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Production of β-Lactoglobulin hydrolysates by monolith based immobilized trypsin reactors

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Abbreviations: β-Lg, β-lactoglobulin; α-La, α-Lactalbumin; CIM®, Convective Interaction Media®; IMTRs, immobilized trypsin reactors; N1, immobilized trypsin reactor based on monolith with pore size 2 µm; N2, immobilized trypsin reactor based on monolith with pore size 6 µm; HCCA, α-Cyano-4-hydroxycinnamic Acid; DHAP, 2,5-Dihydroxyacetophenone; BAEE, Nα-Benzoyl-L-arginine ethyl ester; BET, Brunauer–Emmett–Teller; DH, degree of hydrolysis.

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Abstract: Tryptic hydrolysis of β-Lactoglobulin (β-Lg) is attracting more and more attention due to the reduced allergenicity and the functionality of resulting hydrolysates. To produce hydrolysates in an economically viable way, immobilized trypsin reactors (IMTRs), based on polymethacrylate monolith with pore size 2.1 µm (N1) and 6 µm (N2), were developed and used in a flow-through system. IMTRs were characterized in terms of permeability and enzymatic activity during extensive usage. N1 showed twice the activity compared with N2, correlating well with its almost two times higher amount of immobilized trypsin. N2 showed high stability over 18 cycles, as well as over more than 30 weeks during storage. The efficiency of IMTRs on hydrolyzing β-Lg was compared with free trypsin, and the resulting hydrolysates were analyzed by MALDI-TOF/MS. The final hydrolysis degree by N1 reached 9.68% (86.58% cleavage sites) within 4 h, while only around 6% (53.67% cleavage sites) by 1.5 mg of free trypsin. Peptides analysis showed the different preference between immobilized trypsin and free trypsin. Under the experimental conditions used in this study, the potential cleavage site Lys^{135}-Phe^{136} was resistant against the immobilized trypsin in N1.

Key words: monolith, trypsin, immobilized enzyme reactor, β-Lactoglobulin

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1. Introduction

Among the major whey proteins β-Lactoglobulin (β-Lg) is the main antigen. Enzymatic hydrolysis of this protein is a common approach to reduce allergenicity. The use of the serine protease Trypsin (EC 3.4.21.4), which preferably cleaves the C-terminal peptide bonds of Arginine (Arg/R) and Lysine (Lys/K), not only can significantly reduce its allergenicity [1], but also leads to the release of five biofunctional peptides [2]. In addition, Schmidt and Poll [3] showed that native α-Lactalbumin (α-La) was highly resistant to tryptic digestion while native β-Lg was not. Indeed, Konrad and Kleinschmidt [4], taking advantage of this selective susceptibility of β-Lg, used a combination of membrane technology and trypsin hydrolysis to obtain a yield of α-La of 90 ~ 95% in purity. However, the cost of large-scale use of trypsin in solution is very high, which severely limits its industrial application. Correspondingly, immobilized trypsin represents an alternative approach due to the possibility of reusing the enzyme and producing enzyme-free hydrolysates.

During the past few decades, the immobilization of trypsin onto different stationary supports has been studied resulting in the development of various immobilized trypsin reactors (IMTRs). These IMTRs can generally be divided into three groups: (1) trypsin is immobilized on nano- or microparticles [5-7]; (2) trypsin is retained by membrane [8]; or (3) trypsin is immobilized onto surface of monolithic materials [9-13]. However, available systems still suffer from low processing stability, i.e. high pressure drop over time [14], enzyme leakage due to non-covalent bonding [7, 14], and low capacity in terms of volume throughput [15].

Among the different immobilization supports, monoliths have been of recent interest, because they generally allow for a higher mass flow, and mass transfer of target molecules

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with the enzyme bound to the monolithic channel surfaces is primarily governed by the mobile phase convective flow. Furthermore, the surface of monoliths can be modified with various functional groups, which makes covalent bonding of enzyme much easier. However, most of the developed monolithic IMTRs are designed to accelerate the digestion step for proteomics analysis, which differs from the application of producing protein hydrolysates. Besides high enzymatic efficiency, to reach a higher space-time yield (the amount of substrate converted per biocatalyst reactor), the crucial requirements for enzymatic hydrolysates production are a need for processing larger amount of substrate and longer continuous digestion time in each cycle. These requirements may cause a blockage problem. Theoretically, monoliths with very large pores can minimize this issue [16], but at the expense of the decreased surface area, resulting in significant reduction of the amount of immobilized trypsin.

Based on these considerations, for the first time, we immobilized trypsin on monolith with pore size 6 µm (N2), which is less prone to pore blockage, and compared its efficiency with that with pore size 2.1 µm (N1). This allows us to conclude on the effect of convective flow through the pores at longer operation times in a continuous mode. Short polymethacrylate monolithic columns, commercialized under the trademark Convective Interaction Media® (CIM®), were used as the stationary supports. The immobilized trypsin yield was compared in these two IMTR systems and correlated with their digestion activities. The efficiencies of these two CIM-IMTRs in hydrolyzing β-Lg were compared with each other, as well as with free trypsin. Furthermore, long-term stability of these two IMTRs was monitored throughout the whole study.

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In addition, differences in the hydrolysates may arise from the use of an immobilized enzyme instead of the free one. In fact, immobilization can change, e.g., the accessibility of the enzyme to the substrate or even the affinity of the enzyme towards a specific substrate. In this study, we also explored the peptides compositions in the hydrolysates resulting from immobilized and free trypsin, separately.

2. Materials and methods

2.1 Materials

Bovine β-Lg was fractionated from WPI, a product from Fonterra Co-operative Group Ltd (Auckland, New Zealand) as described by Toro-Sierra, Tolkach, et al. [17]. Trypsin from bovine pancreas (Type I, ~10,000 BAEE units/mg protein), Nα-Benzoyl-L-arginine ethyl ester (BAEE, B4500), Tris (hydroxymethil)-aminomethane (TRIS), sodium cianoborohydride ($NaCNBH_3$), ethanolamine, sodium chloride (NaCl), benzamidine hydrochloride (BAHC), 2-(N-morpholino)-ethanesulfonic acid (MES), sodium hydroxide, sulphuric(VI) acid, calcium chloride and sodium eriodate were purchased from Sigma–Aldrich (St Louis, MO, USA). Deionized water was from MilliQ System (Millipore Corporation, Bedford, USA).

2.2 Preparation of CIM-IMTRs and frame of flow-through system

2.2.1 CIM-IMTRs preparation

Aldehyde activated CIM® radial columns (CIM-ALD, tube dimensions: outer diameter (D) – 1.86 cm, inner diameter (d) – 0.67 cm, height (h) – 0.42 cm; volume 1.0 mL) with nominal pore size diameter of 2.1 and 6 μm were provided by BIA Separations d.o.o. (Ajdovščina, Slovenia).

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The specific surface area of the monoliths before trypsin immobilization was measured via nitrogen adsorption by TriStar II 3020 (Micromeritics Instrument Corporation, Norcross, GA, USA). Nitrogen of 99.999% purity was used. Before analysis the monolith samples were dried in nitrogen flow at 70°C for 1 hour.

Pore size distribution was measured by a Pascal 440 (Thermo-Quest Italia, Rodano, Italy) mercury porosimeter within a range of 15–10,000 nm. Approximately 0.1 g of dried monolith sample was measured before immobilization.

Trypsin was covalently immobilized on CIM-ALD monolithic columns using the following coupling protocol. The immobilization was done in duplicate. 5.0 mg of trypsin was dissolved in 5 mL of immobilization buffer, composed of 0.1 M MES, pH 5.6, $NaCNBH_3$ (3.0 mg/mL) and BAHC (0.4 mg/mL). The CIM-ALD column was washed with 20 mL of 0.1 M MES buffer at pH 5.6, followed by 10 mL of immobilization buffer. Then the trypsin solution was continuously recirculated for 3 h at a flow rate of 0.5 mL/min. Aliquots of trypsin solution were collected at the beginning, during and at the end of the immobilization procedure for subsequent trypsin mass balance analysis. The residual aldehyde groups were deactivated by treating the columns with ethanolamine and $NaCNBH_3$ solution as it is described in Naldi et al.[9]. Afterwards, the column was washed with 20 mL of Tris-HCl buffer (20 mM pH 7.4 containing 1 M NaCl). Before use the prepared CIM-IMTRs were stored at 4°C in aqueous solution of 20 mM acetic acid, pH 3.5 containing 1.0 mM $CaCl_2$.

2.2.2 Determination of immobilized trypsin density

The amount (mg) of immobilized trypsin was calculated according to Eq. 1.

$$m_{immobilized}(trypsin) = \gamma_{load}(trypsin) \times V_{load} - \sum \gamma_n(trypsin) \times V_n \qquad (1)$$

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Where, γ_{load}(trypsin) is the concentration of trypsin in the loading solution; γ_n(trypsin) are concentrations (mg/mL) of trypsin in washing and deactivation fractions after the immobilization; V_{load} is the volume of trypsin solution applied to the column and V_n is the sum of the washing and deactivation fraction volumes, in mL. The determination of trypsin concentration was performed chromatographically as described by Naldi et al. [9].

