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Refolding of difficult-to-fold proteins by a gradual decrease of denaturant using microfluidic chips

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Protein refolding is an important process to obtain active recombinant proteins from inclusion bodies (protein aggregates). However, the conventional refolding method of dialysis or dilution is a time consuming procedure and often, recovering yields of active proteins are low. In this study, we used controllable diffusion through laminar flow in microchannels to control the denaturant concentration. The performance of the designed microfluidic chips was evaluated by the refolding of difficult-to-fold proteins (citrate synthase and the ζ-associated protein 70-kDa protein kinase domain). We demonstrated this by varying the flow rates of the diluting buffer stream(s) and multi-junctions which could control the different flow rate ratios of the buffer stream(s) and the denatured protein stream. By this strategy, we were able to improve the efficiency of protein refolding. Our method achieved refolding within a short period of time at room temperature without the need of any small molecules or chaperone proteins. Moreover, the efficiency of protein refolding by microfluidic chip was found higher than that prepared by dialysis or dilution. These results suggest that microfluidic chips employing this strategy may provide miniaturized tools for rapid and efficient recovery of active proteins from inclusion bodies.

Keywords: denatured protein/inclusion body/microfluidic chip/multi-dilution/protein refolding.

Abbreviations: CAB, carbonic anhydrase B; CS, citrate synthase; Gdn-Cl, guanidinium chloride; NBD-NH_2, *N*-(4-nitrobenzo-2-oxa-1,3-diazolyl)amine; PDMS, poly(dimethylsiloxane); PEEK, poly(ether-etherketone); ZAP-70, ζ-associated protein 70 kDa.

Biologically active recombinant proteins are useful for studies of biological functions of genes and for the development of therapeutic drugs (*1*) and bioelements in the industries (*2*). *Escherichia coli* overexpression system is most convenient and frequently used to produce recombinant proteins (*1*). Recombinant proteins often require the assistance of folding modulators such as chaperon proteins. Under the overexpression system, the rate of protein aggregation is often much greater than that of proper folding and chaperon proteins are rapidly functioned (*3*). These inactive and insoluble protein aggregates called inclusion bodies is often a drawback in the use of *E. coli* expression systems (*2*).

Because inclusion bodies contain relatively pure and intact recombinant proteins, several approaches have been reported to refold these aggregated proteins into a biologically active form. In a typical procedure, aggregated proteins are denatured and solved with high concentration of denaturant such as urea or guanidinium chloride (Gdn-Cl). The chemically denatured protein is then refolded to reduce the denaturant concentration sufficiently to allow protein refolding. The dilution method is carried out for this purpose because the procedure is simple: the denatured proteins are merely diluted directly with the refolding buffer which does not contain denaturants. However, this method requires a large volume of buffer to dilute the denaturant to a concentration that does not disturb refolding. Moreover, there are difficulties encountered in uniform mixing in a large volume, wherein reformation of aggregates occurs. The one-step dialysis (high denaturant concentration to the refolding buffer) is another simple method. Because the concentration of denaturant is decreased with increasing dialysing time, the rate of refolding into the native structures increases. However, the rate of misfolding or aggregation will also increase, possibly due to contact between the exposed hydrophobic surfaces (*2*, *4*). This suggests that quick decrease in denaturant concentration initiates the reformation of aggregates as observed in the dilution method. To solve this problem, the step-wise dialysis has been utilized. Denatured proteins are first brought to equilibrium with high denaturant concentration, then with middle concentration, and with low concentration. This suggests that gradual removal of denaturant from denatured proteins can achieve high refolding efficiency (*5*). Although the step-wise dialysis method may give us the refolded (active) proteins, it is a time-consuming procedure (multi-day) and the

proteins often take the inactive form during the refolding process due to the reformation of aggregates and the presence of other incorrectly folding species (*2*, *5*). Recent studies explain that protein aggregation predominantly occurs at middle concentrations of denaturant (*6*, *7*), suggesting that the refolding procedure in a short period of time may reduce the formation of protein aggregation and achieve efficient protein refolding.

Microfluidic reaction system is widely studied and used in chemistry and biotechnology fields (*8*–*10*). A laminar flow in microchannels can be used to create a well-defined and predictable interfacial region among streams. Additionally, a diffusional mass-transfer property is enhanced in the microchannel. These characteristics inspired us to control gradual removal of denaturants from chemically denatured proteins in the laminar flow. Previous microfluidic chips developed for protein refolding were designed to study the initial folding events by rapid mixing of denatured protein solution and refolding buffer through either turbulent flow (*11*, *12*) or diffusion (*13*). In these kinetic studies, easy-to-fold proteins such as cytochrome *c* which have measured folding occurring within 100 μs (*11*) were used. In contrast, difficult-to-fold proteins which often form inclusion bodies lead to protein aggregation in microchannel by rapid mixing (*14*) due to quick removal of denaturant from denatured proteins. It is suggested that the microfluidic chips with rapid mixing are not applicable for refolding of difficult-to-fold proteins. In addition, there is no study yet on the refolding of aggregated proteins in inclusion bodies using the microfluidic chip. In this study, we designed the microfluidic chips to control the distribution of denaturant concentration and investigated their performance on refolding of difficult-to-fold proteins. The concept of refolding protein through microfluidic chips is based on controllable diffusion of the denaturant from the denatured protein stream to the diluting buffer in a laminar flow.

