Plasmin determination based on enzymatic digestion of a β-casein layer at the air/water interface

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HIGHLIGHTS

• Tensiometry-based label-free determination of proteolytic enzyme activity was developed.
• Change in surface pressure of interfacial protein layers upon reaction was determined.
• Assay linearity of plasmin enzyme against β-casein substrate of 0.7 nM was achieved.
• Sensitivity of the method can be enhanced by increasing the concentration of β-casein.

ARTICLE INFO

Keywords:
Langmuir film
Gibbs monolayer
β-casein
Proteolytic enzyme
Tensiometry
Surface pressure

ABSTRACT

The possibility of enzyme activity determination based on natural substrate digestion at the air/water interface has been investigated through the interaction of a β-casein layer with the protease plasmin. The protein layers were spread from aqueous solutions of β-casein in a 0.1–0.4 g/L concentration range to the air/water interface. 200 μL plasmin was introduced into the water subphase (15 mL) in various initial concentrations (25–1000 nM), corresponding to 0.33–13.3 nM final concentration. The decrease of the surface pressure due to the enzymatic degradation of the β-casein monolayers was followed in time, and calibration curves were obtained by plotting either the total surface pressure change, or the maximum rate of the surface pressure change against the plasmin concentration. The method is suitable for label-free determination of plasmin, with assay linearity down to 0.7 nM as the final concentration of plasmin in the system, corresponding to 50 nM for the initial, undiluted plasmin sample in the given experimental setup.
technique for the study of molecular organization, molecular interactions, (bio)analytical chemistry and sensor development, drug development, etc. The basic concept of the technique is that the very high surface tension of pure water ($\gamma_{\text{water}} \approx 72$ mN/m) is effectively reduced drastically by only a fraction of a monolayer of surface-active molecules. The behaviour of this Langmuir monolayer can be studied under different conditions. Upon any interaction of the interfacial molecules with components of the subphase, the surface tension changes. By measuring the interfacial forces with a fine balance, the surface pressure ($\Pi \equiv \gamma_{\text{water}} - \gamma_{\text{monolayer}}$) can be monitored routinely with a better than 0.05 mN/m precision. Thus, small changes of material amount can be detected.

β-casein is a single-chained milk protein of molecular mass of 24 kDa that consists of 209 amino acids, without a pronounced secondary structure [2]. Comprising both hydrophilic and hydrophobic regions, it is an amphiphilic compound, capable of forming a monolayer at the air/water interface, with an equilibrium surface pressure of $\Pi_{E} \approx 21-22$ mN/m, stable in a wide temperature range ($5-40 \, ^\circ C$) [3]. However, as a protein, it is also water-soluble, thus the stability of this monolayer differs from the stability of true Langmuir monolayers. The amphiphilic character of the molecule is exploited in the food industry, where β-casein is used as a common emulsifier. In the past decades, the interfacial behaviour of the β-casein has been more and more understood. Mitchell et al. found that surface pressure – area ($\Pi-A$) and surface pressure – surface concentration ($\Pi-C$) isotherms of β-casein monolayers at the air/water interface are practically identical, suggesting weak cohesion forces, and a disordered state [4]. Boyd et al. [5] showed that the surface viscosity of β-casein monolayers at the air/water interface is very low and practically does not change with surface concentration. When the subphase is slightly acidic ($\mathrm{pH} = 5$), the $\Pi-A$ isotherms are shifted towards higher $\Pi$ and lower $A$ values as compared to a subphase of $\mathrm{pH} = 7$. Practically no hysteresis of the isotherm is observed upon repeated compression–expansion cycles of the very same β-casein monolayer [3], however, a critical analysis of the available literature [3,4,6,7] reveals a very large discrepancy among the isotherms of different β-casein monolayers, which will be the topic of a forthcoming paper. The monolayer consists of a densely packed, ~2 nm thick layer in the air side of the interface and a loose, ~6 nm thick layer extending into the aqueous phase [8]. Monolayers adsorbed from a solution follow a non-Gibbs adsorption isotherm [9].

However, despite of the relatively vast knowledge gathered, β-casein monolayers have not been used for studying the cleavage by proteases. The objective of this work was to study the proteolysis of β-casein by plasmin at the air/water interface with a view of method development for simple, label-free enzyme activity assay.

