Quick Refolding of High-Concentration Proteins via Microchannel Dialysis

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Abstract

The industrial production of active proteins from E. coli necessitates the refolding of high concentrations of protein over a short period of time. However, it is difficult to simultaneously achieve high concentration and short residence time. The dialysis method can refold high concentrations of proteins, but this process takes a long time. The dilution method can quickly refold proteins, but the resultant proteins are inevitably diluted. In the present study, by designing microchannels in dialysis membranes, which can enlarge the surface area for quickly removing protein denaturant, high concentrations of active carbonic anhydrase—eight times more concentrated than achieved using dilution method—were refolded in 20 minutes, which is orders of magnitude faster than the conventional dialysis method.

Keywords

quick refolding, microchannel dialysis, short residence time, high refolding efficiency

1. Introduction

The production of proteins is fundamental to the fields of bio-pharmaceutical production, functional foods, and biosensors, and demand continues to increase (Menzella, Gramajo, & Ceccarelli, 2002; Rasala & Mayfield, 2015; Romanov, Kostromina, Miroshnikov, & Feofanov, 2016; Wells & Robinson, 2017). Proteins can be fabricated using a range of hosts, including *Escherichia coli* , yeast, mammalian cells, and green algae. Among these, *E. coli* , which is inexpensive and has a high proliferation rate, is a valuable resource for mass culture for protein production. However, *E. coli* often produce insoluble protein aggregates, which do not have active three-dimensional structures, and sometimes produce toxic proteins or self-digestive proteases. To recover protein activity, the aggregate must be solubilized using denaturants such as urea or guanidine hydrochloride (GdnHCl), and then reconstructed to produce the original active structure; this process is called "refolding." A recent steep increase in the demand for highly-functional proteins requires technologies for rapid refolding. From an industrial perspective, the size of facilities required for this refactoring should be minimized, requiring efficient production of high concentrations of proteins (Clark, 2001; Eiberle & Jungbauer, 2010; Zhao et al., 2014).

Current refolding techniques can be generally classified into one of three strategies: dilution; dialysis; or solid-phase treatment (Yamaguchi, Yamamoto, Mannen, & Nagamune, 2013). The dilution method involves refolding proteins by diluting denatured proteins by adding 10 to 1000-fold concentrations of buffer to lower the denaturant concentration to allow protein refolding. While the procedure is simple, the concentration of proteins produced after dilution is generally low, so stirring and storing tanks must be quite large. (Clark, 2001; Eiberle & Jungbauer, 2010) In the dialysis method, proteins denatured using a denaturant are placed in a dialysis membrane bag. As only the denaturant can permeate through the membrane, the protein concentration within the dialysis membrane can be maintained at high levels during the refolding process.

The downside of this method is that the process of refolding can take days, lowering productivity and inducing the aggregation of high concentrations of intermediate proteins (Yamaguchi, Miyazaki, Briones-Nagata, & Maeda, 2010). The third approach is the solid-state method. In this method the denaturant is removed using chromatography, solid particles, or gels (Batas & Chaudhuri, 1996; Lanckriet & Middelberg, 2004; Li et al., 2009). Column chromatography can be used for protein purification and is easily automated. As the denaturant is rapidly removed from the protein, aggregation generally occurs in the top section on the column (Yamaguchi et al., 2013).

For industrial scale refolding, the protein concentration after refolding should be high enough to reduce the amount of processing required. There has been some research into high-concentration refolding (Batas & Chaudhuri, 1996; Li et al., 2009; West, Chaudhuri, & Howell, 1998; Zhao et al., 2014). While protein concentrations are lower during refolding using the dilution or chromatography methods, the dialysis method can produce high levels of refolded proteins because of the reduction in protein dilution achieved by using a dialysis bag. However, this method can be time consuming, because the rate-determining step of the process is the removal of denaturant through dialysis membrane. The membrane surface area—that is, membrane area per volume of denatured protein solution—is small in the conventional method (Kohyama, Matsumoto, & Imoto, 2010; Maeda, Koga, Yamada, Ueda, & Imoto, 1995). If the surface area of the dialysis membrane can be enlarged, higher concentrations of protein could be recovered over shorter time periods.