Experimental procedures

Materials

Porcine heart citrate synthase (CS) in an ammonium sulphate suspension, bovine erythrocytes carbonic anhydrase B (CAB), BSA, acetyl-CoA, oxalacetate, 5,5′-dithiobis nitrobenzoic acid and *p*-nitrophenyl acetate were purchased from Sigma-Aldrich (St Louis, MO, USA). Urea and Gdn-Cl were from Wako Pure Chemical (Osaka, Japan). Poly(etheretherketone) (PEEK) tube (360 μm outer diameter, 100 μm inner diameter) was obtained from Upchrch Scientific (Oak Harbor, WA, USA). All other chemicals were of high-quality analytical grade and were purchased from Wako Pure Chemical.

Fabrication of the microfluidic chips

The microfluidic chips were fabricated in poly(dimethylsiloxane) (PDMS) by replica molding as previously reported (*15*). Briefly, channels were fabricated by molding PDMS (SILPOT 184 W/C, Dow Corning Toray, Tokyo, Japan) on a master fabricated on poly(methylmethacrylate) by mechanical micromachining (*15*). The design of the microfluidic chips are shown in Fig. 1A and Supplementary Fig. S1. Main channel dimensions for chips are 500 μm width and 500 μm depth. Channel *a* in chips are 100 μm wide and 500 μm depth. Each channel structure was confirmed using laser microscope analysis (VK-8510; Keyence Co., Osaka, Japan). The reagents were introduced to the microfluidic chips from syringes (Terumo, Tokyo, Japan) by syringe pumps

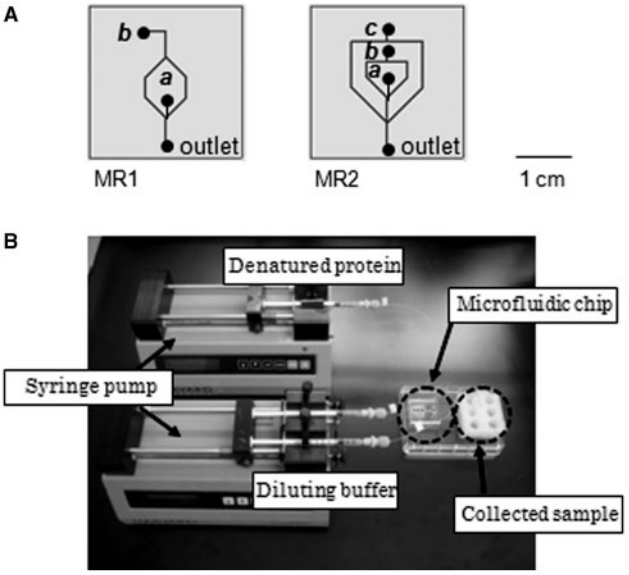

Fig. 1 Schematic representation of protein refolding by microfluidic chips. (A) Microfluidic chips. The microfluidic chips were fabricated in PDMS. The denatured protein was injected into channel *a*. The diluting buffer was injected into channel(s) *b* and/or *c*. Microchannel dimensions for the chips are shown in Supplementary Fig. S1. (B) The assembled microreaction system. The denatured protein and the diluting buffer were introduced into the microfluidic chips from syringes by syringe pumps. PEEK tubes were used to connect syringes and a sample tube with microfluidic chips. The refolded protein was collected in a sample tube. The refolding procedure was carried out at room temperature.

(Pico Plus, Harvard Apparatus, Holliston, MA, USA). PEEK tube was used to connect syringes with microfluidic chips. To suppress non-specific protein absorption, the channel walls, tubes and syringes were coated with NanoBio Blocker (NanoBio Tech, Kanagawa, Japan) following protocols provided by the manufacturer.

Expression and purification of the mouse ζ-associated protein 70-kDa (ZAP-70) protein kinase domain

The plasmid containing mouse ZAP-70 protein kinase domain cDNA was prepared as previously reported (*16*). The resulting plasmid was used to transform *E. coli* JM109 (Invitrogen, Carlsbad, CA, USA). Transformed bacteria were grown on LB/ampicillin (100 μg/ml) plates. A single colony was selected and grown in 6 ml of LB broth containing 50 μg/ml ampicillin overnight with shaking at 37°C. The culture was transferred to 800 ml of LB broth containing 50 μg/ml ampicillin and shaken at 37°C until the absorbance at 600 nm was between 0.5 and 0.6. Following the addition of isopropyl 1-thio-β-D-galactopyranoside (Wako Pure Chemical) to a final concentration of 1 mM, the culture was incubated at 37°C with shaking for 4 h. The cells were harvested by centrifugation at 3,800 rpm at 4°C for 20 min, and the cell pellets were resuspended in 6 ml of phosphate buffered saline with EDTA-free protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany), 8 M urea, 1 mM 2-mercaptoethanol and 0.2% Triton X-100 and then lysed by homogenizer. The cell debris was removed by centrifugation at 11,500 rpm for 30 min at 4°C, and the supernatant was decanted into a 50 ml conical tube to which 1 ml of a 50% slurry of Ni-chelating resin (GE Healthcare, Piscataway, NJ, USA) equilibrated with washing buffer [50 mM phosphate buffer (PB), 500 mM NaCl, 8 M urea, pH 7.0] was added. After incubation with gentle agitation at 4°C for 1 h, the resin was transferred to a column and washed with 5 mM imidazole in washing buffer. The urea-denatured N-terminal histidine-tagged ZAP-70 protein kinase domain was eluted by addition of five bed volumes of 500 mM imidazole in washing buffer. The eluents were dialysed (Slide-A-Lyzer, Pierce, Rochford, IL, USA) against washing buffer for overnight. The obtained protein was denatured ZAP-70.

