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Analytical method for determining relative chaperone activity using an ovalbumin-conjugated column

Makoto Hirano, Yuki Kato, Ayami Imagawa, Kiichiro Totani *

Department of Materials and Life Science, Seikei University, 3-3-1 Kichijoji-kitamachi, Musashino-shi, Tokyo 180-8633, Japan

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ABSTRACT

Investigating the relative efficiencies of molecular chaperones is important for understanding protein biosynthesis inside a cell. We developed an analytical method for estimating relative chaperone activity under physiological, multi-chaperone conditions using a protein-conjugated column. A chaperone mixture was subjected to chromatography on a column conjugated with denatured ovalbumin, and the elution positions of target chaperones were compared using western blotting to determine the relative affinity of each chaperone for the denatured protein. Because molecular chaperones should be eluted according to their strength of association with the denatured ovalbumin in the column, the elution position must accord with the chaperone activity and can be used as an indicator of relative chaperone activity. We found that the column procedure was effective in an assay of a mixture of calreticulin and BiP, the molecular chaperones in the endoplasmic reticulum; the assay showed that calreticulin associated with denatured ovalbumin more strongly than BiP.

1. Introduction

Most proteins must fold into proper higher order structures to gain functional activity. However, in the cellular environment, nascent polypeptides are at great risk of misfolding and aggregation. To avert these dangers, various molecular chaperones associate with the folding intermediates to prevent aggregation and promote efficient folding [1,2]. Molecular chaperones are usually classified into the small Hsp, Hsp40, Hsp60, Hsp70, Hsp90, Hsp100, and calnexin/calreticulin (CNX/CRT) families [2], and at least Hsp60 and Hsp70 are known to be necessary for protein biosynthesis [3].

General assay procedures for chaperone activity have been widely studied and include the following: (1) protein aggregation and refolding monitored by measuring light scattering at 360 nm [4–7], (2) luciferase refolding assay [6,8], (3) real-time fluorescence assay using green fluorescent protein [9], and (4) molecular probe-based assays [10–12]. However, an analytical method for determining relative chaperone activity in the presence of multiple chaperones has never been reported. In fact, various chaperones cooperate to generate higher order protein structure inside a cell; for example, BiP (Hsp70 family), ERdj1-7 (Hsp40 family), and CNX/CRT coexist in the endoplasmic reticulum (ER) [13]. Thus, knowing the relative contribution of each chaperone during protein folding will be important for a complete understanding of protein biosynthesis.

Herein, we describe an analytical method for determining relative chaperone activity using a protein-conjugated column (Fig. 1A). Although protein-conjugated columns have been used for affinity purification of chaperones [14–17], essential use of the column to assay relative chaperone activity has never been reported. First, denatured protein is immobilized on agarose beads and the beads are packed into an empty column to manufacture a denatured protein-conjugated column. Next, a chaperone mixture is applied to the column and eluted with buffer solution. The elution fractions are then analyzed by western blotting to detect the elution position of each chaperone. Because molecular chaperones should be eluted according to their strength of association with the denatured proteins in the column, the elution position must accord with the chaperone activity and can be used as an indicator of the relative chaperone activity. Furthermore, quantification of the detected bands can be used to determine the 50% elution volume, which can be useful for elucidation of the relative chaperone activity. To examine the effectiveness of this method, relative chaperone activities of BiP and CRT were analyzed using a column filled with denatured ovalbumin (d-OVA)-conjugated beads.

* Corresponding author. Fax: +81 422 37 3871.
E-mail address: ktotani@st.seikei.ac.jp (K. Totani).