To address this problem, in the work reported in the present study we developed a dialysis refolding method using microchannels, which can produce a large surface area to volume ratio. There have been studies on the application of microchannels to refolding, but all of them have been developed for dilution methods (Kashanian, Masoudi, Shamloo, Habibi-Rezaei, & Moosavi-Movahedi, 2018; Yamaguchi & Miyazaki, 2015; Yamaguchi et al., 2010; Yamamoto et al., 2010; Zaccai, Yunus, Matthews, Fisher, & Falconer, 2007), rather than dialysis methods. In research combining dialysis and microchannels, there have been reports on areas such as the development of bioassays (Imura, Yoshimura, & Sato, 2013), separation of single- and double-stranded DNA (Sheng & Bowser, 2014), and pH adjustment of microemulsions (Hood, Vreeland, & DeVoe, 2014). There has been no application to protein refolding.

In this study, to facilitate the preparation of microchannels for dialysis refolding, rational design involving the permeability of denaturant through dialysis membranes was used. First, the permeation coefficient of the denaturant through the dialysis membrane was determined. Then, the details of the microchannels, which can reduce the denaturant concentration in a designated time, were designed using this coefficient. Finally, using the fabricated microchannels, the reductions in denaturant concentration and the refolding of a model protein with a short residence time were investigated. As a model protein, Carbonic Anhydrase, the enzymatic reaction of which can be traced by hydrolysis of *p* -nitrophenyl acetate to *p* -nitrophenol, was used (Ikai, Tanaka, & Noda, 1978).

2. Materials and Methods

2.1. Materials

GdnHCl, *p* -nitrophenyl acetate, acetone, ethanol, and hydrochloric acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). Coomassie Plus (Bradford) Assay^{TM} kits for measuring the protein concentration were purchased from Thermo Fisher Scientific K.K. (Rockford, USA). Carbonic Anhydrase from bovine erythrocytes was purchased from Sigma-Aldrich Co. LLC (St. Louis, USA). Sylgard184 was obtained from Dow Corning Corp. (Midland, USA), and cellulose tubes (MWCO 12–14 kDa, pore size 5 nm, thickness 39.2 μm, diameter 28.6 mm) were purchased from Nihon Medical Science, Inc. (Tokyo, Japan).

2.2. Methods

The permeation coefficient of the denaturant through the dialysis membrane was obtained as described in Section 2.2.1, and this coefficient was used to design microchannels with a high specific surface area, in order to enable dialysis with short residence times, as described in Section 2.2.2. Using these microchannels, the reduction of the denaturant concentration with a predetermined residence time was measured, as described

in Section 2.2.3. Finally, refolding of the model protein was performed using the microchannels, and refolding with a short residence time was investigated as described in Section 2.2.4.

2.2.1.Measurement of the permeation coefficient of GdnHCl through dialysis membranes

The characteristics of mass transfer through a dialysis membrane in both conventional dialysis and microchannel dialysis are the same when the same dialysis membrane is used. Therefore, the permeation coefficient of the denaturant through the dialysis membrane obtained by normal dialysis can be used for microchannel design. The permeation coefficient was determined using a custom permeation cell (Fig. 1). The dialysis membrane was sandwiched between the feed chamber and the permeation chamber, and 1 M GdnHCl aqueous solution was poured into the feed chamber, while pure water was poured into the permeation chamber. In each chamber 117 mL of the appropriate solution was introduced; the effective area of the dialysis membrane was 9.62 cm^2. Each chamber was thoroughly stirred with a stirrer tip. The absorbance of the solution in the permeation chamber, collected at a predetermined time, was measured to determine the GdnHCl concentration using an ultraviolet-visible (UV-vis) spectrophotometer (JASCO, V-650 spectrometer).