2.2.3 Frame of the flow-through system

IMTR was inserted in ÄKTA system (GE Healthcare Bio Sciences) consisting of sample pump (P-960), system pump, auto-sampler and detectors for UV, pH, temperature and conductivity. The whole system was controlled by Unicorn Software 5.31.

2.3 Pressure drop and permeability calculation

The backpressure without and with IMTR was recorded at increasing flow rates, namely 0.5, 2.0, 5.0, 10 and 15 mL/min, by pumping deionized water. The difference between these two values at a defined flow rate was considered to be the pressure drop created by the inserted reactor. According to the study of Podgornik, et al. [18], the permeability of monolith was calculated according to Eq. 2:

$$B = \frac{F}{\Delta P} \times \frac{\eta \times \ln(\frac{D}{d})}{2\pi h} \qquad (2)$$

Where, ΔP (MPa) is the pressure drop; η is the viscosity of mobile phase (0.87685 MPa*s for deionized water); B (m^2) is the calculated permeability of monolith; F (mL/min) is the volumetric flow rate; D (m) and d (m) are outer and inner tube diameters, and h (m) is monolith height.

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2.4 Activity measurements of free and immobilized trypsin

The activity measurement is a spectrophotometric rate determination (A_{253nm}, Light path = 1 cm) in buffer solution based on the following reaction:

$$BAEE + H2O \xrightarrow{Trypsin} N\alpha - Benzoyl - L - arginine\ (BA) + ethanol$$

All the measurements were conducted at 25 ± 1°C. 0.1 M Tris-HCl buffer was used, and its pH was adjusted to different values, namely 7.2, 7.8, 8.1, 8.5 and 9.2, to assess the effect of pH.

2.4.1 Free trypsin activity measurement

For measuring free trypsin activity, a continuous spectrophotometric rate determination was performed following the protocol given by the manufacturer (Sigma-Aldrich, 2016). The activity of free trypsin was expressed as BAEE units. One BAEE unit is defined to produce a ΔA253 of 0.001 per minute in a reaction volume of 3.20 mL.

2.4.2 Immobilized trypsin activity measurement

Inserted IMTR was washed by deionized water firstly and then equilibrated by 0.1 M Tris-HCl buffer (varied pHs) at 5 mL/min. 10 mM BAEE in 0.1 M Tris-HCl buffer was continuously pumped through this IMTR at 10 mL/min. The first 4 mL of sample was discarded because of the dead volume of Äkta system and following 2 mL was collected by the auto-sampler. The collected samples were diluted and further measured by spectrophotometer at 253 nm. The activity unit U* (μmol/min), defined as the amounts of BAEE one CIM-IMTR converted to BA in 1 min at specific conditions, was calculated using Eq. 3:

$$U^* = \frac{\Delta A \times F \times Di \times 10^3}{L \times \varepsilon} \qquad (3)$$

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ΔA (Au) is the absorbance difference of BAEE solution before and after hydrolysis; Di is dilution factor; L is the light path, here is 1 cm; ε is the molar extinction coefficient, corresponding to the differential molar absorbance of BAEE against BA at 253 nm, here is 808 mol^{-1} cm^{-1} determined by Kedzy et al [19]. U* can be easily converted to BAEE units, using the conversion factor 270 determined by Bergmeyer et al. [20].

2.5 Hydrolysis of β-Lg

Between experiments IMTRs were stored at 4°C in storage solution (19 mM $CaCl_2$ in 10% ethanol/water solution, pH 3 adjusted by 0.1 M HCl). This solution was required to be washed out prior to starting the next cycle. Therefore, before each hydrolysis experiment, the CIM-IMTR was washed by deionized water. The washing step was also used to characterize the IMTR in terms of the pressure drop as described in 2.3. If the pressure drop would be higher than base line, this would have been an indication of pore blockage. Afterwards, it was equilibrated by 0.1 M Tris-HCl buffer at pH 7.8 (this step was excluded when pH-stat method was applied). After each hydrolysis of β-Lg, a cleaning step using an alkaline solution (0.5 mM NaOH + 5 mM NaCl, pH 10.8) was used, and deionized water was applied to remove the alkaline prior to filling the system with storage solution. All the hydrolysis experiments were conducted at 25 ± 1°C.

2.5.1 **Single flow-through approach**

Native β-Lg in 0.1 M Tris-HCl buffer (1.5, 3 and 6 mg/mL) at pH 7.8 was continuously pumped through the IMTR (N1 or N2) at different flow rates, ranging from 0.5 to 10 mL/min. The first 4 mL of hydrolysates were discarded. The following 6 mL were collected by the auto-sampler for the native protein content measurement. The amount of native β-Lg was

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determined using RP-HPLC as described by Toro-Sierra, Tolkach, et al. [17]. The depleting rate of β-Lg was calculated according to Eq. 4:

$$R = \frac{C_d \times v}{v_{/F}} = C_d \times F \qquad (4)$$

Where, R (mg/min) is the depleting rate of β-Lg, C_d (mg/mL) is the difference of β-Lg concentration before and after hydrolysis, v (mL) is the collected volume, and F (mL/min) is the flow rate.

2.5.2 Recirculation flow approach

50 mL of native β-Lg in 0.1 M Tris-HCl buffer (3 mg/mL) at pH 7.8 was recirculated through N1 for 2 h at 0.5, 5, 10 and 15 mL/min, respectively. Samples at different time intervals (0, 10, 20, 30, 60, 90, 120 min) were drawn for native protein content analysis [17].

2.5.3 Comparison of IMTRs and free trypsin on hydrolyzing β-Lg

Native β-Lg dissolved in deionized water (25 mL, 10 mg/mL, pH was adjusted to 7.8 using 1M NaOH) was hydrolyzed by free trypsin or IMTRs. For the hydrolysis by IMTRs, the recirculation flow approach at 10 mL/min was applied.

TitroLine alpha plus autotitrator (Schott AG, Mainz, Germany) measured the change of pH every 3 s during the hydrolysis process and kept pH constant by addition of 0.1 M NaOH. The data was acquired by the TitriSoft 2.5 software. The degree of hydrolysis (DH) is defined as the percentage of peptide bonds cleaved (h) compared to the total available peptide bonds (h_{tot}) in the protein substrate (Eq. 5). Instantaneous DH at any given time was calculated using Eq. 4 according to the pH-stat method [21].

$$DH = \frac{h}{h_{tot}} \times 100\ \% = \frac{V_b \times N_b}{\alpha \times M_p \times h^*_{tot}} \times 100\% \qquad (5)$$

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where, V_b (mL), base consumption; N_b, normality of the base; α, average degree of dissociation of the NH groups; M_P (g), mass of protein; h_{tot}^*, total number of peptide bonds in one gram protein substrate, in this work we calculated this value to be 7.63 meqv/g for β-Lg.

From Eq. 5, h was derived directly, and the velocity of reaction was calculated by Eq. 6.

$$V = \frac{dh}{dt} \quad (6)$$

Where, V (meqv/min) is the rate of reaction; h (meqv) is the amount of peptide bonds cleaved; t (min) is the hydrolyzing time.

2.6 Peptides composition analysis using MALDI-TOF mass spectrometry

Collected samples were analyzed for mass composition using MALDI-TOF/ MS system (ultrafleXtreme MALDI-TOF/TOF, Bruker Daltonics, Bremen, Germany) with two matrices separately, α-Cyano-4-hydroxycinnamic Acid (HCCA, Bruker Part-No. #201344) and 2,5-Dihydroxyacetophenone (DHAP, Bruker Part no. #201346). Generally, HCCA is used for the measurement of peptides and proteins in the low mass range (≤4,000 Da), whereas DHAP is mainly used for proteins in the higher mass range. The detailed method is described in supporting information.

3. Results and discussion

3.1 IMTRs characterization

3.1.1 Yield of immobilized trypsin in IMTRs

Development of CIM-IMTR for proteomic applications clearly demonstrated that decrease of pore size down to 0.6 µm was preferable due to higher immobilized trypsin density on the

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monolithic surface and consequently higher enzymatic conversion [9]. Unfortunately, the decrease of pore size by a factor of 2 results in a 4 times higher pressure drop and a drastic decrease of permeability, which is not an option for hydrolysates production. Therefore, an alternative approach was considered, where monoliths were with larger pores and consequently higher permeabilities. The monolithic surface area is one of the most important parameters influencing the maximum amount of immobilized protein. BET surface areas of CIM monoliths (before trypsin immobilization) were measured to be 5.0 and 2.0 m^2 per g of dry monolith for 2.1 µm (N1) and 6 µm (N2) pore sizes, respectively. In our immobilization protocol, 5 mg of trypsin was available for binding to a monolith with volume of 1 mL. Mass balance calculations showed a complete depletion of trypsin from the immobilization solution for N1, while 2 mg of trypsin remained unbound with the N2. Correlating BET surface area and amount of immobilized trypsin, at least 2 mg more trypsin could be additionally bound to the monolithic surface in case of N1 to obtain the maximum possible ligand density.