Preparation of folded proteins

Folded CS was prepared by dialysis against 20 mM Tris–HCl (pH 7.5) to remove ammonium sulphate. CAB and BSA were reconstituted in 50 mM Tris–sulphate (pH 7.5) and in 20 mM PB (pH 7.5), respectively. Folded ZAP-70 protein kinase domain was prepared by the step-wise dialysis (urea concentration: 8 M to 4 M to 0.8 M) in 50 mM PB (pH 7.0). All proteins were filtered with a 0.45 μm polypropylene cellulose syringe filter (Minisart RC4, Sartorius Stedim Biotech, Göttingen, Germany). Concentrations of proteins were determined by Coomassie Plus Bradford assay (Pierce) and were adjusted with each buffer to 250 μg/ml.

Preparation of denatured proteins

Denatured CS was prepared by dissolving Folded CS in 20 mM Tris–HCl (pH 7.5) and 2.5 M urea at 4°C for 1 h. CAB was denatured in 50 mM Tris–sulphate (pH 7.5) and 5 M Gdn-Cl at 4°C for 16 h. BSA was dissolved in 20 mM PB (pH 7.5) and 6 M Gdn-Cl at 4°C for 16 h. All denatured proteins were filtered with a 0.45 μm polypropylene cellulose syringe filter before use.

Refolding of denatured protein using microfluidic chip

Refolded proteins were prepared by injecting the denatured proteins (250 μg/ml) into the centre channel *a* in Fig. 1A. The refolding buffer which depended on the target protein and did not contain any denaturant or chaperone molecules was injected into the channel(s) *b*, *c*, *d* and/or *e* (see Figs 1 and 5). The refolded proteins were collected in a sample tube for 10 min with a total volumetric flow rate of 100 μl/min (collected volume: 1 ml) at room temperature. The denatured proteins were diluted 10-fold by buffer using microfluidic chips in this study. Concentrations of the refolded proteins were determined by Coomassie Plus Bradford assay before analysis.

CS enzymatic activity measurement

Folded, refolded or denatured CS was added to a reaction mixture containing 20 mM Tris–HCl (pH 7.5), 300 μM acetyl-CoA, 100 μM oxalacetate and 100 μM 5,5′-dithiobis nitrobenzoic acid (*17*). The final concentration of CS was 20 nM. Enzymatic reaction was carried out at 30°C for 20 min. The absorbance at 405 nm was measured at room temperature using a spectrophotometer (Multiskan JX, Thermo, Waltham, MA, USA).

CAB enzymatic activity measurement

CAB activity was estimated by its elastase activity as reported previously (*18*). Enzymatic reaction was carried out in 50 mM Tris–sulphate (pH 7.5) at 30°C for 20 min. *p*-Nitrophenyl acetate was used as a substrate. The final concentrations of CAB and *p*-nitrophenyl acetate in the reaction mixture were 70 and 200 μM, respectively. The absorbance at 405 nm was measured at room temperature using a spectrophotometer.

Circular dichroism spectrum measurement

Circular dichroism (CD) spectra were measured on a JASCO J-820 spectropolarimeter (Tokyo, Japan) using a quartz cuvette with a path length of 5 mm at room temperature. Protein solutions (20 μg/ml) were prepared in 20 mM Tris–HCl (pH 7.5) containing 0.25 M urea (for CS), in 20 mM PB (pH 7.5) containing 0.6 M Gdn-Cl (for CAB and BSA) or 50 mM PB (pH 7.0) containing 0.8 M urea (for ZAP-70). An average of 10 scans was obtained for each sample. The averaged blank spectrum (each buffer containing denaturant) was subtracted from the sample.

Microscopic imaging

The hydrodynamically focused flow in microfluidic chips were imaged with a confocal laser scanning fluorescence microscope (TE2000-U, Nikon, Tokyo, Japan) equipped with a 25 mW HeNe laser. Image processing software was provided by the vendor (EZ-C1, Nikon).

Results

Design of microfluidic chips

Gradual decrease of denaturant concentration from the denatured protein within a short period of time may lead to an efficient protein refolding. In the laminar flow in our designed chips (see Figs 1A, 5A and 6A), the fluid stream to be mixed flows along the central stream (denatured protein) and meets two buffer streams at a junction (see Fig. 2A). In general, it was shown that the mean square displacement ($\langle x^2 \rangle$) of molecules in solution is proportional to mixing time t,

$$\langle x^2 \rangle = 2Dt \tag{1}$$

where D, the diffusion coefficient, is of the order of $10^{-7}\,\mathrm{cm}^2/\mathrm{s}$ for proteins ($0.5–8.7 \times 10^{-7}\,\mathrm{cm}^2/\mathrm{s}$) (*19*) and the order of $10^{-5}\,\mathrm{cm}^2/\mathrm{s}$ for small molecule like urea ($1.4 \times 10^{-5}\,\mathrm{cm}^2/\mathrm{s}$) (*20*), indicating that urea diffuse two order of magnitude faster than proteins. In addition, at low Reynolds number ($Re < 20$ in our experimental conditions), the central stream of denatured protein is squeezed into a narrow stream between the two adjacent buffer streams. The width of the focused stream depends on the flow rate of the diluting buffer (*21*, *22*).

$$t = \frac{\langle x^2 \rangle}{\left[9D(1 + 1/R)^2\right]}, \tag{2}$$

where R is the ratio of flow rate of the denatured protein to the flow rate of the refolding buffer (*21*). The denaturant in the central stream of the denatured protein then enables mixing with the buffer by diffusion and denaturant concentration is decreased, meaning that the flow rate's ratio of the refolding buffer can control denaturant concentration in the microchannel.