By considering the mass balance of GdnHCl in the feed and permeation chambers, and through the dialysis membrane, the following formula can be derived.

$$\frac{V_p + V_f}{V_p \cdot V_f} \cdot \frac{\mathrm{PA}}{L} dt = d(\ln(\Delta c)), \quad (1)$$

where the concentration difference between the two chambers $\Delta c = c_{\mathrm{f}} - c_{\mathrm{p}}[\mathrm{mol/m^3}]$, c_{f} and c_{p} [mol/L] are the GdnHCl concentration in the feed and permeation chambers, and V_{f} and V_{p} [$\mathrm{m^3}$] are the volume of the feed and permeation chambers, P [$\mathrm{m^2/s}$] represents the permeation coefficient of the dialysis membrane, L[m] represents its thickness, and A[$\mathrm{m^2}$] is its area.

In the early stages of the permeation experiment, the GdnHCl concentration in the permeation chamber, c_{p}, was low enough to assume that the concentration difference, Δc , was almost equal to the initial concentration of the feed chamber,c_{f0}, and the mass balance equation can be approximated as

$$\frac{\mathrm{PA}}{L} c_{f0} \cdot t = c_p \cdot V_p \quad (2)$$

The permeation coefficient of GdnHCl through dialysis membrane was calculated using eq. (2). Atomistic molecular dynamics simulations predicted that the diffusivity of GdnHCl depends on the concentration of GdnHCl; for example, diffusivity at 5 M GdnHCl is around one third of that at 1 M GdnHCl (Gannon, Larsson, Greer, & Thompson, 2008). The permeation coefficient, P , used for the microchannel design was modified accordingly.

2.2.2. Design and fabrication of microchannels

From the permeation coefficient determined in Section 2.2.2 and the designated dialysis residence time, the specific surface area of the dialysis membrane—the channel area with respect to the channel volume—was determined. As shown in Fig. 2, a microchannel is assumed to have a dialysis membrane sandwiched between a feed-side channel and a permeation side channel, and GdnHCl permeates from the feed side to the permeation side through the dialysis membrane. When GdnHCl solution was supplied with the concentrations c_{f0} [mol/L] and c_{p0} [mol/L] and flow ratesv_{f} [$\mathrm{m^3/s}$] andv_{p} [$\mathrm{m^3/s}$] from the inlet of the feed-side channel and the permeation side channel, the following equation can be derived for the mass balance of GdnHCl, assuming thatv_{f} and v_{p} are constant throughout the flow channel:

$$\frac{v_p + v_f}{v_p \cdot v_f} \cdot \frac{P \cdot WZ}{L} = \ln \frac{c_{f0} - c_{pZ}}{c_{fZ} - c_{p0}}, \quad (3)$$

where W [m] and Z [m] represent the width and length of the microchannel, and c_{fZ} [mol/L] and c_{pZ} [mol/L] are the concentrations of GdnHCl at the feed side and permeation side outlets, respectively. The following formula can be established for the mass balance of the inlet and outlet:

$$(c_{fZ} - c_{f0}) \cdot v_f + (c_{pZ} - c_{p0}) \cdot v_p = 0 \quad (4)$$

At the flow channel design stage, assuming that a sufficient permeate flow rate is applied ($v_p >> v_f$), eq. (3) can be simplified to eq. (5).

$$\frac{1}{v_f} \cdot \frac{P \cdot WZ}{L} = \ln \frac{c_{f0} - c_{pZ}}{c_{fZ} - c_{p0}} \quad (5)$$

When using the channel height on the feed side d [m] and the residence time τ [s], the channel volume is $WZd = v_f \cdot \tau$, and eq. (5) becomes

$$\frac{\tau}{d} \cdot \frac{P}{L} = \ln \frac{c_{f0} - c_{pZ}}{c_{fZ} - c_{p0}} \quad (6)\,.$$

In this study, the standard residence time, τ, of the microchannel was set to 20 minutes, much shorter than the residence time of conventional dialysis processes, which usually take several days, and longer than the time required for the folding of the model enzyme, CA, which is around 10 minutes (Ikai et al., 1978). Within this residence time, the channel height d ($= WZd\,/\,WZ$) required to reduce the GdnHCl concentration from $c_{f0} = 5$ M for protein unfolding to $c_{fZ} = 0.5$ M required for refolding (Cleland & Wang, 1992; Wetlaufer & Xie, 1995) was determined to be 500 μm, using eqs. (4) and (6).