Considering BET surface areas and the molar mass of trypsin from bovine pancreas (23.8 kDa), each trypsin molecule in N1 and N2 occupies approximately 17 and 11 nm^2 of surface, respectively. Based on Saha et al. [22] the molecular dimension of native trypsin from porcine pancreas is 4.8 nm x 3.7 nm x 3.2 nm. Assuming that the spatial dimensions of bovine-derived trypsin are similar and that enzyme molecules are surrounded by a hydration layer of approximately 0.2 nm, one trypsin molecule would occupy between 10 and 20 nm^2 surface. It is concluded that the highest possible surface density of immobilized trypsin was achieved with N2 (100 ± 15%), while approximately 65 ± 10% coverage was obtained for N1.

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3.1.2 Initial pressure drop and permeability

The pressure drop created by freshly prepared N1 and N2 at different flow rates was recorded in Figure S1. A linear increase of pressure drop depending on flow rates for both reactors was observed as expected for constant permeability, proving that porous monolithic structure is stable and no compression occured even when the flow rate was up to 15 mL/min. According to Eq. 2, the calculated initial permeabilities of N1 and N2 were 2.45 x 10^{-12} m^2 and 1.91 x 10^{-11} m^2, respectively. Due to the same porosity of N1 and N2 (60%), 7.80 times higher permeability of N2 could be attributed mainly to the pore size difference. According to the Blake–Kozeny equation, the permeability of a porous bed depends on the particle size forming the bed, as well as on its porosity. Instead of particle diameter a pore size (d_v) is used, and the expression takes the following form [18]:

$$B = K_v \times \varepsilon \times d_V^2 \quad (7)$$

Where, K_v is a structural constant of particular bed. Assuming same porosity and structural constant for N1 and N2, an Eq. 8 is derived:

$$\frac{B_{N1}}{B_{N2}} = \frac{d_{V1}^2}{d_{V2}^2} \quad (8)$$

The exact pore sizes of both monoliths were determined by mercury porosimetry before the immobilization (2.15 µm for N1 and 5.80 µm for N2) as described above. The measured permeability ratio (N1/N2 = 7.80) fits very well with the squares of the experimentally determined d_v ratio, thus confirming that N1 and N2 were structurally similar materials.

3.1.3 Activity measurements at increasing pH values

BAEE was dissolved in 0.1 M Tris-HCl buffer and used as substrate to assess the activity of immobilized and free trypsin at increasing pH values. As shown in Figure 1, N1 showed

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almost twice the activity compared with N2, which correlates well with the almost two times higher amount of immobilized trypsin in N1. With increasing pH up to 9.2, the activity of both IMTRs was quite stable and even exhibited a slight increment. Actually, the immobilized trypsin could survive without any decrease of activity at pH 10.8, which was decided to be the pH of washing solution applied in this study. On the contrary, the activity of free trypsin started to decrease above pH 8.1.

Not surprisingly, such stabilization of immobilized trypsin with pH increase was attributed to the applied multipoint covalent immobilization, which has been claimed by Fernandez-Lafuente [23]. However, immobilization often results in a decrease of enzymatic activity due to steric hindrance and reduced accessibility to the trypsin active site. Comparing the specific activity of per mg immobilized trypsin in IMTRs with per mg free one, a decrease of 74~ 48% for N1 and 73~41% for N2 at varied pH values was observed.

Based on these results, the characteristics of CIM-ALD columns and corresponding IMTRs are summarized in Table 1.

3.2 Hydrolysis of β-Lg

3.2.1 Efficiency evaluation of IMTRs at different flow rates

The flow rate is one of the most important factors influencing the flow-through bioreactor performance. To evaluate the performance of IMTRs in depleting β-Lg at different flow rates, both single flow-through approach and recirculation flow approach were applied, which are more practical to improve the hydrolysates yield compared with a classical zonal approach (with injection volume only up to microliter or milliliter).

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For the immobilized trypsin, the optimal pH is 8.7 or even higher, while activities of the free one started to decrease when the pH value was above 8.1 (see Figure 1). To compare the efficiencies between the free and the immobilized trypsin, we decided to perform the hydrolysis at pH 7.8.

In a single flow-through approach, β-Lg solutions (in 0.1 M Tris-HCl buffer, pH 7.8) with stepwise increased concentrations were used to avoid underestimation of IMTRs' performance due to the insufficient replenishment of substrate. As shown in Figures 2A and B, 3 mg/mL native β-Lg was found already to be a saturating concentration for all explored flow rates, since no more β-Lg was depleted when the substrate concentration further raised to 6 mg/mL. As expected, for both N1 and N2 more native β-Lg was depleted at lower flow rates because of the corresponding longer contact time between immobilized trypsin and substrate. However, regarding to the efficiency (i.e. the depleting rate of β-Lg) at each flow rate, a clear increasing dependence on flow rate was found, as can be seen from the curves in Figures 2A and B. As compared to IMTRs used in proteomic applications, in continuous operation we rate the performance of IMTR technology from a different perspective. A higher depleting rate would be a decisive criteria regarding processing efficiency. To further assess the effects of flow rates on IMTR's efficiency, a recirculation flow approach within a long given time is preferred, which provides the possibility to compare the depleted β-Lg limited to the same given time while at different flow rates. According to the results in Figures 2A and B, the efficiency values at 5 mL/min seem to be in the middle range. Hence, 50 mL of β-Lg solution (in 0.1 M Tris-HCl buffer, pH 7.8) at 3 mg/mL were recirculated through N1 at 0.5, 5, 10 and 15 mL/min for 2h, respectively. The residual native β-Lg in substrate solution at different time intervals, as shown in Figure 2C,

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decreased significantly faster when flow rates were at or above 5 mL/min during recirculation, compared with that at 0.5 mL/min. The growth of depleting efficiency with increasing flow rates is presumably attributed to the increased mass transfer of β-Lg molecules towards the immobilized trypsin [24]. In addition, the work of Jungreuthmayer et al. [25] demonstrated that the flow in monolith showed a lateral velocity component, which may contribute to the transport of molecules to the monolith wall. This confirmed lateral velocity can be speculated to promote the contact between substrate and fixed trypsin. From this result, it is assumed that the fluctuating flow behavior in monolith may be more severe at higher flow rates, since the efficiency in depleting β-Lg was higher at increased flow rates. However, this increase of efficiency depending on flow rates can reach a plateau, as shown in Figure 2C, no increment was found when the flow rate further raised to 15 mL/min from 10 mL/min. Actually, a clear plateau was observed in Figure 2D, where the depleted β-Lg in first 10 min was plotted versus applied flow rates. This dependence of efficiency on flow rates is quite different from that in the single flow-through approach (Figures 2A and B), where the efficiency improved significantly with increasing flow rates. It was noted that the substrate concentration was assumed constant in the calculation by single flow-through approach, while it declined continuously in a recirculation flow approach. Hence, for further experiments, the recirculation flow rate was fixed at 10 mL/min.

3.2.2 Comparison of the hydrolysis efficiency by IMTRs and free trypsin

The efficiencies of IMTRs in producing β-Lg hydrolysates at recirculation flow approach were compared with each other, as well as with free trypsin (1.5 mg and 15 mg) in a stirred solution. The same amount of substrate (25 mL, 10 mg/mL β-Lg in water, pH 7.8) was used.

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Additionally, it was noted that the activity of 1.5 mg free trypsin equals the activity of N1 in term of BAEE units (Figure 1).

As shown in Figure 3A, the DH using 1.5 mg free trypsin increased much faster at the beginning than that using N1 or N2. From the curves of velocity versus time in Figures 3B-1, B-3 and B-4, the maximum reaction velocity (V) by free trypsin at the given conditions in this study reached around 1.28 meqv/min, 5-fold of that by N1 and 7-fold of that by N2, respectively. Obviously, free trypsin is more flexible to contact with β-Lg compared with immobilized trypsin in IMTRs, forming enzyme-substrate complex, and then contributed to a significantly higher reaction velocity. On the other hand, the steric effects that are involved by non-oriented immobilization can also lead to a lower velocity.