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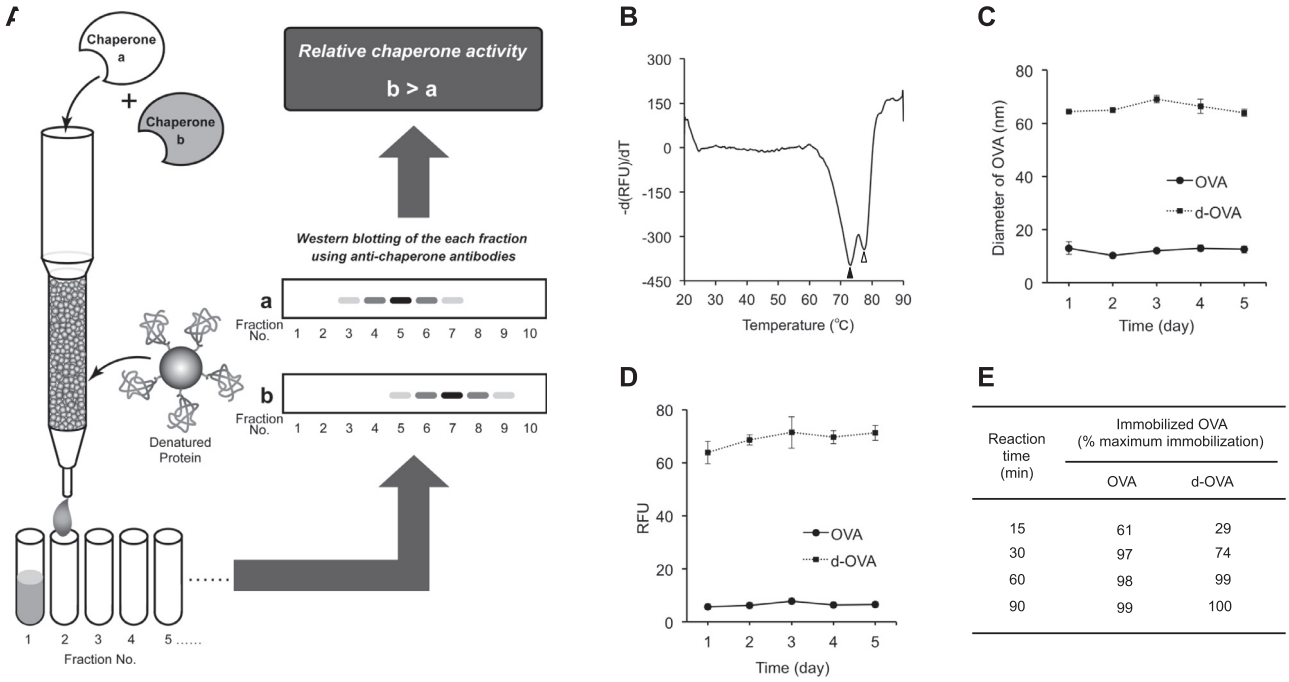


Fig. 1. Working hypothesis and samples used in this work. (A) Schematic diagram of this work. (B) Denaturation peak of OVA obtained by the Thermofluor assay. Arrowheads indicate the denaturation temperatures (T_d) of OVA. (C) Time course for diameters of OVA obtained by dynamic light scattering analysis. Data are the mean of three experiments with standard deviation. (D) Time course for fluorescence intensities of OVA in the presence of SYPRO Orange. Data are the mean of three experiments with standard deviation. (E) Time course of immobilization of OVA and d-OVA onto the beads.

2. Materials and methods

2.1. General

OVA from Wako Pure Chemical Industries; BSA and agarose beads from Sigma–Aldrich; SulfoLink coupling resin from Thermo Scientific; SYPRO Orange and streptavidin-HRP from Invitrogen-Life Technologies; BiP, rabbit anti-CRT monoclonal antibody and rabbit anti-BiP polyclonal antibody from Abcam; and HRP-conjugated anti-rabbit IgG from PerkinElmer. Human CRT was expressed in *Escherichia coli* as a GST fusion protein, purified with glutathione–Sepharose (GE Healthcare), and treated with PreScission Protease (GE Healthcare) to remove GST as described previously [18].

