enzymes. Certainly, plasmin is partially free in milk system as some are bounded to the surface of casein micelles. Such binding characteristic illustrates the likelihood of the enzyme to interact with plasmin specific antibodies immobilized at ELISA plate. In addition, the immunoassay, in some cases, present resistance to inhibitory and interfering compounds. On the other hand, activity-based assays are largely affected by reaction mixture composition and sample preparation in use. Collin et al. (1988) reported the higher plasmin concentration quantified by ELISA method than fluorometric assay employing synthetic substrate due to the inhibition effect partially free in milk system as some are bounded to the surface of casein between active (only those plasmin molecules that are involved in the production in the dairy industry. For example, milk containing high crucial for the analysis of the usefulness of the milk for a certain type of lower plasmin activity is more appropriate for sweet milk drinks or other products.

3.4. Effect of β-casein concentration

The effect of β-casein concentration, within the concentration range of 1–8.64 mg.mL⁻¹ on the activity of plasmin at a constant concentration of 5 nM in 0.1 M PB pH 7.8 at 37 °C was investigated. Fig. 4A illustrates the enhancement of both initial rate, $d\Delta\alpha/dt$, and amplitude of relative change of velocity increased with substrate concentration. Likewise, the initial rate of hydrolysis was determined using the similar approach as discussed above. The corresponding $c_{eb}$ and $d\alpha$ were recalculated from ultrasonic velocity profile using Eq. (3). The calculated $d\alpha$ exhibited inverse proportionality with the initial β-casein concentration. At the highest concentration of β-casein (8.64 mg.mL⁻¹), the $d\alpha$ was 0.92% (equivalent to 2 peptide bond cleaved) obtained at 120th min.; whereas, at 1 mg.mL⁻¹ β-casein, the $d\alpha$ was 2.7% (equivalent to 5–6 peptide bonds cleaved). Such negative effect of substrate concentration on $d\alpha$ is generally observed in proteolysis of milk proteins owing to the competitive substrate inhibition.

It is important to note that the varying the concentration of non-globular proteins, such as β-casein, influences its aggregation state. In this case, the initial enzyme affinity and accessibility might become limited at high aggregation number of β-casein micelle due to masking of cleavage sites. The effect of concentration on β-casein association was previously studied using light scattering technique (O Connell, Grimberg & de Kruif, 2003), Huang, Tai & Kegeles (1984) reported CMC of β-casein to be 0.46% (w/v) in phosphate buffer (ionic strength of 0.2) at 25 °C. Furthermore, increasing the temperature induces the β-casein aggregation through hydrophobic interactions and yielding a more compact aggregate structure. It was predicted, using the results of Portnaya et al. (2006) on the effect of temperature and concentration and of Sinaga et al. (2017) on the effect of pH, that the CMC of β-casein at our hydrolytic condition is within 0.2–0.25% (w/w). Thus, this explains the hydrolysis of all the main cleavage sites at 0.1% (w/w) β-casein concentration. The size of the aggregate structure may have the additional contribution to $\Delta\alpha$. The formation of compressible void volumes and soft hydrophobic cores in the aggregate structures increases the compressibility changes affecting the ultrasonic velocity. With our ultrasonic attenuation measurement (e.g. Fig. 1B) at 15.5 MHz, it was estimated that the scattering contribution at changing β-casein aggregate size to $\Delta\alpha$ is within the level of coefficient’ experimental uncertainty, $\Delta\alpha$. Therefore, the constant $\Delta\alpha$ was used in the calculation.

3.5. Kinetic aspects of hydrolysis

The time profile of hydrolysis presented in Fig. 4A displays a high rate of increase in velocity within the initial stage of hydrolysis due to the presence of high substrate concentration. The hydrolysis curve was followed by deceleration until steady slow increase was observed. The characteristic time curve of hydrolysis is in accordance with the exponential model of enzymatic hydrolysis (González-Tello, Camacho, Jurado, Páez & Guadix, 1994). The key features of the model is the
empirical description of the hydrolysis rate deceleration by these three factors – decrease in substrate concentration ruled by the principle of law of mass action; substrate inhibition of plasmin by intermediate peptides; and possibly time-dependent enzyme destabilization during the hydrolysis. Substrate inhibition is responsible for the lower $d_0$ obtained at higher concentration of protein substrate. The exponential model was previously applied to provide both kinetic and mechanistic description of trypsin hydrolysis of β-casein at different pH and temperature (Melikishvili, Dizon & Hišnak, 2021). The real-time profiles acquired signifies suitability for fitting and verification of the kinetic and mechanistic aspect of the model.

The effect of substrate concentration on plasmin hydrolysis also tends to follow the conventional Michaelis-Menten behavior (Fig. 4B). The kinetic model parameters, Michaelis-Menten constant ($K_M$) and maximum rate ($V_{max}$) of peptide bond (as the substrate) hydrolysis were determined by performing non-linear least-square fitting method with Eq. (5):

$$v = \frac{d_0 V_{max} c_P}{c_P + K_M}$$

where $c_P$ is the concentration of the protein substrate. The maximum initial rate, $V_{max} = \frac{V_{max}}{1}$ expressed in molal concentration; and $K_M$ were calculated to be $(6.30 \pm 2.21) \times 10^{-5} \text{mol} \cdot \text{L}^{-1}$ and $(10.33 \pm 3.50) \times 10^{-5} \text{mol} \cdot \text{L}^{-1}$, respectively. Our experimental value of $K_M$ at pH 7.8 and 37°C was even assumingly higher than those obtained for plasmin hydrolysis of a synthetic substrate at 25°C (Wiman & Colleen, 1978). The $K_M$ is expected to be relatively lower at high range of plasmin concentration used and when synthetic substrate (small molecules) is used due to higher enzyme affinity than towards the sterically hindered peptide bonds in proteins. Furthermore, the $V_{max}$ (where $k_{cat} = \frac{V_{max}}{c_P}$) provides catalytic efficacy of plasmin during hydrolysis of β-casein and was calculated to be $428 \text{L} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$. Our experimental value was much lower than the value for the plasmin cleavage of a ferrocenyl peptide substrate (FcPS) in homogenous solution (Ohtsuka, Maekawa, Waki & Takenaka, 2009).