Normally, the maximum velocity at given conditions is determined by measuring the initial reaction velocity in a catalyst system, because it requires a high concentration of substrate. As shown in Figure 3B-1, in a system with free trypsin, the maximum velocity was achieved immediately once the reaction started. However, it took around 30 min to reach the maximum velocity for N1 (Figure 3B-3). Since the measurements of DH depended on consumed volumes of NaOH, it is assumed that the initial delay of calculated DH in N1 case is not because of a slower enzymatic reaction, rather because of the residual charge on monolithic surface. The surface of N1 has properties of a weak base (secondary amine) due to ethanolamine deactivation of residual aldehyde groups after trypsin immobilization [26]. The DH experiments were performed in water, therefore the monolith charge was not shielded by the buffer and obviously it served as the neutralizing agent or as a buffering agent in the beginning of the reaction, thus decreasing the consumption of NaOH. In Figure 2C, where β-Lg was dissolved in 0.1 M Tris-HCl buffer, no delay of β-Lg depletion was

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observed with N1 in the beginning of the reaction, indirectly confirming the interpretation of DH experiment in Figure 3C. We also developed two IMTRs using another approach, where ethanolamine was excluded during deactivation, and no delay of initial reaction velocity was observed (data not shown here). One would expect similar observations with N2 as well, but the amount of ethanolamine groups on the N2 surface was considerably lower, which almost eliminated this delay. The reasons are: 1) higher surface coverage by trypsin (see Section 3.1.1.) leading to lower amount of residual aldehyde avaliable for the reaction with ethanolamine; 2) 2.5 times lower surface area of N2 leading to at least 2.5 times lower amount of charged functional groups.

Along the hydrolysis process, the reaction velocity continuously decreased, especially using free trypsin. The final DH within 4 h by 1.5 mg free trypsin was only around 6%, while its theoretical DH_{max} is 11.18% [2]. The effects of autodigestion, reduction of substrate and potential inhibition of products can account for this result. Correspondingly, the decrease of reaction velocity by immobilized trypsin was much slower mainly because of the absence of autodigestion. Since autodigestion of enzyme is a time-dependent process, more trypsin provided, longer effective hydrolyzing time can be achieved. Hence, 10-fold amount of free trypsin was used to hydrolyze the same amount of substrate. A sharp increase of maximum reaction velocity was observed (Figure 3B-2) and DH increased extremely fast in a short time, close to the theoretical DH_{max}. The DHs by 15 mg free trypsin and N1 finally reached plateaus so that they were considered as the practical maximum DHs. Accordingly, the hydrolysates were collected for further analysis. In N2, the DH gradually increased, reaching 7% after 6 h. Presumably, this hydrolysis process can reach its maximum DH when enough time is provided.

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3.2.3 Peptides compositions analysis

The peptides compositions in collected samples were analyzed using MALDI-TOF/MS, as shown in Figure 4. (the mass spectrum exported directly from flex analysis software is shown in Figure S2). Mass signals in the range between 500 and 8,000 Da were clearly observed in the sample from N1, even intact β-Lg peaks around 18,000 Da were still visible (Figure 4A). On the contrary, most peaks were below 4,000 Da in the sample hydrolyzed by free trypsin. Correlating to the practical DH_{max}, the final DH by N1 (9.68%) was indeed lower than that by free trypsin (11.07%).

β-Lg contains 161 peptide bonds, of which 18 peptide bonds are theoretically cleavable by trypsin, leading to the release of 19 peptides after complete trypsinlysis, as shown in Figure S3. However, the masses below 500 Da are not possible to be detected by our method in this study. Regarding other detectable peptides, only f(48-60) was missing in both samples, f(125-135) and f(61-69) only missed in the sample hydrolyzed by N1. By searching polypeptides in the hydrolysates from N1, no matched peptide starting from Phe^{136} or end at Lys^{135} was found. Hence, it is speculated that the potential cleavage site Lys^{135}-Phe^{136} was resistant against the immobilized trypsin in N1 under our experimental conditions.

Along the hydrolysis process, it is important to point out the competition for the active site between the original protein substrate and the released polypeptides. Analysis of these polypeptides in the final hydrolysates provides information on the preference between immobilized and free trypsin. From the mass spectrum in Figure 4, f(41-60), f(71-100) and f(70-75) remained in final hydrolysates by free trypsin, as well as by N1. The existence of f(125-138) and f(92-138) further confirms Lys^{135}-Phe^{136} was indigestible by N1. Other polypeptides with quite high intensity, such as f(41-70), f(41-69), f(101-124) and f(92-101),

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only existed in the hydrolysates from N1. By calculating the content of hydrophobic amino acids, these four polypeptides contain 39.9%, 41.38%, 41.7% and 60.0%, respectively. These high contents of hydrophobic amino acids may account for the non-digestion. Because the hydrolysis by immobilized trypsin in methacrylate monolith requires a transportation of the substrate to the monolith wall, these hydrophobic peptides seem less prone to be transported and then are hard to contact with the active site of immobilized trypsin.

3.3 Operational stability

During operation of IMTRs, pressure drop and activity were monitored through the whole study. To confirm the durability of IMTRs in one cycle, 10 mg/mL β-Lg solution was continuously recirculated through N1 or N2 at 10 mL/min for 6 h, respectively. As shown in Figure S4, a slight increment of recorded pressure drop was noted for N1, namely from 0.30 MPa to 0.33 MPa, whereas the pressure drop by N2 completely kept constant. The adsorption of β-Lg molecules on the surface of monolith should be responsible for this slight increase of N1.

On the other hand, cumulative action from each cycle to the next one is a potential issue. N2 was used to hydrolyze β-Lg (10 mg/mL) for 18 cycles (except for 6 h in the first cycle, others were continued for 3 h each time), both enzymatic activity and pressure drop remained constant (the monitored permeability and activity was recorded in Figure S5). Regarding to N1, the same substrate solution and recirculation time in each cycle were applied. Figure S6 records the pressure drop, calculated permeability and activity over 18 cycles. No decrease was observed of either permeability or activity in the first 10 cycles. Hereafter, the permeability declined gradually while the enzymatic activity was still unchanged until the 18th cycle. Because of the continuous increase of pressure drop, 20 mL

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of 0.05 M NaOH solution was used to flash N1 at 5 mL/min after the 18th cycle, which resulted in an increase of permeability, while accompanied by a complete loss of activity. After the application of 1 M NaOH, the permeability nearly returned to its initial value. This result confirms the blockage problem in N1 due to the cumulative action. It should be noted that the cycles were not always conducted over consequent days, and IMTRs were stored at 4 ^{o}C in storage solution between cycles. After these operations, N2 was completely stable during storage over 30 weeks, in terms of BAEE activity and pressure drop, monitored over 3 weeks.

4. Concluding remarks

Trypsin immobilized on CIM monolith columns showed significant activity towards β-Lg. Different from hydrolysis by free trypsin in stirred solution, the efficient contact time between immobilized trypsin and substrate is much shorter than the recirculation time due to 0.6 mL pore volume of IMTRs. Nevertheless, the hydrolysis by N1 reached a much higher DH (9.68%) than that by 1.5 mg free trypsin (6.02%) in 4 h, mainly due to the autodigestion of free trypsin. To avoid this problem, a higher amount of enzyme is necessary to achieve DH_{max}, leading to higher costs. Furthermore, monoliths with large pores and consequently higher permeabilities make long-term use of IMTRs possible. At least 18 times' reuse of N1 significantly reduces costs in total. In order to additionally improve the operational stability, IMTR based on CIM monolith with pore size of 6 µm (N2) was developed and characterised as highly permeable alternative.

It is noted that hydrolysis conditions, such as pH, ionic strength, temperature, etc., are not optimized in this study. The efficiency of these IMTRs in production of β-Lg hydrolysates is

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highly potential to be improved after the optimization of processing parameters. Furthermore, these parameters play great roles not only in the effectiveness, but also in the selectivity. For example, Cheison's work [27] confirms that trypsin hydrolysis in solution at pH 8.5 and 25^{o}C offers the complete removal of β-Lg while the retention of 67.87% native α-La. Hence, optimization of these parameters for IMTRs could allow a better process control such that the desired hydrolysis of the target β-Lg would preferably take place, while α-La remained largely unaffected, at lower levels of denaturation.

Acknowledgements

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The authors have declared no conflict of interest.