2.2. Preparation of OVA-conjugated beads

All washing steps consisted of suspension of beads in 50 μL of buffer, sedimentation of the beads by centrifugation (1000*g*, 4 °C, 1 min), and removal of the supernatant. First, SulfoLink coupling resin (50 μL) was washed twice in coupling buffer (5 mM EDTA, 50 mM Tris HCl, pH 8.5). The beads were then treated with 150 μL of OVA (2 mg/mL) in the coupling buffer. After 15–90 min (optimum: 60 min) of mixing by inversion (6 times/min) at 25 °C, the beads were sedimented by centrifugation (1000*g*, 4 °C, 1 min) and the supernatants were collected to measure the residual protein concentration by BCA protein assay to calculate percent immobilization. The beads were then washed once in 10 mM NaCl and 3 times in the coupling buffer to give OVA-conjugated beads. Next, the beads were treated with 50 mM cysteine (50 μL) in the coupling buffer to block unreacted iodoacetyl groups. After 15 min of mixing by inversion (6 times/min) at 25 °C, the beads were sedimented by centrifugation (1000*g*, 4 °C, 1 min), washed 3 times in TBS (pH 7.4), and resuspended in TBS to give blocked OVA-conjugated beads. Subsequent denaturation of the OVA-conjugated beads by heating at 95 °C for 10 min provided d-OVA-conjugated beads.

2.3. Interaction analysis of OVA-conjugated beads

Blocked OVA-conjugated beads (10 μL, 0.57 nmol of immobilized OVA), blocked d-OVA-conjugated beads (10 μL, 0.57 nmol of immobilized OVA), or agarose beads (10 μL) were added to human CRT (0.15 nmol) or BSA (0.15 nmol) in TBS (pH 7.4), 10 mM $CaCl_2$. After 17 h of mixing by inversion (6 times/min) at 4 °C, the supernatant was removed (supernatant fraction). The beads were washed with buffer (40 μL) containing 10 mM $CaCl_2$ and TBS (pH 7.4) and the supernatant was removed as the wash fraction to give the bead fraction. The resulting supernatant, wash, and bead fractions (5 μL) were analyzed by SDS–PAGE; gels were stained with Coomassie Brilliant Blue to detect the protein bands.

2.4. Chaperone activity assay using the OVA-conjugated column

Molecular chaperone (CRT and/or d-CRT and/or BiP) (5.5 pmol) was added to BSA–biotin (5.5 pmol) and the resulting mixture was subjected to chromatography on an OVA- or d-OVA-conjugated column (5.5 mm $\phi \times 42$ mm, 1 mL of beads, 65 nmol of OVA) equilibrated with TBS (pH 7.4), 10 mM $CaCl_2$, at a flow rate of 20 μL/min at 4 °C. The eluted fractions (120 μL) were analyzed by SDS–PAGE followed by western blotting with anti-CRT and anti-BiP antibodies or ligand blotting with streptavidin-HRP. Each fraction (20 μL) was resolved on SDS–PAGE and transferred onto a PVDF membrane. After blocking with Blocking One (Nacalai Tesque), the membrane was incubated with an anti-CRT antibody, anti-BiP antibody, or streptavidin-HRP for 12 h at 4 °C. After washing with TBS-Tween (3×), the membrane treated with anti-CRT or anti-BiP antibody was incubated with HRP-conjugated anti-rabbit IgG for 1 h. After

washing with TBS-Tween (3×), the bands on each membrane were visualized by chemiluminescence (Immobilon Western, Millipore).