The channel length was determined by setting a feed volume flow rate $v_f = 0.05$ mL/min and a channel width of 5 mm, to be 40 cm. Using these values, serpentine type microchannels were fabricated (Figure 3). A GdnHCl solution with or without CA was fed into the feed-side inlet, and pure water or folding buffer was fed from the permeation side inlet. As the permeate side channel, poly (methyl methacrylate) (PMMA), which has strength, transparency, and chemical resistance, was used. As the feed-side channel for introducing proteins, poly (dimethylpolysiloxane) (PDMS), which has transparency, low damage, and low adsorptivity to protein, was used. The PDMS flow path was prepared by first fabricating a machined PMMA (convex) mold, into which PDMS prepolymer mixture (Sylgard 184) was poured, and an incubator LTI-601SD (EYELA, Tokyo, Japan) was used for curing the PDMS for one hour at 60 °C (Briones et al., 2006).

2.2.3. Permeation denaturant through microchannels

Using the prepared microchannels, the denaturant (GdnHCl) solution was dialyzed, in order to confirm whether the denaturant concentration could be reduced to the desired concentration within a predetermined residence time, τ.

Using a syringe pump, $c_{f0} = 5$ mol/L GdnHCl solution was fed from the inlet of the feed-side channel, and pure water ($c_{p0} = 0$ mol/L) was fed from the inlet of the permeation side channel at $v_p = 0.5$ mL/min. The flow rate of the GdnHCl solution on the feed side was changed from $v_f = 0.1$ mL/min (retention time $\tau = 10$ min), 0.05 mL/min ($\tau = 20$ min), to 0.033 mL/min ($\tau = 30$ min). The feed flow rate was changed at dialysis time $t = 50$, 150 minutes. The solutions were collected from the outlets of the feed-side channel and the permeate side channel, and the absorbances were measured using a UV-vis spectrophotometer, to determine the GdnHCl concentration.

2.2.4. Refolding of denatured proteins

Conventional dilution refolding and batch dialysis refolding were carried out to produce reference data for the microchannel dialysis refolding. CA denatured with GdnHCl as described below was used for all methods.

(1) Preparation of denatured proteins

Denatured CA (0.25 mg/mL) was prepared by dissolving CA in 0.05 M Tris-HCl (pH 7.5) and 5 M GdnHCl at room temperature overnight and filtering with a 0.45 μm polysyringe philtre (product name) before use, to produce denatured CA solution.

(2) Refolding of denatured proteins

Conventional 10-fold dilution method

10 mL of denatured CA solution was diluted 10-fold with 0.05 M Tris-HCl buffer (pH7.5) at room temperature. The diluted solution was centrifuged at 4,000 rpm for five minutes, and left for 10 minutes, after which the concentration and enzyme activity of the supernatant were measured.

Conventional batch dialysis method

A10 mL volume of denatured CA solution was placed in a dialysis bag and dialyzed overnight at room temperature against 90 mL of 0.05 M Tris-HCl buffer. The collected solution was centrifuged at 4,000 rpm for five minutes, and the concentration and activity of the supernatant was measured.

Microchannel dialysis method

The denatured CA solution was fed from the feed channel inlet of the microchannel in Fig. 3 at a rate of 0.05 mL/min (τ = 20 min), and 0.05 M Tris-HCl refolding buffer was fed from the inlet of the permeation channel at a rate of 0.5 mL/min. The solution was collected from the outlet of the feed-side channel for each 30 minutes, and the amount of collected samples, CA concentrations, and enzyme activities were measured.