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Table 1: Characteristics of CIM-ALD columns (column volume 1 mL) and corresponding IMTRs

Column	Pore size (µm)	BET surface (m^2/g)	Permeability in di-water (m^2)	Amount of immobilized trypsin (mg per mL)	Trypsin surface density (molecules per nm^2)	Activity U* (µmol/min) (substrate BAEE) at pH 7.8 and 10 mL/min
N1	2.15 ± 0.1	5.0 ± 0.4	2.45×10^{-12}	5.0±0.2	11±4	63.86 ± 6.08
N2	5.80 ± 0.3	2.0 ± 0.2	1.91×10^{-11}	3.0±0.3	17±5	36.56 ± 5.56

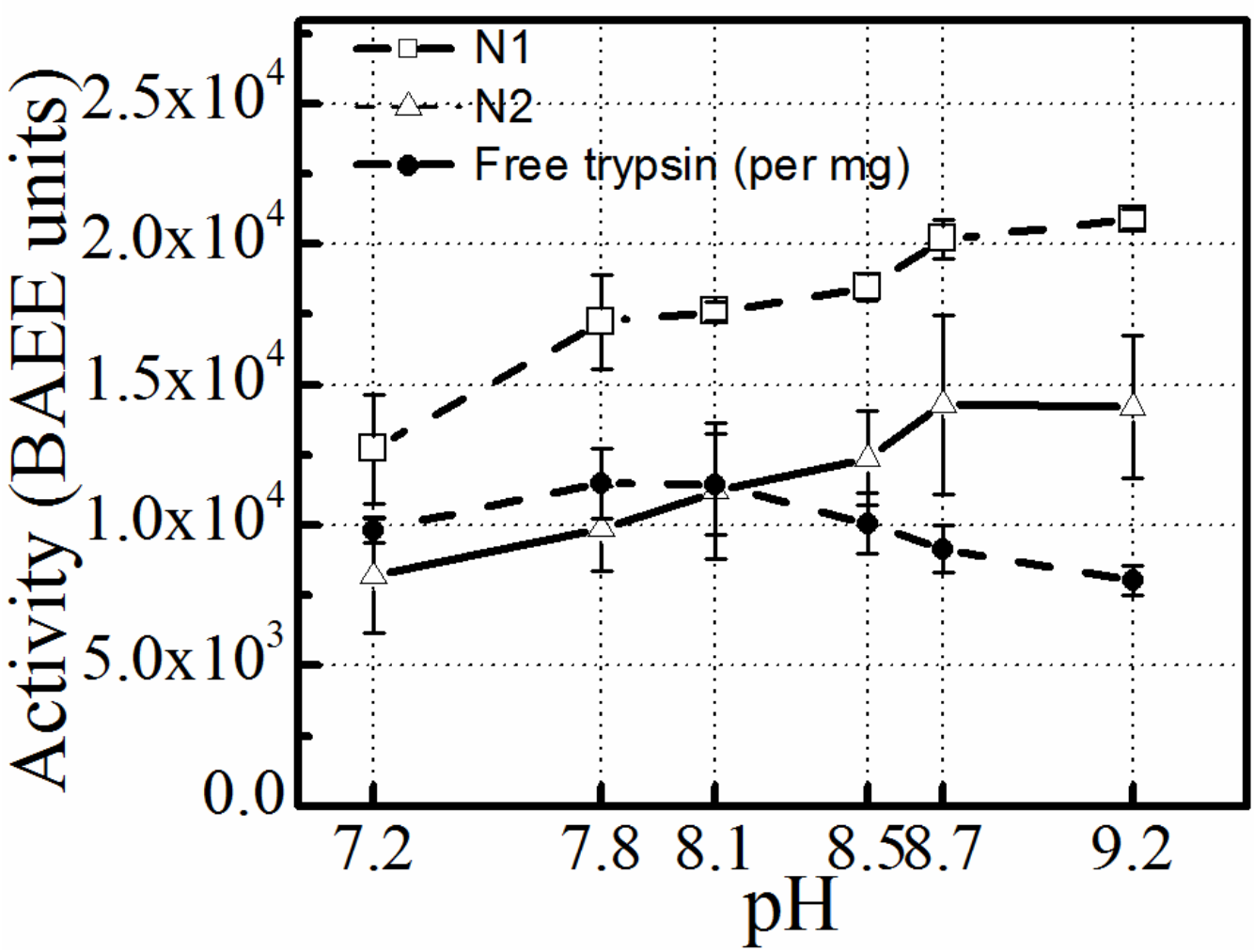
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Figure 1. Activity profiles for IMTRs and free trypsin at increasing pH values and 10 mL/min. The activity measurements at each pH value was repeated at least for three times.



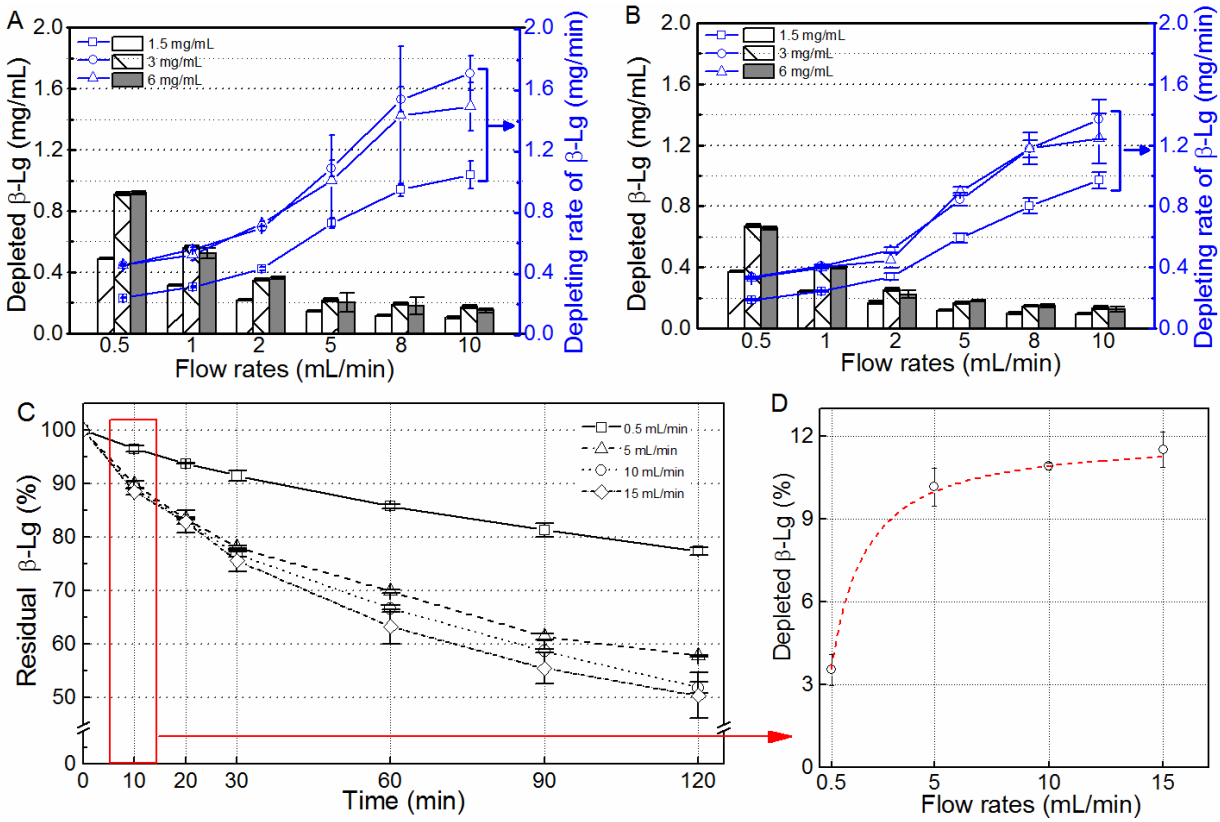
Received: 05 03, 2017; Revised: 06 25, 2017; Accepted: 07 03, 2017

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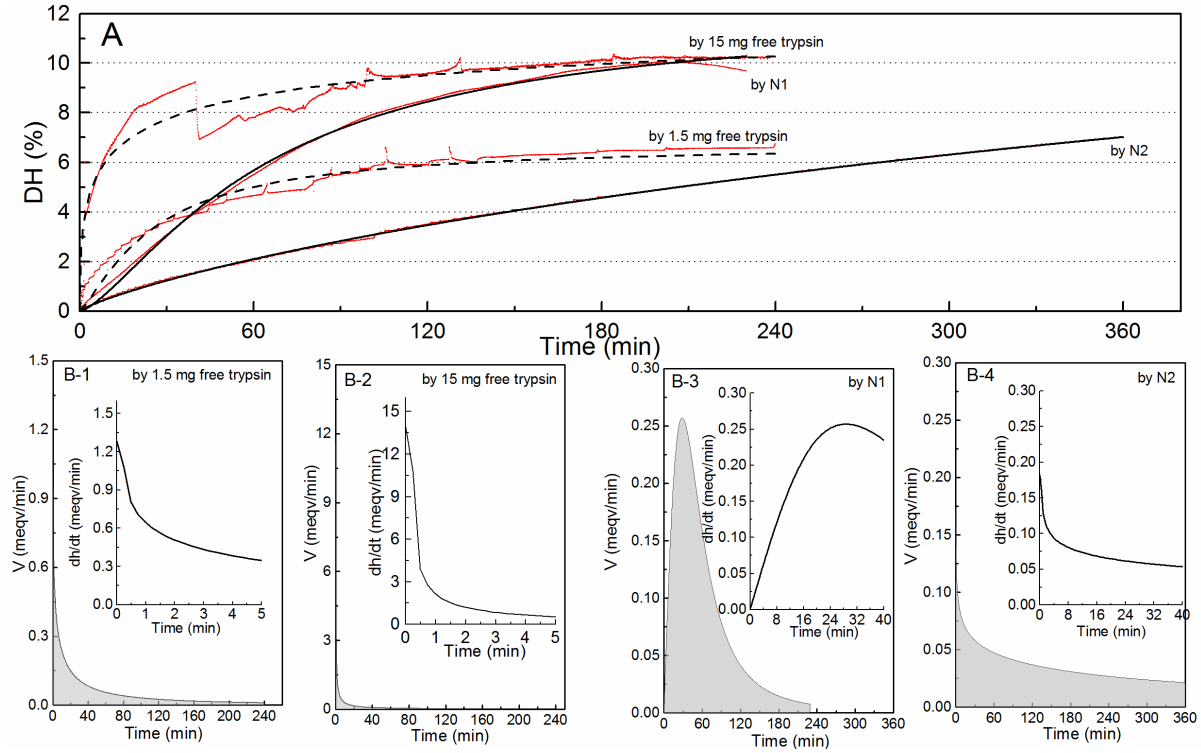
Figure 2. Efficiency of IMTRs in depleting β-Lg: (A) & (B) the depleted amount of β-Lg by N1 and N2 depending on flow rates in single flow-through approach; (C) the residual amount of β-Lg during recirculation at different flow rates by N1; (D) The depleted amount of β-Lg by N1 in first 10 min during recirculation at different flow rates