To refold the protein, denaturants in the denatured proteins should be diluted at least 10-fold in our

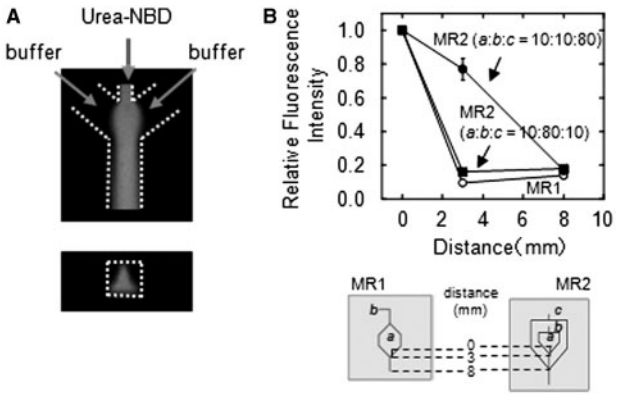

Fig. 2 Hydrodynamic focusing. (A) Confocal fluorescence microscope image at the junction in MR1 showing laminar flow of the urea stream by the diluting buffer streams. The focused urea stream contains NBD-NH_2 as an indicator. Top view (upper) and vertical cross view (bottom) of a channel are shown. Concentrations of urea and NBD-NH_2 were 2.5 M and 10 μM, respectively. All solutions were prepared in 20 mM Tris–HCl (pH 7.5). (B) Relative fluorescence intensities of NBD-NH_2 in the urea stream as a function of the distance from inlet (0 mm). Fluorescence intensities were measured at the junctions in MR1 and MR2. Flow rate: channel *a* (urea-NBD), 10 μl/min; channel *b* (buffer), 90 μl/min for MR1 (opened circle). MR2 (closed circle): channel *a* (urea-NBD), 10 μl/min; channel *b* (buffer), 10 μl/min; channel *c* (buffer), 80 μl/min. MR2 (closed square): channel *a* (urea-NBD), 10 μl/min; channel *b* (buffer), 80 μl/min; channel *c* (buffer), 10 μl/min. Twenty millimolar Tris–HCl (pH 7.5) solution was used as the diluting buffer. The graph shows the mean ± standard error for at least three experiments.

experimental conditions. However, it is expected that a direct 10-fold dilution at the junction in the microchannel may induce misfolding or aggregation. Although the varying flow rate of the diluting buffer can control the distribution of denaturant concentration as described in Eq. (*2*), the chip with only a single junction cannot generate gradual decreasing of denaturant for the efficient protein refolding. It is expected to control the concentration of denaturant with increasing number of junctions (buffer streams) better than the chip with only one junction. Therefore, the chip with multi-junctions was designed to generate the gradual decrease of denaturant concentration. Based on these ideas, we designed two types of microfluidic chips, MR1 and MR2 (Fig. 1A). The reagents were introduced to the microfluidic chips from syringes by syringe pumps (Fig. 1B). PEEK tube was used to connect syringes with microfluidic chips. The denatured protein was injected into the centre channel *a* in Fig. 1A. The denatured proteins directly dilute by the buffer in MR1 (one junction), which is expected to have a similar mechanism with the one-step dialysis. On the other hand, MR2 has two junctions which can control the different flow rate ratios of buffer streams (channels *b* and *c* in Fig. 1A).

Distribution of denaturant concentration in the designed microchannel

To confirm whether the laminar flow in the designed chips can control the distribution of denaturant concentration, the urea stream in the microchannel was studied by a confocal fluorescence microscopy (Fig. 2A). The microfluidic chips in this study were fabricated in PDMS. PDMS used to perform these experiments may transmit and does not scatter or reflect right, obstructing fluorescence measurement. Urea solution (2.5 M) in buffer was injected into channel *a* of each chips in Fig. 1A. Because common denaturants such as urea, does not have fluorescent property, hydrophilic [*N*-(4-nitrobenzo-2-oxa-1,3-diazolyl)amine] NBD-NH_2 (10 μM) that has similar molecular size and the diffusion coefficient to urea was added to urea stream as a fluorophore. It is possible that the fluorescence intensity of NBD-NH_2 may be affected by urea concentration. Therefore, the fluorescence intensities of NBD-NH_2 were measured in 0.25 and 2.5 M urea concentrations. The results revealed that the intensity in 2.5 M urea solution was ~10% higher than that in 0.25 M urea solution (data not shown). Based on these results, we estimated the relative fluorescence intensity in the following experiments. The flow rate of the urea stream (channel *a*) was constantly maintained at 10 μl/min (0.67 mm/s). In MR1, the flow rate of the diluting buffer (20 mM Tris–HCl, pH 7.5) at channel *b* was 90 μl/min (6.03 mm/s), while the flow rates of buffers at channels *b* and *c* in MR2 were varied with the different flow rate ratios. The focus of the microscope was adjusted at a position of inlet (at 0 mm in Fig. 2B). Therefore, the diluted NBD-NH_2 by the buffer at the junction shows decreasing of its fluorescence intensity as compared to that at the inlet, meaning that the urea concentration is decreased.

We first examined the urea distribution at the junctions in MR1 and MR2 using the fluorescence intensity of NBD-NH_2. Figure 2A shows fluorescence image of the laminar flow of the urea stream at the junction in MR1. Because 2.5 M urea solution has a higher relative density (1.03 g/ml) than that of the dilution buffer (1.00 g/ml), a vertical cross view of the channel showed that the urea stream was a triangular distribution, not a typical top/bottom symmetrical distribution. Similar reorientation of the liquid–liquid interface between fluids of different densities in the microchannels was previously reported (*23*, *24*). Figure 2B shows the urea concentration at the junctions along the central line for the inlet and the outlet. The fluorescence intensity of NBD-NH_2 in the urea stream in MR1 was quickly decreased at the junction (distance of 3 mm) as compared to that of the inlet and was similar to that of the outlet (distance of 8 mm), suggesting that high concentration of urea was quickly diffused in MR1 channel. In contrast, when the flow rate ratio of channel *b* and *c* was 1:8, the fluorescence intensities were gradually decreasing from the first junction (at 3 mm) to the second junction (at 8 mm), suggesting that a gradual decrease in urea distribution was generated in MR2. While, when the flow rate ratio of channel *b* and *c* was 8:1, urea was quickly diluted at the first junction (Fig. 2B). The decreasing of the urea concentration at the flow rate ratio of 8:1 (channel *b* : *c*) was similar to that of MR1. These results indicate that by controlling the flow rate ratio of the dilution buffer streams, the gradient of the denaturant concentration is generated.