3. Results and discussion

The immobilized protein of our proposed assay should be denatured by a simple method, and the resulting denatured state should be stable for a long period. To ensure these, we adopted OVA [19] as the protein to be immobilized on agarose beads. OVA is a singly *N*-glycosylated glycoprotein of 45 kDa that represents 65% of egg white protein, which is a well described and easily denatured by simple heating method [20,21]. To produce thermal denatured OVA, we first measured the denaturation temperature of OVA with the Thermofluor assay [22,23] (Fig. 1B). A mixture of OVA and SYPRO Orange [24], which accumulates and fluoresces at hydrophobic surfaces, was gradually heated using real-time PCR equipment while the resulting fluorescence intensity was monitored. The differential of the fluorescence was plotted on the ordinate and the temperature was plotted on the abscissa to obtain the denaturation peak (denaturation temperature). Two peaks at 73.0 and 77.6 °C were observed. Ordinary OVA and its stable form (S-OVA) are known to exist, and their denaturation temperatures obtained by differential scanning calorimetry are 78.0 and 85.8 °C, respectively [25]. It has been reported that commercially available OVA contains both forms of OVA [26]; therefore, we conclude that the two peaks obtained in our experiment correspond to OVA and S-OVA, and that the difference between our denaturation temperatures and the previously reported values could be because of the different measurement procedures.

To examine the stability of d-OVA, which is obtained with sufficient thermal treatment (95 °C, 10 min), the time course of d-OVA particle size was analyzed by dynamic light scattering (Fig. 1C). The particle size of d-OVA was approximately 5 times larger than that of native OVA, showing that the protein loses its original folding state. Moreover, d-OVA maintained this particle size for 5 days after denaturation. We also examined differences in the surface hydrophobicity of OVA and d-OVA by comparing the fluorescence intensity after treatment with SYPRO Orange (Fig. 1D). The intensity from d-OVA was consistently higher than that from OVA over a 5-day period. These results show that the prepared d-OVA stably maintained the hydrophobic denaturation state, allowing its use as the immobilized protein.

Inversely, bovine serum albumin (BSA) was not sufficient for a binding agent of our chaperone assay. Although the heat denaturation of BSA (95 °C, 10 min) increased the particle size with losing its original folding state (Supplementary Fig. S1A), both the denatured- and the native-form showed higher hydrophobicity compared to OVA (Fig. 1D and Supplementary Fig. S1B). In view of general chaperone action reduces hydrophobicity of a protein, the hydrophobic nature of BSA even in its native form [27] is not suitable for the immobilized protein.

We next examined the immobilization of OVA on agarose beads. Because OVA has four free cysteines in its native structure [28], we used the thiol groups of the cysteines for the immobilization of OVA. OVA-conjugated beads were prepared using SulfoLink coupling resin (Thermo Scientific); the terminal iodoacetamide groups of the beads coupled with the thiol groups of OVA. Percent immobilization of OVA, with complete conversion of the iodoacetamide groups considered to be 100%, was calculated from the concentration of OVA remaining in the reaction mixture after the immobilization procedure (Fig. 1E). For immobilization of native OVA, a 30-min incubation was sufficient for completion of the coupling reaction. In the case of d-OVA obtained by thermal denaturation (95 °C, 10 min), the coupling reaction proceeded more slowly, and a 60-min incubation was necessary to complete the reaction. Breaking the higher order structure of OVA may cause changes in the accessibility of the thiol groups. Because OVA and d-OVA showed different rates of immobilization, d-OVA-conjugated beads for further investigations were prepared by thermal denaturation of OVA-conjugated beads to ensure a constant immobilization rate.

We next examined the interaction of immobilized d-OVA with CRT, a molecular chaperone in the ER. CRT has both lectin and chaperone activity: it binds with the high-mannose type glycan $Glc_1Man_9GlcNAc_2$ [29] and also shows chaperone activity toward non-glycosylated proteins [7]. We have been working on functional analysis of CRT-mediated ER glycoprotein quality control [30]. One of the reasons for choosing CRT as a model chaperone is that we intend to apply the proposed assay for chaperone activity to further analysis of the quality control system. Moreover, several interaction analyses of CRT with denatured ovalbumin have been reported [31,32], also inspired us to focus on CRT. Although the immobilized OVA is *N*-glycosylated and has high-mannose, hybrid, and complex types of glycans, the main component of its high-mannose structure is $Man_{5/6}GlcNAc_2$, and the CRT-binding glycan $Glc_1Man_9GlcNAc_2$ has never been reported [33,34]. Consequently, CRT can be used for our chaperone assay.