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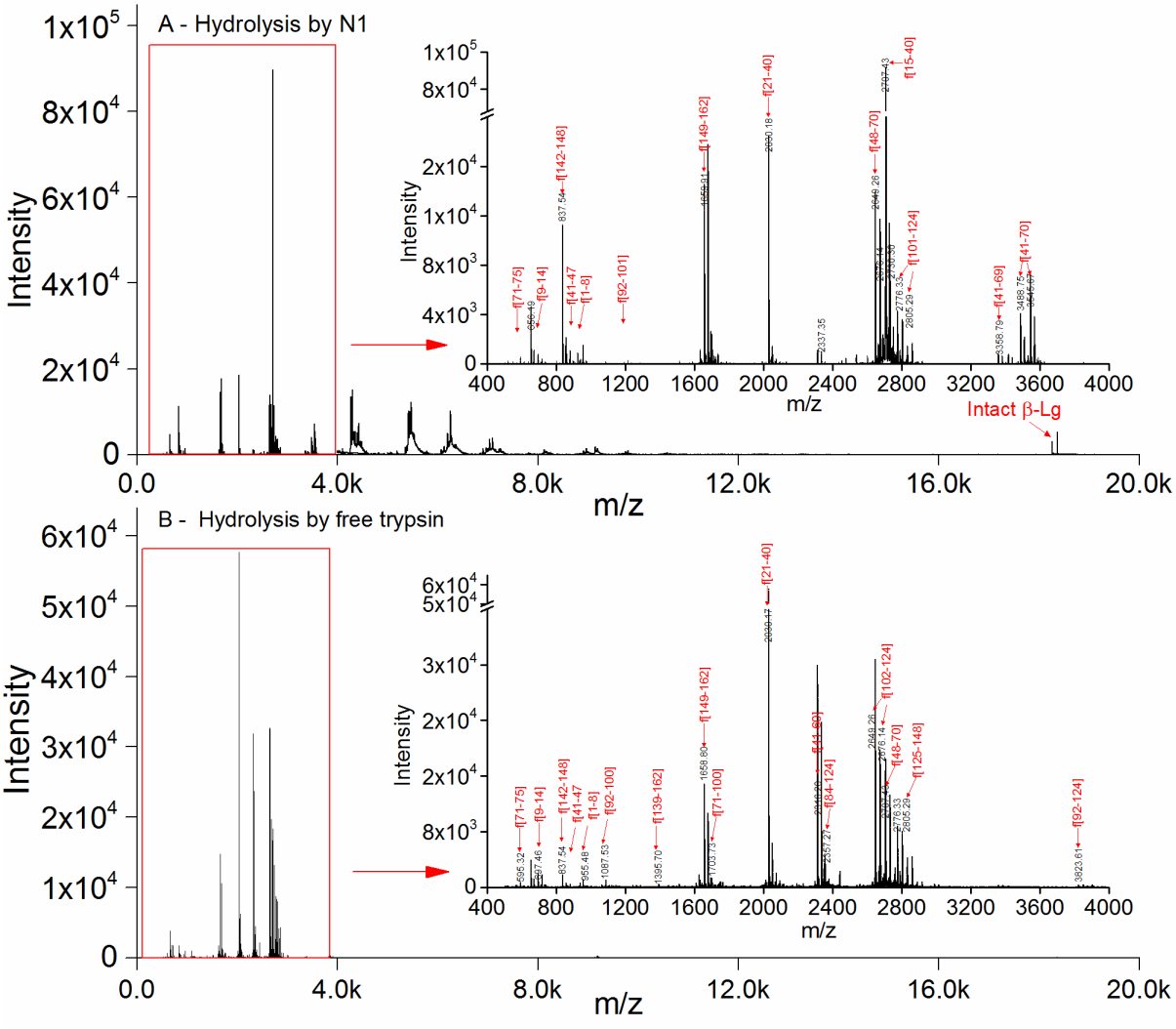
Figure 3. (A): the evolution of DHs depending on time for four hydrolysis experiments; (B): the velocities of these four hydrolysis processes, B-1 ~ B-4 regards to the hydrolysis by 1.5 free trypsin, 15 mg free trypsin, N1 and N2, respectively, in addition, the first 5 min for free trypsin and 40 min for IMTRs were highlighted.



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Figure 4. Mass spectra of samples analyzed using the α-Cyano-4-hydroxycinnamic Acid (HCCA) and 2,5-Dihydroxyacetophenone (DHAP) matrix: (A) sample hydrolyzed by N1; (B) sample hydrolyzed by 15 mg free trypsin



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Supporting Information

Production of β-Lactoglobulin hydrolysates by monolith based immobilized trypsin reactors

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Abbreviations: β-Lg, β-lactoglobulin; α-La, α-Lactalbumin; CIM®, Convective Interaction Media®; IMTRs, immobilized trypsin reactors; N1, immobilized trypsin reactor based on monolith with pore size 2 µm; N2, immobilized trypsin reactor based on monolith with pore size 6 µm; HCCA, α-Cyano-4-hydroxycinnamic Acid; DHAP, 2,5-Dihydroxyacetophenone; BAEE, Nα-Benzoyl-L-arginine ethyl ester; BET, Brunauer–Emmett–Teller; DH, degree of hydrolysis.

Key words: monolith, trypsin, immobilized enzyme reactor, β-Lactoglobulin

Peptides composition analysis using Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry

Sample preparation

Bovine β-Lg has five Cys residues, four of them form disulfide bridges between Cys66 and Cys160, and between Cys106 and Cys119. Cys121 is free and therefore available for reaction. It is well documented that MALDI-TOF mass spectra often shows the expected peaks corresponding to the disulfides accompanied by their reduction products [19]. To avoid the interference from disulfide bonds, reduction with DTT (Dithiothreitol) and Cys-alkylation with CAA (Chloroacetamide) were processed to all samples.

Samples were diluted in water to a protein concentration around 0.5 mg/ml, and the pH after dilution stayed between 7 and 8. 128.6 µl 80 mM DTT was given to 900 µl diluted sample, the mixture was shaked at 37 ^{o}C for 45 min, afterwards 141 µl 400 mM CAA was added and samples were stored in dark for 30 min. To acidify the reduced samples, 6 µl 0.5% formic acid was mixed with each sample. The final pH of samples ready for spotting was below 3. A blank sample (using 900 µl distilled water instead of protein solution) was prepared following same steps as well.

HCCA was dissolved in solvent containing 60% ACN diluted in distilled water and 0.1% TFA. The mixture was ultrasonicated for 10 min to achieve maximum solubility and then centrifuged (3 min, 10,000 rpm) to separate the saturated solution and the rest of the insoluble HCCA. The supernatant was used for spotting. Exactly same procedure was applied to prepare DHAP matrix.

The external standards (protein standard I and PAS, Bruker Daltonics GmbH, Bremen, Germany) were dissolved in 500 µL of the same solvent that was used for the DHAP and HCCA. For the calibration.

1 µl sample (including blank sample) or standards was mixed with 1 µl HCCA matrix or DHAP matrix directly on anchor target (stainless steel MTP 384) by pipetting up and down several times. Every sample was spotted at least three times. Spots on target were dried in fume hood at room temperature.