Protein refolding by microfluidic chips

The performance of designed chips for protein refolding was studied. All proteins were chemically denatured by high concentration of urea or Gdn-Cl in this study. CD spectra measurements revealed that all proteins were denatured under our experimental conditions (see Figs 3A and 6, and Supplementary Fig. S2). To avoid the protein aggregation, refolding must be performed at low protein concentrations to favour refolding over reaggregation in the microchannel (*14*). In our refolding procedure, the denatured proteins (250 μg/ml) were diluted 10-fold in microchannel by buffer. Under this experimental condition, we did not observe any protein aggregation during refolding process (data not shown). The flow rate of the denatured protein stream (channel *a*) was constantly maintained at 10 μl/min (0.67 mm/s). One millilitre of refolded protein was collected in a sample tube for 10 min with a total constant volumetric flow rate of 100 μl/min (6.68 mm/s) at room temperature. The batch sample as a control was directly diluted 10-fold by buffer in a sample tube at room temperature. The enzymatic activity and/or secondary structure of the collected proteins were measured to evaluate the refolding efficiency.

We first tested the refolding of BSA. BSA is known as the easy-to-fold protein, and this is well-used in protein refolding kinetics studies (*25*). CD measurements showed that there are no differences in the spectra of BSA between batch sample prepared by dilution

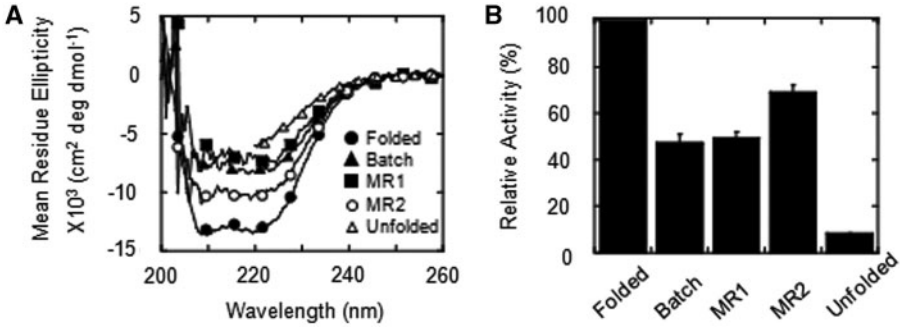

Fig. 3 CS refolding by microfluidic chips. (A) CD spectra of the refolded CS in 20 mM Tris–HCl (pH 7.5) and 0.25 M urea at room temperature. Due to high noise level, ellipticities between 200 and 220 nm were omitted from the spectrum in the unfolded CS. (B) The recovered enzymatic activities of CS using different refolding approaches. The graph shows the mean ± standard error for at least three experiments. Flow rate: channel *a* (denatured CS), 10 μl/min; channel *b* (buffer), 90 μl/min for MR1. MR2: channel *a* (denatured CS), 10 μl/min; channel *b* (buffer), 10 μl/min; channel *c* (buffer), 80 μl/min. Twenty millimolar Tris–HCl (pH 7.5) solution was used as the diluting buffer. Folded CS was prepared by dialysis. Unfolded CS was assayed in 20 mM Tris–HCl (pH 7.5) and 2.5 M urea. The batch sample was directly diluted by buffer in a sample tube. Concentrations of CS were 20 μM (for CD measurement) and 20 nM (for activity measurement), respectively.

and MR1-treated sample (shown in Supplementary Fig. S2A). These CD spectra were similar to that of the reported native BSA (*26*), indicating that denatured BSA easily formed native conformation due to quick refolding as reported previously (*25*). We next tested the refolding of CAB, known as one of the difficult-to-fold protein (*18*, *27*). CD spectrum of refolded CAB by MR2 showed that the spectrum was similar to those of the folded and batch proteins prepared by dilution (shown in Supplementary Fig. S2A). The recovered activity of CAB by MR2 was >90% and this value was also similar to that of the batch sample (shown in Supplementary Fig. S2B). Recent refolding studies using lysozyme and α-glucosidase reported that the use of an initial low protein concentration often suppresses protein aggregation with consequent improvements in refolding yields (*14*). Protein concentration of CAB in the previous refolding studies (*18*, *27*) was 30 mg/ml, while 250 μg/ml of CAB was used in our refolding procedure. At 30 mg/ml of CAB concentration, visible protein aggregates were observed on the wall of the MR2 microchannel or the batch sample by dilution (data not shown), possibly due to contact between the exposed hydrophobic surfaces at high CAB concentration. Therefore, it is suggested that initial concentration is important for the efficient refolding of CAB in our refolding system. Similar observation was shown in the kinetics study of CAB in the microchip (*25*).

Because our goal in this study is to obtain efficient refolding of difficult-to-fold proteins, we next studied CS. CS is recognized to be of low refolding yield by dialysis or dilution, therefore, CS has been employed as a test case for refolding strategies (*17*, *27*). In addition, CS is a dimeric protein which is composed of two identical subunits, suggesting that CS is a good model protein to study not only secondary and tertiary structures but also quaternary structure.