We first examined the direct interaction of CRT with agarose beads (Fig. 2A). Agarose beads were mixed with CRT or BSA (as a negative control protein having no chaperone activity), and the supernatant, wash, and bead fractions were analyzed by SDS–PAGE. No protein bands were detected in the bead fractions, showing that CRT and BSA do not directly interact with agarose beads. We next analyzed the interaction of BSA and CRT with OVA- and d-OVA-conjugated beads in the same way (Fig. 2B and C). No protein bands were observed in the bead fractions from the BSA–OVA and BSA–d-OVA interaction experiments, showing that BSA did not associate with the proteins on the beads (Fig. 2B). Conversely, in the case of interaction with CRT, both OVA and d-OVA bead

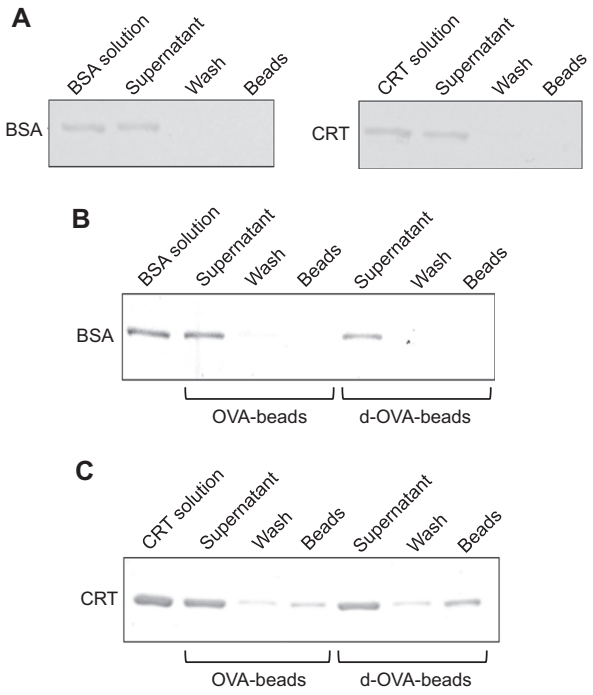


Fig. 2. Interaction analysis of CRT with OVA-conjugated beads. SDS–PAGE of fractions obtained from interaction experiments of (A) agarose beads with BSA and CRT, (B) BSA with OVA- and d-OVA-conjugated beads, and (C) CRT with OVA- and d-OVA-conjugated beads.

fractions yielded protein bands in the SDS–PAGE analysis, indicating that CRT associated with the proteins on the beads (Fig. 2C). Comparison of the intensity of the protein bands from the bead fractions showed that the band from the d-OVA bead fraction was more concentrated than the band from the OVA bead fraction, indicating that CRT binds with d-OVA, while association with OVA seems to be relatively weak. These results suggest that CRT interacts with the denatured protein on the bead owing to its chaperone activity. Thus, the d-OVA-conjugated beads are suitable as affinity chromatography beads for molecular chaperones.

OVA- or d-OVA-conjugated beads were packed into a mini column S (Muromachi Technos) to provide OVA- and d-OVA-columns, respectively. A mixture of CRT and BSA–biotin was applied to both columns, the columns were eluted with buffer solution at a rate of 20 μL/min, and 120-μL elution fractions were collected. Each fraction was analyzed by SDS–PAGE followed by western blotting (for CRT) or ligand blotting (for BSA–biotin) (Fig. 3A and D).