Mass Spectrometry

MALDI-TOF/MS was managed using flexControl 3.0 Software (Bruker Daltonics, Bremen, Germany). Spectra based on the HCCA-matrix were obtained at reflectron mode, and the method PepMix.par was chosen and modified. The samples with DHAP-matrix was analyzed at linear mode by method ProtMix.par. The laser strength was optimized at 30% for HCCA-matrix and 60% for DHAP-matrix. Each spot was shot at least 5 times manually at different parts.

Peptides analysis

The peaks in blank sample was picked using flexAnalysis 3.0 (Bruker Daltonics, Bremen, Germany) firstly and imported into mass control list of theoretical trypsin auto digests, then this new mass control list was set as background peaks. These background peaks were removed from the picked peaks of samples before analysis. A theoretical peptides database from β-Lg digested by trypsin was built using the Sequence Editor (Bruker Daltonics, Bremen, Germany). By comparing the mass of picked peaks in sample with this database using a written code in software R 3.3.3 (open source software), the sequences were assigned to each peak.

Supporting information tables

Supporting information Table 1A Identification of peptides obtained by free trypsin hydrolysis of β-Lg

Calculated mass [a)]	Observed mass [b)]	S/N	Intensity	Assigned Sequence
595.36	595.38	48.80	1053.26	[71- 75]
674.42	674.45	18.50	405.58	[78- 83]
695.39 [Na]	695.39	51.00	1126.14	[9- 14]
739.46 [K]	739.34	14.30	320.95	[70- 75]
837.48	837.49	124.60	2983.49	[142-148]
880.02	881.44	29.80	729.78	[41- 47]
938.47	938.46	38.10	958.32	[84- 91]
955.54 [Na]	955.53	68.20	1739.05	[1- 8]
1064.13 [B]	1064.45	13.10	364.25	[61- 69]
1087.58	1087.57	55.30	1571.36	[92-100]
1122.17 [A]	1122.46	11.10	325.95	[61- 69]
1245.59	1245.82	17.10	1055.66	[125-135]
3178.64	1395.64 *	14.10	553.41	[139-162]
1679.78 [Na]	1680.77	266.20	14016.42	[149-162]
3401.93	1702.75 *	30.10	1596.03	[71-100]
2029.06	2030.07	895.90	55472.75	[21-40]
2313.26	2313.24	451.60	30620.22	[41- 60]
4716.30 [B]	2357.20 *	62.20	4136.44	[84-124]
4744.33 [A]	2373.16 *	11.40	754.73	[84-124]
2646.06 [B]	2647.15	517.60	27628.98	[102-124]
2674.11 [A]	2675.18	313.10	16361.50	[102-124]
2705.32 [Na]	2705.23	193.10	9817.88	[48- 70]
2707.38	2708.33	103.20	5252.95	[15- 40]
2804.45 [k]	2803.28	148.00	6964.99	[125-148]
3821.43 [B]	3821.68	10.10	119.51	[92-124]
3849.48 [A]	3849.70	14.20	161.92	[92-124]
3873.33 [B]	3873.78	11.40	731.00	[102-135]
3901.39 [A]	3901.63	12.10	130.30	[102-135]

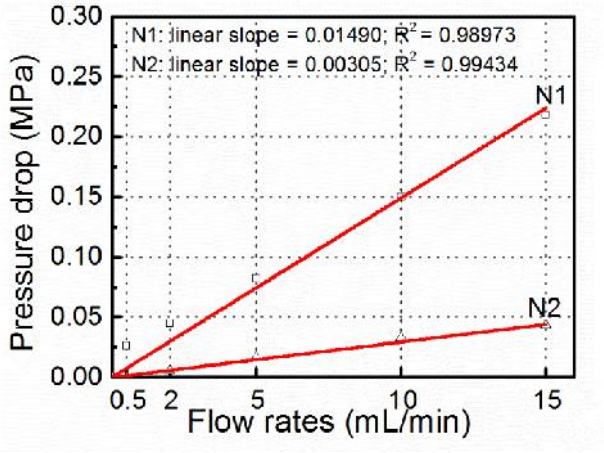
Supporting information Table 1B Identification of peptides obtained by N1 hydrolysis of β-Lg

Calculated mass [a)]	Observed mass [b)]	S/N	Intensity	Assigned Sequence
573.36	573.50	22.80	3804.20	[71- 75]
673.39	673.53	15.90	2696.44	[9- 14]
674.42	674.55	19.00	3183.10	[78- 83]
739.456 [K]	739.38	11.80	160.99	[70- 75]
837.48	837.53	159.80	2240.08	[142-148]
880.02	881.48	36.90	525.56	[41- 47]
903.57	925.59	43.30	628.99	[76- 83]
933.54	933.68	22.10	3344.63	[1- 8]
938.47	938.49	17.70	260.71	[84- 91]
1087.58	1087.70	25.80	3442.63	[92-100]
1193.68	1193.84	20.60	1420.95	[92-101]
1635.78	1635.86	13.90	316.23	[125-138]
1680.83 [Na]	1680.86	154.80	3520.47	[149-162]
3401.93	1702.848 *	25.70	597.50	[71-100]
2029.06	2030.18	27.50	7352.10	[21-40]
2313.26	2313.36	18.70	569.61	[41- 60]
2647.31 [B]	2647.19	276.90	7653.24	[48- 70]
2707.38	2707.39	1460.10	38111.90	[15- 40]
5435.60 [B]	2719.221 *	13.50	556.91	[92-138]
2722.98 [A]	2723.38	21.00	748.42	[48- 70]
2707.38	2729.37	214.20	5708.41	[15- 40]
5463.63 [A]	2733.271 *	101.40	2807.11	[92-138]
2772.27 [B]	2772.33	17.60	562.44	[101-124]
2802.28 [A]	2803.34	77.60	1997.31	[101-124]
3357.68 [B]	3358.63	25.30	439.38	[41- 69]
3415.68 [A]	3416.64	19.40	322.41	[41- 69]
3485.78 [B]	3486.72	98.30	1401.68	[41- 70]
3543.78 [A]	3544.74	166.60	2226.37	[41- 70]
18265.39 [B]	9142.34*	9.10	1834.00	Intact β-Lg
18351.42 [A]	9183.16*	7.30	1456.00	
18265.39 [B]	18282.29	8.10	425.00	
18351.42 [A]	18367.33	6.40	338.00	

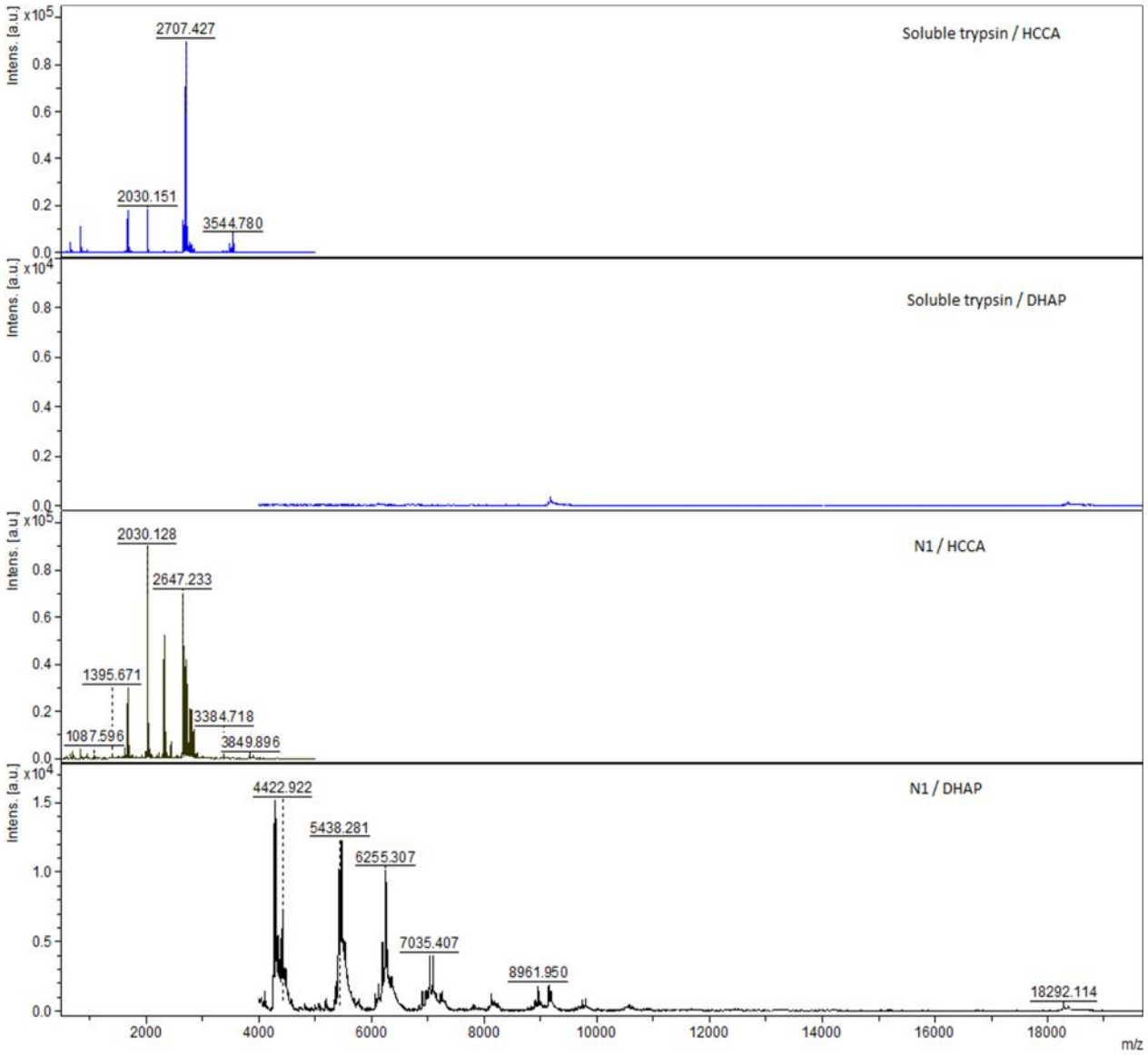
a): Monoisotopic mass with single charge, calculated from amino acid sequence. A represents this sequence particularly from β-Lg A, and B is from β-Lg B. There are two amino acids different as shown in Figure S3. Na and K represent sodium and potassium adduct, respectively.