2 Experimental

As an enzyme substrate, β-casein (from bovine milk, BioUltra, ≥98% Sigma-Aldrich, M = 24,000 g/mol) was used. The inactivezymogen, plasminogen from bovine plasma (lyophilized powder, ≥2.0 units/mg protein, Sigma-Aldrich) was transported in dry ice (~78.5 °C) and stored in deep freeze (~30 °C). The activation was done according to [28,29], using urokinase from human urine (EMD Millipore), yielding 200 μL 1000 nM for each aliquot, as described in [24] based on the spectrophotometric plasmin activity determination of [30]. The enzymatic degradation experiments were run in a home-built round PTFE vessel of 50 mm diameter and 8 mm depth (19.6 cm² surface area). The surface pressure was monitored by a computer-controlled NIMA PS4 (Coventry, UK) surface pressure sensor, using chromatographic paper strips ($\pi = 10$ mm, $t = 0.3$ mm). The water used as, respectively for the preparation of, subphases was ultrapure water obtained by reverse osmosis (ELGA LabWater, Veolia Water Technologies, UK), β-casein layers were formed by spreading 2 × 25 μL of 0.100–0.411 g/L (~4.16–17.13 μM) β-casein solutions in phosphate buffered saline (PBS; from tablets; Sigma-Aldrich; pH = 7.4, composition: 10 mM Na₂HPO₄, 2 mM KH₂PO₄,
3. Results and discussion

In Fig. 2, typical kinetics of the β-casein monolayer formation and the change of surface pressure following the addition of plasmin is shown. The baseline is recorded and levelled in AB. At point B, the β-casein is added to the air/water interface. Consequently, the surface pressure rises, steeply at the beginning (BC, immediate spreading as a Langmuir monolayer), and slowly afterwards (CD, probably readsorption from the subphase, as a Gibbs monolayer), until an equilibrium surface pressure is reached. Then, in point D, the activated enzyme solution is introduced, which causes an exponential decrease of the surface pressure (DE), until the new stable surface pressure is reached. This exponential decrease is attributed to the reorganization of the interface following the dissolution of some of the peptide products. This is possible because 1) the main cleavage sites of the β-casein for PL are located in the hydrophilic regions of the molecule, thus most likely facing the aqueous phase and the PL dissolved therein; and 2) some of the peptide fragments are fully hydrophilic [1], with high potential for dissolution in the aqueous subphase. However, the majority of the peptide residues are fully or predominantly hydrophobic, thus being expected to remain at the interface. This explains why the ΔΠ surface pressure change is always small relative to the surface pressure of the intact β-casein monolayer, ultimately limiting the sensitivity of the method. To obtain calibration curves, either the ΔΠ surface pressure difference, or the \( r_{\text{max}} \) opposite of the maximum rate of surface pressure decrease (1) is then plotted against the enzyme concentration.

\[
r_{\text{max}} = -(\Delta \Pi/\Delta t)_{\text{max}}
\]

The calibration curves obtained by fitting the data points with (2) reveal a maximum sensitivity at low PL concentrations that levels off at higher PL concentrations (Fig. 3). The sensitivities and the plateau values are higher for higher β-casein concentrations. The three fitting parameters, \( A \), \( \alpha \) and \( y_0 \) are plotted in Fig. 4 against the β-casein concentration.

2.7 mM KCl and 137 mM NaCl) with a Hamilton microsyringe. This range of amount of spread β-casein was chosen so that the obtained nominal specific area (assuming all β-casein molecules being at the interface), 0.4–0.1 m\(^2\)/mg (corresponding to an area per molecule of 1565–381 Å\(^2\)) fall in the liquid condensed region of the surface pressure – area isotherms [7]. For the enzymatic degradation experiments, 15 mL subphase was used. After the surface pressure reached a constant value (in typically 3–6 h), 200 μL undiluted or diluted enzyme solution was added to the subphase by a Hamilton microsyringe through a small channel. In the followings, \( c_{\text{PL}} \) will be used to denote the initial concentration of the injected plasmin solution, whereas \( c_{\text{f PL}} \) will be used for the corresponding final concentration calculated for the entire volume (15 mL) after injection. The temperature of air was 27 °C. After each experiment, the subphase was aspirated dry by the help of a vacuum pump (KNF Neuberger Laboport, Freiburg, Germany), the vessel was cleaned with lint-free cleaning wipers (Kimtech Pure 7624) soaked in chloroform. During the experiments, the subphase was continuously stirred with a small magnetic bar positioned at maximum distance from both the vessel wall and the paper strip of the surface pressure sensor. The experimental setup is presented in Fig. 1. Data analysis, fitting and plotting was done in OriginPro (OriginLab Co., USA).
Interestingly, adding β-casein to the air/water interface in two different volumes and concentrations so that their product, i.e., the amount of substance is kept equal, produces two very different calibration curves. The one corresponding to the higher concentration and lower volume substrate shows higher sensitivity and higher plateau value than the one corresponding to the lower concentration and higher volume substrate (Fig. 5). The discrepancy is most likely related to the spreading mechanism of the β-casein at the air/water interface, which partially resembles the spreading of a true, insoluble Langmuir film, and partially the spreading of a soluble, Gibbs monolayer.