Bands for BSA–biotin were observed in fractions 7–12 from both the OVA- and the d-OVA-column (Fig. 3A), and the BSA–biotin elution profiles from the two columns were nearly identical (Fig. 3B). We propose the 50% elution volume of the target chaperone as an index of interaction with the conjugated protein. The 50% elution volume for BSA, derived from Fig. 3B, was 1125 μL for the OVA-column and 1170 μL for the d-OVA-column, and thus was nearly identical for the two columns. Therefore, the elution of BSA–biotin from the protein-conjugated columns was not affected by the folding state of OVA, indicating that BSA did not interact with OVA. These results show that BSA–biotin is suitable as a negative control protein for the column chromatography method.

Conversely, bands for CRT were observed in fractions 9–13 from the OVA-column (Fig. 3D, top) and fractions 11–14 from the d-OVA-column (Fig. 3D, middle). These results suggest that CRT interacts with d-OVA by its chaperone activity during elution of the column. We examined the elution of denatured CRT (d-CRT) from the d-OVA-column to confirm the interaction is derived from the chaperone activity of CRT. We detected bands for d-CRT in fractions 8–12 (Fig. 3D, bottom), which was nearly identical to the elution position of CRT from the OVA-column (Fig. 3D, top). Comparing elution profiles, we found that the elution of CRT from the d-OVA-column was clearly delayed in comparison with that from the OVA-column (Fig. 3E). Moreover, the elution profile of d-CRT from the d-OVA-column was nearly equal to that of CRT from the OVA-column. The 50% elution volume of CRT from the OVA-column was 1230 μL, similar to the value of 1170 μL obtained for d-CRT from the d-OVA-column (Fig. 3F). Because these values are near those obtained for BSA (Fig. 3C), no practical interaction seems to be observed in the case of CRT with the OVA-column or d-CRT with the d-OVA-column. In contrast, the 50% elution volume of CRT from the d-OVA-column was 1450 μL, indicating that CRT interacted with d-OVA during elution of the column. Thus the chaperone activity of CRT can be estimated using the d-OVA-column. When we directly reused the same d-OVA-column for analysis of chaperone activity of CRT, the resulting 50% elution volumes gradually became more similar to that obtained from the OVA-column (data not shown). These results indicate that d-OVA immobilized on the beads were partially refolded during repeated interaction experiments with CRT, because any proteins were not detected in the elution from the reused d-OVA column after SDS