Folded CS was prepared from an ammonium sulphate suspension by dialysis. CD measurements revealed that the folded CS formed helical structure with typical double minimum bands at 208 and 222 nm (Fig. 3A), and the spectrum is comparable to that of the reported native CS (*17*), indicating that the folded CS in this study has a native conformation. Thus, this folded CS was used as a positive control in the CD and enzymatic activity studies. The denatured CS was prepared in 2.5 M urea using folded protein. At this urea concentration, CS did not form any ordered structure (Fig. 3A) and completely lost its enzymatic activity (Fig. 3B).

The refolded CS by MR1 with one junction showed a similar recovered enzymatic activity (<50%) to that of the batch sample which was prepared by dilution, and lower helical structure compared with that of the folded CS (Fig. 3A and B), suggesting that rapid diffusion of urea from the denatured CS leads to misfolding. In contrast, the refolded CS by MR2 with two junctions showed increasing negative ellipticities at 208 and 222 nm in contrast to the batch sample or MR1-treated sample, suggesting that CS has a higher helical structure than those of the CS prepared by dilution or MR1 (Fig. 3A). Moreover, the recovered activity was also enhanced in CS by MR2 compared with the batch sample or MR1 (Fig. 3B). These results indicate that the denatured CS was refolded efficiently by MR2 compared with those prepared by dilution and MR1. The recovered enzymatic activity of CS by MR2 (>70%) is of similar value as those of the artificial chaperone-assisted system, which is a successful technique to recover active proteins from denatured forms (*17*, *27*). The refolding by MR2 was achieved within a short period of time at room temperature. The estimated throughput of our CS refolding method by MR2 was 150 μg/h. This value is one-order higher than those of the artificial chaperone-assisted system [9–14 μg/h in ref. (*27*)]. In addition, refolding by MR2 did not need any additives or chaperone proteins that suppress protein aggregation. These results suggest that gradual decrease of the denaturant concentration in the microchannel can provide the equilibrium between unfolding and refolding (native conformation) and not misfolding and/or aggregation.

As shown in Fig. 2, laminar flow conditions allowed control of the urea distribution in MR2's microchannel, thus, we studied the different flow rate ratios of the

refolding buffers on the CS refolding yield. In this experiment, the flow rate of denatured CS was constantly 10 μl/min (channel *a*) and a total flow rate of the refolding buffer was 90 μl/min (channels *b* and *c*) with different ratios. As shown in Fig. 4A, changes of the flow rate ratio of the buffers demonstrated the different decreasing patterns of urea concentrations. Lower flow rate of buffer at channel *b* elicited a slow decrease in urea concentration, leading to higher recovered enzymatic activities (Fig. 4B). We also tested the microchips with three junctions (MR3) or four junctions (MR4) for CS refolding (Figs 5 and 6). The results also showed that the gradual decrease in urea concentration (Fig. 5A for MR3 and Fig. 6A for MR4) was correlated with the recovery of enzymatic activities (Fig. 5B for MR3 and Fig. 6B for MR4). The recovered activities by MR3 (73%) and MR4 (74%) were similar to that of refolded CS by MR2 (76%). This suggests that the chip with two junctions (MR2) is sufficient to bring out CS refolding. Because the surface area between the denatured protein stream and the diluting buffer stream in the microchannel correlates with the diluting rate of denaturant, we also designed the MR2 chips with several channel depths. To generate a stable laminar flow, the chips having main channel dimensions of 300 μm width and 100, 200, 300 or 400 μm depth were used in this experiment. Deeper depth of the channel elicits a wide surface area. As shown in Fig. 7, the chip with a wide surface area (400 μm of depth) and a gradual decrease in urea concentration (closed circles in Fig. 7) showed higher recovered activity (80%) than the chips with a narrow surface area (100 μm of depth) and rapid decrease in urea concentration (opened circles in Fig. 7). Although the recovered activity of CS was not 100%, these results suggest that an efficient microfluidic design and appropriate flow rate condition can control the gradual decrease of denaturant concentration in microchannel and promote efficient protein refolding.

Refolding of protein from inclusion body by microfluidic chips

The designed chips have been evaluated for their refolding performance on protein from inclusion bodies. ZAP-70 is a tyrosine kinase (*28*). Because the overexpressed ZAP-70 in the bacterial expression system makes inclusion bodies, it is usually expressed in the mammalian or insect expression system (*16*, *28*). However, these expression systems cost a great deal compared with the *E. coli* system, and the recovered yield of the protein is generally low. In this study, urea-denatured ZAP-70 protein kinase domain (mouse residues 337–597), which was purified from *E. coli* inclusion bodies, was applied to the microfluidic chips to evaluate protein refolding.

The folded ZAP-70 kinase domain was prepared by the step-wise dialysis over 2 days and has showed helical conformation as reported previously (*16*, *28*). As shown in Fig. 8, the CD spectrum of refolded ZAP-70 by MR1 showed similar spectrum as batch sample by dilution. In contrast, the CD spectrum of ZAP-70 prepared by MR2 was similar to that of the folded ZAP-70. Helical content of the ZAP-70 by MR2 was higher than those of ZAP-70 prepared by MR1 and dilution. Although we could not measure the kinase activity of ZAP-70 due to the fact that the kinase domain involved in this study does not have the enzymatic activity (*28*), these CD results show that protein refolding by microfluidic chip can be applied to recover folded protein from inclusion bodies.