Both the exponential and Michaelis-Menten model discussed above may provide an inadequate representation of the actual enzymatic mechanism since the several transition steps during the hydrolysis may have different enzyme affinity. The model approach to hydrolysis of proteins is based specifically on the assumptions that all peptide bonds are hydrolysed with the same kinetic constants and can be freely attacked by proteases. Alternatively, a more complex mechanism of proteolytic hydrolysis of β-casein was previously described to undergo two-step stage – demasking and hydrolysis steps (Vorob’ev, 2009). β-casein is known to be an amphiphilic structure-unordered protein. However, in solutions, it normally exists as in the form of casein micelles or aggregates which yields limited openness and accessibility of the enzyme to the peptide bonds, i.e. demasked state. Demasking is a proteolytic process that results in the degradation of aggregates and structural opening to expose peptide bonds making them accessible for enzyme attack, thus, a transition from masked to demasked state. It is often the rate-limiting step for the hydrolysis of globular or aggregated proteins. Once demasked, the process proceeds to further hydrolysis of the remaining peptide bonds at its maximum rate. The ratio between the demasking and the hydrolysis rate determines the mechanism of the proteolysis. Vorob’ev et al. (2013) estimated that the demasking rate constants for trypsin hydrolysis of β-casein are at least one order of magnitude lower than the maximum hydrolysis rate. Furthermore, these steps are quantitatively characterized by kinetic parameters which are functions of $d_0$ due to the contribution of enzyme inactivation, progressive unmasking of specific sites and variance in the productive binding of enzymes with peptide intermediates. In parallel, the initial stage is subjected to intermediate peptide accumulation and subsequently aggregation due to the exposure of hydrophobic region of the protein fragments resulting in series of hydrophobic interactions.

Overall, inadequate representation of the true mechanism will limit its general applicability, while a complicated model cannot be analysed statistically with sufficient precision by the often relatively simple kinetic experiments.

4. Conclusion

Based on the results reported here, HR-US present an alternative tool to determine milk protease concentration and activity in a wide range of process samples and conditions. The evolution of ultrasonic velocity is related to the hydration effect of the released atomic groups and the decrease in ultrasonic attenuation is caused by the reduction in β-casein aggregate size during hydrolysis. In the absence of specific interactions and other solvent effects, ultrasonic velocity can be translated into profile of number of peptide bond hydrolysed. The ultrasonic frequency range 8–20 MHz were recommended, which excluded the relaxation contribution on the ultrasonic parameters, for measurement of hydrolysis particularly in highly absorbing buffers such as phosphate. Also, the contribution of scattering effects caused by ultrasonic velocity within this frequency is small and can be neglected. The capability of HR-US to probe the rise of relaxation contribution during the hydrolysis through ultrasonic attenuation may present a potential new method of monitoring chemical reactions.

The use of HR-US for development of sensitive, time-effective, and direct enzymatic activity-based assay for quantification of naturally occurring bovine plasmin levels in different milk samples has shown to be feasible. The quantification was based on the ultrasonic analysis of concentration dependence of plasmin activity towards the natural substrate rather than synthetic substrate. The non-linear initial rate activity curve with respect to the plasmin concentration (0.2–25 nM) can be fitted with inverse Michaelis-Menten model. The difference in plasmin concentration measured by HR-US and ELISA methods owed to the difference in the principle of detection as well as the complexity of plasmin behaviour in solution. Moreover, the ultrasonic analysis on the effect of β-casein concentration (1–8.65 mg.mL⁻¹) further confirmed the Michaelis-Menten description of the proteolysis. The HR-US assay determined 5–6 peptide bonds cleaved by plasmin at 1 mg.mL⁻¹ of β-casein, corresponded to the number of main cleavage sites reported in the literature.

Overall, since most of the biocatalytic processes are associated with hydration changes which can be measured precisely by HR-US, such ultrasonic methodology presents an efficient technology for routine analysis of proteolytic reactions in the dairy laboratories. Its applicability in homogenous and buffered media was demonstrated. This can be further extended to real-milk samples but may require a comprehensive analysis of the effect of each interacting additive as well as secondary reactions (e.g. aggregations). Rather, this study presents importance as a reference as most activity-based assay is carried out in homogenous solution and phosphate buffer has been widely used buffer in enzymatic assays as well as other bioprocesses.

Credit authorship contribution statement

Mark Dizon: Investigation, Writing - original draft, Writing - review & editing, Methodology, Formal analysis. Marek Tatarko: Investigation, Formal analysis. Katalin Szabo: Investigation, Formal analysis. Tibor Hišnak: Supervision, Writing - original draft, Writing - review & editing, Conceptualization, Methodology, Funding acquisition, Project administration, Resources.

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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