Reference system (native CA)

As a reference, native CA as purchased was dissolved in 0.05 M Tris-HCl buffer to a concentration of 0.25 mg/mL, and its concentration and activity were measured.

(3) Measurement of activity and concentration of enzyme

The enzyme activity of native and refolded CA was determined via esterase activity assessment (Ikai et al., 1978). A mixture of 1.5 mL of pure water and 1 mL of p -nitrophenyl acetate solution (3 mL in acetone) was placed in a quartz cell. After 60 seconds 0.4 mL of 0.05 M Tris-HCl buffer was added, and after another 60 seconds 0.1 mL of each of the CA solution samples prepared as described in Section (2) was added. The increase in absorbance at 400 nm due to hydrolysis of$p$ -nitrophenyl acetate was monitored.

The concentration of CA was measured using Coomassie Plus (Bradford) Assay kits (Thermo Fischer Scientific. co., Waltham, USA) and was normalized using 0.25 mg/mL native CA.

3. Results and Discussion

3.1. Design and fabrication of microchannels

Using the diffusion cell shown in Fig. 1, the permeability of the denaturant GdnHCl through the dialysis membrane was determined. Fig. 4 shows the time course of the GdnHCl concentration in the permeation chamber. Eq. (2) and the concentration from 0 to 60 minutes, in which the gradient of the GdnHCl solution concentration difference in the two chambers can be assumed to be constant. These data were used to determine the permeation coefficient P , which was calculated as 1.1×10^{-10} m^2/s. The diffusion coefficient in the solution was about 5 x $10^{-10} m^2/s$ (Gannon et al., 2008). After 1440 min the concentrations in the feed and permeation chambers became almost equal. As the diffusion coefficient of GdnHCl was reported to be concentration dependent, and that at 1 M was around three times that at 5 M (Gannon et al., 2008), which is the concentration of feed used for the microchannel, P used for the microchannel design was collected with the scale factor.

3.2. Permeation of denaturant via microchannels

Using the value of P determined in as described in Section 3.1, a microchannel with channel height d = 500 μm, width W = 5 mm, total length Z = 40 cm was designed and fabricated. A 5 M GdnHCl solution was fed from the feed channel inlet, pure water was fed from the permeation channel inlet, and the GdnHCl concentrations at the feed and permeation channel outlets were measured (Fig. 5). By changing the flow rate of the feed side, the residence time, τ , in the channel was changed to be 10 minutes (in a time range of 0–50 minutes), 20 minutes (in a time range of 50–150 min), or 30 minutes (in a time range of 150–300 minutes). After switching the flow rate, the GdnHCl concentrations converged to a constant value for both the feed and permeate sides for each residence time. The expected GdnHCl concentration at the feed outlet, c_{fZ}, calculated using eq. (3) is as shown in Table 1. The results shown in Fig. 5 are almost identical to the values calculated using eq. (3), and confirmed that the microchannel functioned as designed. The GdnHCl concentration required for model protein refolding was 0.5 mol/L or less. The concentration was attained at a residence time of 20 minutes at the feed outlet. It therefore appears that the refolding process can be carried out in only 20 minutes.

3.3. Refolding of denatured proteins

To investigate the refolding of the model protein, a 0.25 mg/mL CA solution unfolded using 5 M GdnHCl was fed at a flow rate of 0.05 mL/minute, with a residence time, τ , of 20 minutes, from the feed-side inlet of the microchannel, and 0.05 M Tris-HCl buffer was fed from the permeation side inlet. Refolding was carried out by removing GdnHCl from the feed side to the permeation side through the dialysis membrane. The result of the microchannel refolding was compared with that of refolding using the conventional 10-fold dilution and batch dialysis methods (Fig. 6).