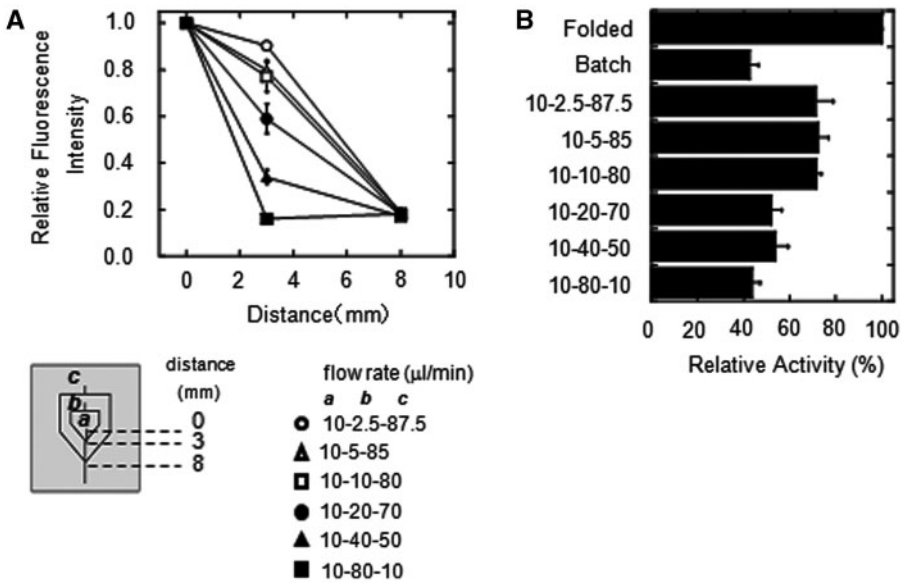

Fig. 4 Effect of the different flow rate ratios of diluting buffers on CS refolding. (A) Relative fluorescence intensities of NBD-NH_2 in the urea stream at the junctions in MR2 and (B) the recovered enzymatic activities of refolded CS in MR2. The flow rates of buffers (channels *b* and *c*) were changed with a total volumetric flow rate of 90 μl/min. The flow rate of urea-NBD (channel *a*) was constantly maintained at 10 μl/min. The flow rates of solutions were shown in (A). Other experimental conditions were the same as those in Fig. 2 (for fluorescence microscopy) and those in Fig. 3 (for activity measurement). The graph shows the mean ± standard error for at least three experiments.

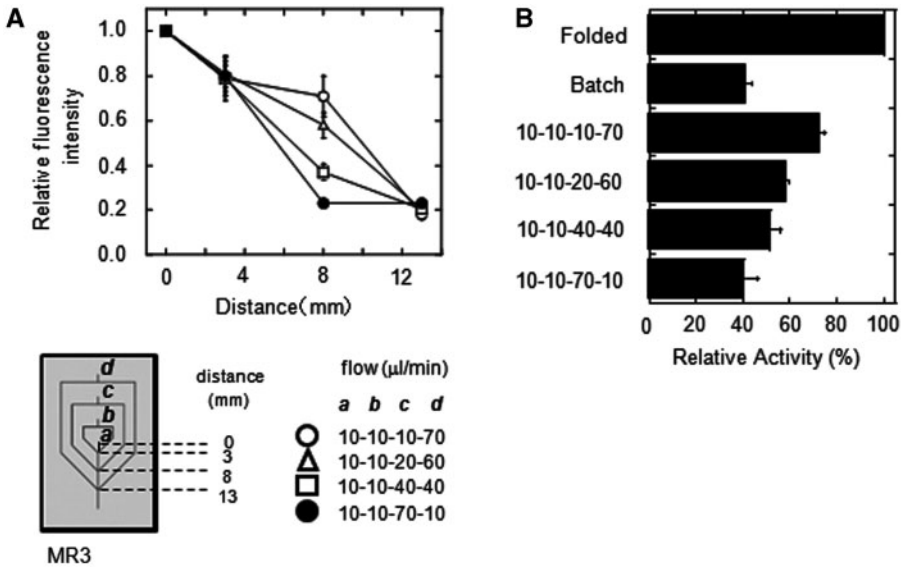

Fig. 5 CS refolding by microfluidic chips with three junctions (MR3). (A) Relative fluorescence intensities of NBD-NH_2 in the urea stream at the junctions and (B) the recovered enzymatic activities of refolded CS. The microfluidic chips have dimensions of 500 μm in depth and 500 μm in width. The flow rates of the diluting buffers (channels *b*, *c* and *d*) were changed to a total volumetric flow rate of 90 μl/min. The flow rate of the denatured CS was constantly maintained at 10 μl/min. The flow rates of solutions were shown in (A). Other experimental conditions were the same as those in Fig. 4.