b): Observed mass with single charge except for those marked with *, * means double charged mass.

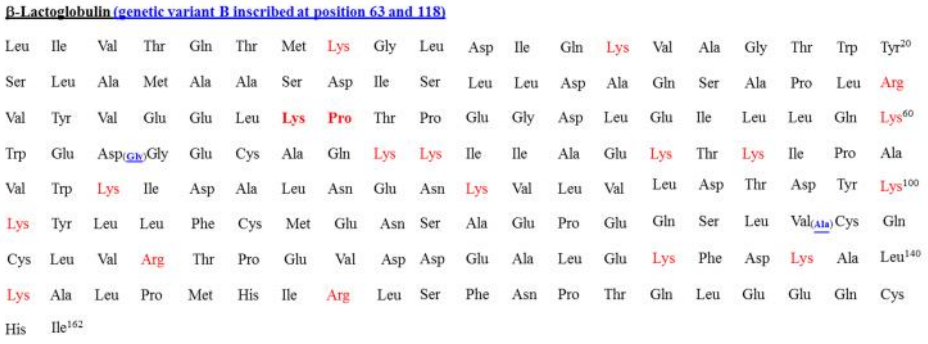
Supporting information figures



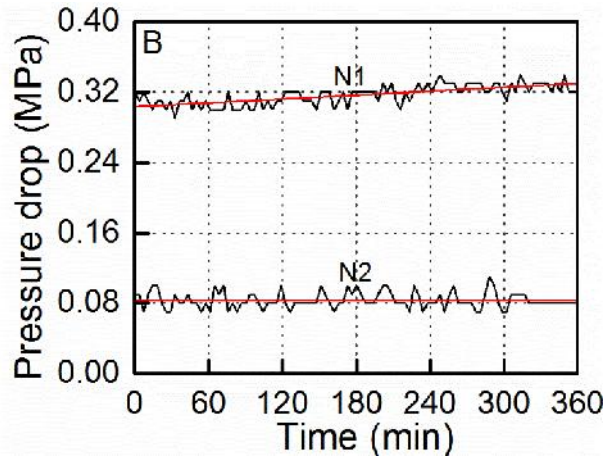
Supporting Information Figure 1. Pressure drop of freshly prepared IMTRs depending on flowrates



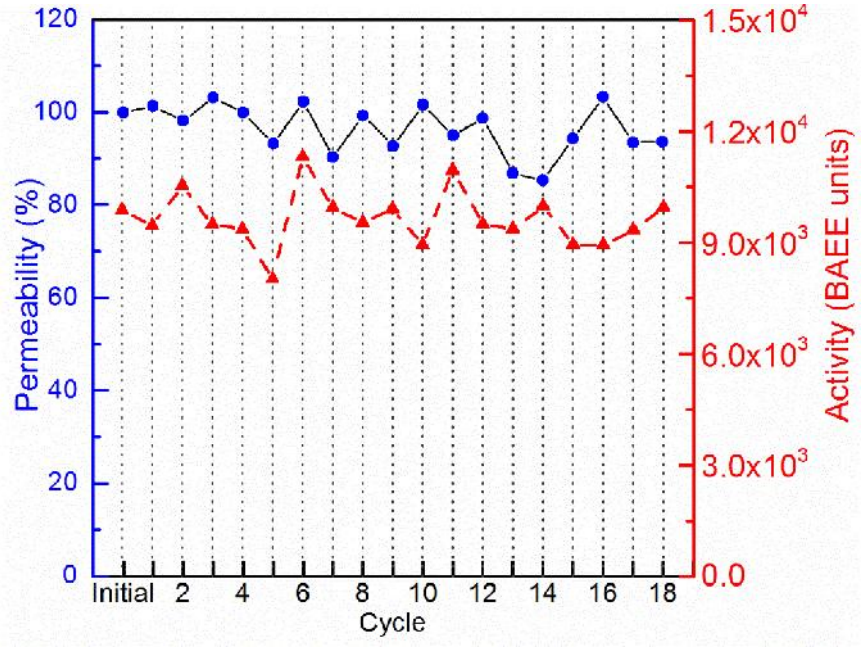
Supporting Information Figure S2. Mass spectrum of samples in different matrix exported directly from Flex analysis software



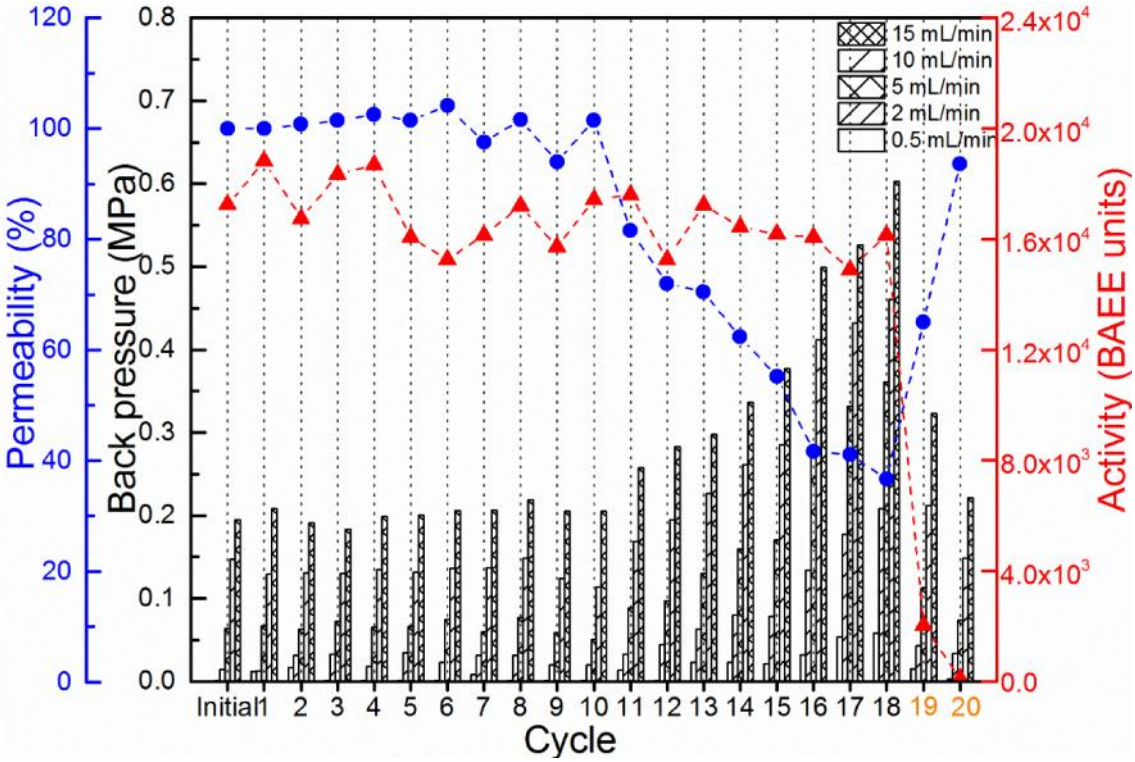
Supporting Information Figure S3. Amino acid sequence of bovine β-Lg (variants A and B). The cleavage sites for trypsin (Lys and Arg) are highlighted.



Supporting Information Figure S4. Pressure drops of N1 and N2 during 6 hours' recirculation with 10 mg/mL β-Lg solution at pH 7.8



Supporting Information Figure S5. Records of pressure drop, permeability and activity of N2 over cycles



Supporting Information Figure S6. Records of pressure drop, permeability and activity of N1 over cycles