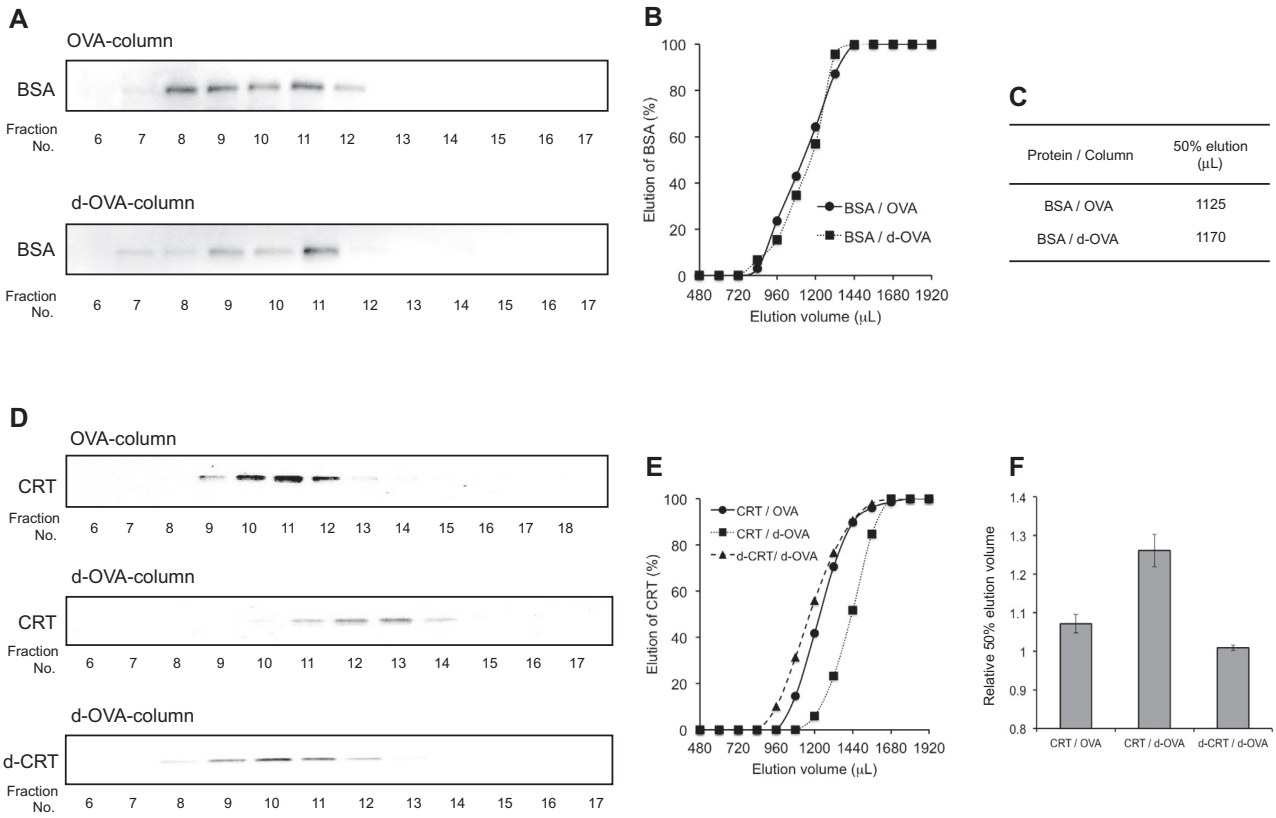
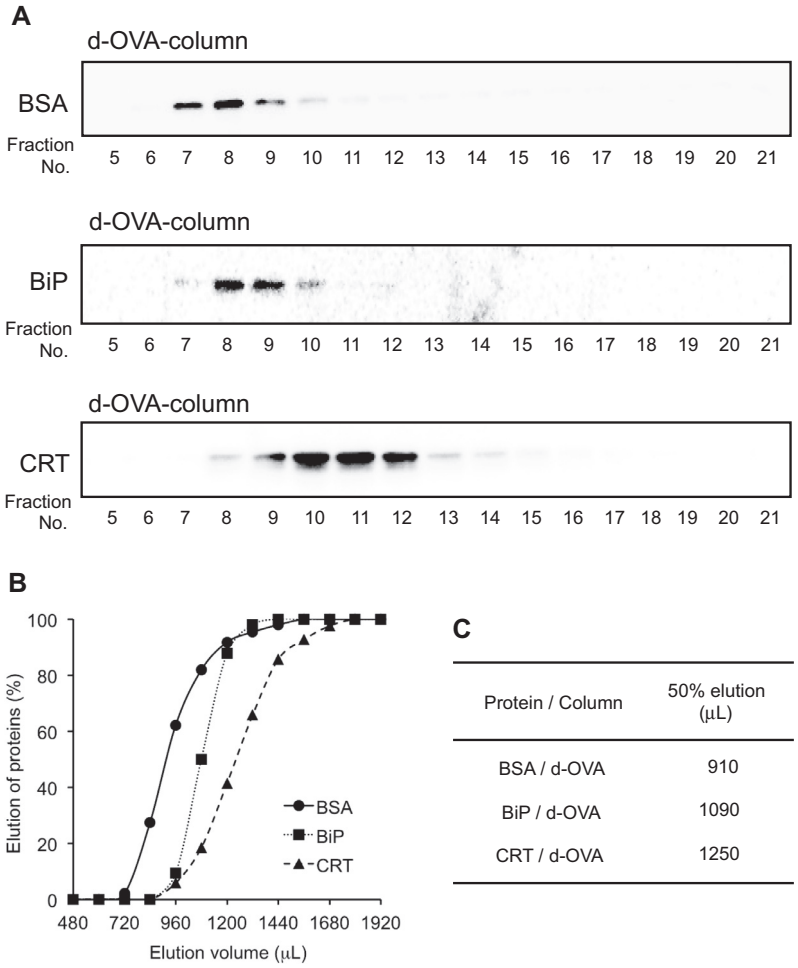