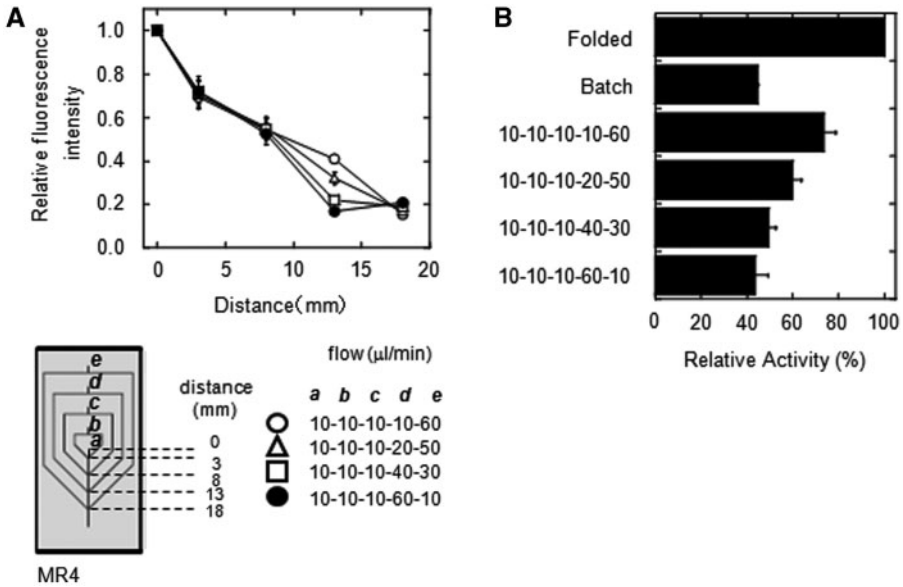

Fig. 6 CS refolding by microfluidic chips with four junctions (MR4). (A) Relative fluorescence intensities of NBD-NH_2 in the urea stream at the junctions and (B) the recovered enzymatic activities of refolded CS. The flow rates of the diluting buffers (channels *b*, *c*, *d* and *e*) were changed to a total volumetric flow rate of 90 μl/min. The flow rates of solutions were shown in Fig. 6A. Other experimental conditions were the same as those in Figs 4 and 5.

Discussion

Effective protein refolding methodology is important not only for the analysis of protein structure and function (*29*) but also for the development of therapeutic drugs (*1*) and bioelements in the industries (*2*). Refolding is a change of protein conformation from unfolding to folding, which is dependent on the denaturant concentration. Because rapid decrease in denaturants leads to misfolding and/or aggregation (*4*, *5*), gradual decrease in denaturant concentration from the denatured protein within a short period of time may lead to efficient protein refolding. The present microfluidic chips with multi-junctions can control gradual decrease in denaturant concentration in the microchannel by varying flow rate ratios of the refolding buffer. In addition, quick and high throughput protein refolding was achieved at room temperature. Because the step-wise dialysis is time-consuming (multi-days), rapid and effective refolding is a superior advantage of our refolding procedure over dialysis.

Several methods for refolding of aggregated proteins were reported, such as size-exclusion chromatography (*30*), reversed micelles system (*31*), zeolites absorbing

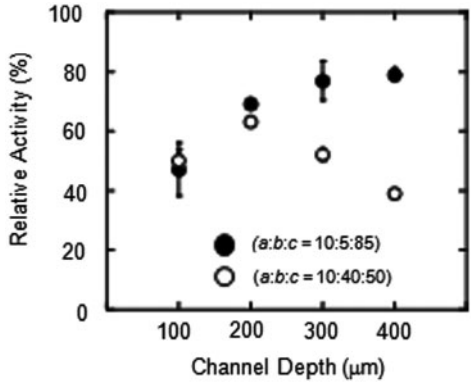

Fig. 7 The recovered enzymatic activities of refolded CS by MR2 with several channel depths. The microfluidic chips have dimensions of 300 μm in width and 100, 200, 300 or 400 μm in depth. Flow rate: channel *a* (denatured CS), 10 μl/min; channel *b* (buffer), 5 μl/min; channel *c* (buffer), 85 μl/min for closed circle. Open circle: channel *a*, 10 μl/min; channel *b*, 40 μl/min; channel *c*, 40 μl/min. Other experimental conditions were the same as those in Fig. 3.

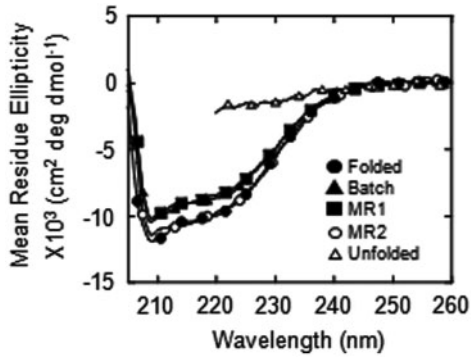

Fig. 8 CD spectra of refolded ZAP-70 kinase domain by microfluidic chips. Spectra were measured in 50 mM PB (pH 7.0), 150 mM NaCl and 0.8 M urea at room temperature. The flow rates of solutions in each chip were the same as those of Fig. 3. Folded ZAP-70 was prepared by the step-wise dialysis. Unfolded ZAP-70 was assayed in 50 mM PB (pH 7.0) and 8 M urea. Fifty millimolar PB (pH 7.0) solution was used as the diluting buffer. Due to high noise level, ellipticities between 200 and 220 nm were omitted from the spectrum in unfolded sample. Concentration of ZAP-70 was 20 μM.

system (*32*) and artificial chaperone-assisted system (*17*). The refolding efficiency of CS obtained by the present microfluidic chip is similar to those of the artificial chaperone-assisted system (*17*, *27*) or the natural GroEL–GroES chaperon protein (*33*). Because the present procedure is much simpler than the other refolding methods and does not add any chaperon protein, i.e. the denatured protein is just passed through the microchannel, it is not necessary to purify the target protein from the refolding system.

Since many proteins refold optimally under very different buffer conditions, no universal protein refolding buffer exists. For example, low molecular weight additives such as polyamines (*34*), detergents (*2*) or polyethylene glycol (*35*) that prevent protein aggregation and enhance protein refolding have been used as folding assistants in the refolding buffer. Identifying the refolding buffer is still one of the major bottlenecks of protein production for structural studies. The approach using microfluidic chip can be automated and offers a generic method to screen refolding conditions. Therefore, microfluidic system can also accelerate the analysing rate at which the proteins can be functionally and structurally investigated.

In conclusion, the microfluidic chip with multi-junctions has been designed to enable refolding of denatured proteins. This refolding strategy takes into account the hydrodynamic diffusion of denaturants. Microfluidic chips employing this strategy may serve as strong miniaturized tools for recovery of difficult-to-refold proteins from inclusion bodies and for screening system of protein refolding.

Supplementary Data

Supplementary Data are available at *JB* Online.

Conflict of interest
None declared.

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