As the other calibration possibility, $r_{\text{max}}$ values, determined by derivation of the DE sections of the measurement curves (Fig. 2), were plotted against PL concentration (Fig. 6). In spite of the experimental difficulties (those related to the non-ideal spreading of the substrate, the eventual differences of the enzyme mass transport from experiment to experiment due to the possible irregularities of the convection, the high number of the cleavage sites, etc.), the conspicuously linear trends of the calibration data are impressive. Not surprisingly, as in the case of surface pressure change calibration plots, the sensitivity (the slope of the lines)
increases with activity measurements down to 50 nM final, corresponding to 0.7 nM generally increases with increasing substrate concentration. Based on Fig. 8. The sensitivity of the PL determination (slope of the lines in Fig. 6) increases with $c_{\beta\text{-casein}}$ linear (red) and parabolic (blue) fits. Left axis: sensitivity expressed in experiment-relevant units; right axis: sensitivity in basic SI units. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Fig. 7 is another representation of the same calibration data, where the $r_{\text{max}}$ values are plotted against the substrate concentration, grouped for different enzyme concentrations. The curves, partially extrapolated, show a sigmoidal run, with maximum steepness at intermediate substrate concentrations, decreasing maximum steepness with decreasing PL concentration and maximum $r_{\text{max}}$ for highest PL and substrate concentrations.

A final plot summarizing the sensitivity of the method is shown in Fig. 8. The $r_{\text{max}}/c_{\text{PL}}$ ratios, that are in fact the slopes of the calibration lines of Fig. 6, are plotted against the substrate concentration. The obtained points follow a parabolic trend that is very close to linearity, telling that the sensitivity of the method after all increases with substrate concentration. It should be noted, however, that the sensitivity most probably cannot be increased beyond a certain critical value by using increasingly higher substrate concentrations, which is expected to be near the critical micelle concentration of the $\beta$-casein, approx. 0.5 g/L.

4. Conclusions

$\beta$-casein monolayers at the air/water interface have been digested with plasmin, a proteolytic enzyme, injected into the subphase, and the surface pressure drop was observed in the function of applied enzyme concentration. The presented method allows the determination of plasmin with an assay linearity down to 50 nM sample concentration in the given experimental setup, corresponding to 0.7 nM final concentration. The advantage of the method consists in its simplicity. The several hours needed to reach the starting equilibrium surface pressure of the substrate interfacial film definitely constitutes a limitation, just as the measurement variant based on the surface pressure difference determination, the variant based on the maximum rate of surface pressure change being considerably faster. Ultimately, the source of all weaknesses of the method lies in the limited film-forming ability of the $\beta$-casein substrate. Although being amphiphilic, $\beta$-casein is also soluble in the aqueous subphase which is spread upon, consequently does not form a proper, i.e. insoluble, Langmuir monolayer at the interface, but it is partially dissolved in, and (again partially) readsorbed to the interface. Nevertheless, here we reported for the first time a plasmin determination method based on tensiometric measurements of an interfacial layer of a natural substrate of the enzyme, $\beta$-casein.

Further ongoing work is aimed at optimising experimental variables, offering a substantial decrease of the detection limit of enzyme activity as well as enhancing the robustness of the method.

CRediT authorship contribution statement

Loránd Románzki: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. Tibor Hianik: Conceptualization, Methodology, Resources, Writing - review & editing, Project administration, Funding acquisition, Supervision. Zsófia Keresztes: Conceptualization, Methodology, Resources, Writing - review & editing, 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.

Acknowledgements

The research leading to these results has received funding from the European Commission within the project Innovative technology for the detection of enzyme activity in milk (FORMILK) under grant agreement number 690898/H2020-MSCA-RISE-2015, and the <GS1>BIONANO_GINOP-2.3.2-15-2016-00017 project. T.H. acknowledges funding from the Science Grant Agency VEGA, project No.: 1/0419/20.

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