The CA concentrations shown in Fig. 6a represent the CA concentrations after refolding, and the protein recovery data shown in Fig. 6b is the ratio of the amount of soluble CA after folding (CA concentration [mg/mL] × recovered liquid amount [mL]) to the amount of soluble CA before refolding (CA concentration [mg/mL] × feed solution volume [mL]). The relative recovery shown in Fig. 6c represents the CA activity after refolding (activity per concentration) when the enzyme activity of native CA is considered to be 100%. The total recovery shown in Fig. 6d is the product of relative activity (Fig. 6c) and CA recovery amount (Fig. 6b), and represents the total recovery rate of active CA for each refolding method when that of native CA is taken to be 100%.

From the data shown in Fig. 6a, the CA concentration after refolding was 0.025 mg/mL using the conventional dilution method, which was almost the same as the dilution factor (10-fold), whereas in batch dialysis and microchannel dialysis, the concentration was about 0.20 mg/mL, about 20%–30% lower than the initial value. Table 2 shows the relationship between the amount of solution supplied from the microchannel inlet and that recovered at the outlet for microchannel dialysis. The solution amount increased by around 1.3 times during the dialysis. For batch dialysis, the solution amount before dialysis was 10.0 g and that after dialysis was 11.4 g, although not all of the solution was recovered. The results indicate that for both methods, the amount of solution increased during dialysis.

The water possibly moved from the permeate side to the feed side through the dialysis membrane due to the osmotic pressure of GdnHCl. When a permeation experiment was attempted with a 5 M GdnHCl feed solution, as described in Section 2.2.1, the amount of solution in the feed side increased, and that in the permeate side decreased, strongly suggesting the effect of osmotic pressure (data not shown). Nevertheless, the concentration of CA remained high in both dialysis processes, as shown in Fig. 6a.

The CA recovery rate for this process is shown in Fig. 6b. Almost 100% of CA was recovered using the 10–fold dilution and microchannel dialysis methods, and protein aggregation did not occur. Using the conventional dialysis method, the recovery rate is slightly lower, at about 80%.

A comparison of the enzymatic activity after refolding is shown in Fig. 6c. Since the enzymatic activity depends on the CA concentration, activity normalized by CA concentration was compared. As a reference point, the activity of native CA was taken to be 100%. The recovery rate of the activity using the 10-fold dilution method was about 40%, which is consistent with previous reports (Yamaguchi et al., 2010). CA is

known to be a protein that is relatively difficult to refold. Activity reached 100% in batch dialysis refolding, and 10% higher activity in microchannel dialysis refolding on average. The raw data for enzymatic activity measurement are shown in the Supporting Information. Conventional batch dialysis was reported to cause collisions and agglomeration of intermediates during relatively long refolding times, (Yamaguchi et al., 2010) while in microchannel dialysis, the residence time during which these intermediates can be produced is short. It is possible that the activity normalized by enzyme concentration exceeds 100%, as has been reported in the literature (Batas & Chaudhuri, 1996). The state after refolding may be better than the native folding state, which was prepared by dissolving the purchased enzyme in buffer.

The total esterase activity of the CA as a product of the CA recovery (in Fig. 6b) and the normalized enzymatic activity (in Fig. 6c) was shown in Fig. 6d. Microchannel dialysis was shown to be superior among the three refolding methods.

As shown in Fig. 6a–d and Table 2, microchannel dialysis produced stable results with respect to the final concentration, activity, and flow rate, in 30–150 minutes. The CA concentration on the permeate outlet side was always less than 0.01 mg/mL, which was negligible sufficiently smaller than that on the feed side; the permeation of CA through the dialysis membrane was almost negligible (data not shown).

Table 3 summarizes the results of three protein refolding methods: conventional 10-fold dilution, conventional batch dialysis, and microchannel dialysis. These data clearly show that microchannel dialysis achieved high protein concentrations with sufficient recovery of active proteins, with a short residence time.

One problem with the use of microchannel dialysis is the limitation of the amount of solution which can be processed, due to the small size of the flow path. However, this problem can be addressed by increasing the number of the channels by using, for example, hollow fiber membrane. The concentration of 0.20 mg/mL produced by microchannel dialysis is about eight times higher than that of the dilution method. Nevertheless, even higher concentrations are expected industrially, and the issue of producing higher concentrations of active protein in a short time is currently under investigation.

4. Conclusions

In the present study, a system for microchannel dialysis capable of producing high concentrations of active refolded proteins with a low residence time was designed and demonstrated. Using a short treatment time of around 20 minutes, which is at least an order of magnitude faster than conventional dialysis, it was possible to recover sufficient amounts of active CA, a protein which is difficult to refold. Although the incorporation of a dialysis membrane into a microchannel is technically challenging at present, the concept of shortening the dialysis time by increasing the specific surface area is generally applicable, and can greatly reduce the time required for the preparation of molecules such as purified proteins and synthetic polymers.

Nomenclature

A [m^2]: area of dialysis membrane

c_{f} [mol/L]: GdnHCl concentration at feed side

c_{f0} [mol/L]: GdnHCl concentration at feed channel inlet (for microchannel) / initial GdnHCl concentration in feed chamber (for batch cells)

$c_{\mathrm{f}Z}$ [mol/L]: GdnHCl concentration at feed channel outlet

c_{p0} [mol/L]: GdnHCl concentration at permeation side

c_{p0} [mol/L]: GdnHCl concentration at permeation channel inlet

$c_{\mathrm{p}Z}$ [mol/L]: GdnHCl concentration at permeation channel outlet

d [m]: height of microchannel

L [m]: thickness of dialysis membrane

P [m^2/s]: permeability of GdnHCl through the dialysis membrane

τ [min]: residence time in the microchannel in the feed side

$v_{\,f}$ [mL/min]: flow rate of the feed side

$v_{\,p}$ [mL/min]: flow rate of the permeation side

W [m]: width of microchannel

Z [m]: length of microchannel

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Table 1. Assumed GdnHCl concentration via microchannel GdnHCl dialysis calculated by eq. (3).

v_f [mL/min]	τ [min]	$c_\mathrm{fZ,calc}$ [mol/L]
0.1	10	1.10
0.05	20	0.384
0.033	30	0.125

Table 2. Measured flow rate [g/min] at inlet and outlet of feed microchannel. x –y means time span of x to $y$min.

inlet	outlet	outlet	outlet	outlet
	30–60	60–90	90–120	120–150
0.0521	0.0653	0.0654	0.0655	0.0662

Table 3. Comparison of the results of conventional 10-fold dilution, conventional batch dialysis, and microchannel dialysis methods. Residence time required for the processing, final CA concentration, and active recovery.

	10-fold dilution	batch dialysis	microchannel dialysis
residence time	2–3 min.[*1]	24 h	20 min.
final CA conc.	0.025 mg/mL	0.190 mg/mL	0.197 mg/mL[*2]
active recovery	43%	99%	112%[*2]

*1. It takes another 10 min for refolding.

*2. The average value for 30–150 min.

Figure legends

Figure 1. Schematic of permeation cell for the measurement of the permeation coefficient of GdnHCl through a dialysis membrane.

Figure 2. Schematic of microchannel dialysis setup. Feed and permeation flow channel were set in counter current.

Figure 3. Design of microchannel dialysis setup; GdnHCl solution with/without enzyme was fed from feed flow channel (PDMS) inlet and pure water/refolding buffer was fed from permeation flow channel (PMMA) inlet.

Figure 4. Time course of GdnHCl concentration in permeation experiment. Inset represents the magnification of first 100 min.

Figure 5. Time course of GdnHCl concentration in microchannel permeation. Feed flow rate was 0.1 mL/min ($\tau = 10$ min) in 0–50 min, 0.05 mL/min (20 min) in 50–150 min, 0.025 mL/min (30 min) in 150–300 min.

Figure 6. (a) CA concentration after refolding (CA concentration before refolding is 0.25 mg/mL), (b) recovered soluble CA after refolding. CA amount (CA concentration [mg/mL] × solution amount [mL]) before refolding is set as 100%, (c) relative activity of CA after refolding. CA esterase activity normalized by CA concentration was compared. (d) total activity of CA after refolding, which is the product of protein recovery and relative activity.

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