Fig. 3. Analysis of CRT chaperone activity using OVA-conjugated columns. (A) Ligand blot analysis of BSA–biotin in elution fractions obtained from an OVA- or d-OVA-column with applied BSA–biotin and CRT. (B) Elution profiles of BSA–biotin from OVA- and d-OVA-columns. (C) The 50% elution volumes of BSA–biotin from OVA- and d-OVA-columns. (D) Western blot analysis of CRT in elution fractions obtained from an OVA- or d-OVA-column with applied BSA–biotin and CRT or d-CRT. (E) Elution profiles of CRT or d-CRT from OVA- and d-OVA-columns. (F) Relative affinity of CRT or d-CRT to OVA- and d-OVA-columns, calculated from the 50% elution volumes. Data are the mean of three experiments with standard deviation.



Protein / Column	50% elution (μL)
BSA / d-OVA	910
BiP / d-OVA	1090
CRT / d-OVA	1250

Fig. 4. Analysis of chaperone activity of a CRT–BiP mixture using d-OVA-columns. (A) Ligand or western blot analysis of BSA–biotin, BiP and CRT in elution fractions obtained from a d-OVA-column with applied BSA–biotin, BiP, and CRT. (B) Elution profiles of BSA–biotin, BiP, and CRT from a d-OVA-column. (C) The 50% elution volumes of BSA–biotin, BiP, and CRT from a d-OVA-column.

treatment. Namely, CRT could associate with d-OVA by its chaperone activity in the column chromatography experiment. We also found that affinity of the used d-OVA column to chaperones is recovered with thermal treatment (95 °C, 10 min).

We next applied the d-OVA-column method to analyze relative chaperone activities under multi-chaperone conditions, which has not previously been done. In this study, we focused on protein folding in the ER to verify the relative efficiencies of the important chaperones CRT and BiP [35]. A mixture of CRT, BiP, and BSA–biotin was applied to the d-OVA-column and the elution fractions were analyzed by western blotting to obtain the elution positions of each protein (Fig. 4A). The proteins were eluted from the column in the order BSA, BiP, and CRT. The 50% elution volumes of BSA, BiP, and CRT from the elution profiles (Fig. 4B) were 910 μL, 1090 μL, and 1250 μL, respectively (Fig. 4C). The 50% elution volume of BSA in Fig. 4C is smaller than that in Fig. 3C; this might be because the total protein concentration applied to the column in this experiment was higher than the concentration applied in the previous experiment. BiP and CRT were eluted significantly more slowly than BSA, indicating that these chaperones interact with d-OVA immobilized on the bead. The slower elution of CRT compared with BiP indicates that CRT interacts more efficiently with d-OVA under competitive conditions of equal molar concentrations of BiP and CRT. Thus, our proposed column chromatography method provides a novel index for estimating relative chaperone efficiency under multi-chaperone conditions.

In view of the importance of molecular chaperones for protein biosynthesis, we developed an analytical method to estimate relative chaperone activity in the presence of various molecular chaperones. Our method was effective in an assay of a chaperone mixture containing CRT and BiP, and we found that CRT associated with d-OVA more strongly than BiP. Of course, the index resulting from this procedure is based on the interaction efficiency, and we understand that this value does not directly represent the folding capacity of each molecular chaperone. However, knowing which molecular chaperone preferentially binds to a substrate protein will provide an important clue to understanding protein biosynthesis under physiological conditions. Comparison of relative chaperone activities among various cellular conditions, such as ER stress [36] and some diseases, may indicate changes in protein folding mechanisms connected with variations in cell status. Moreover, the use of columns with denatured proteins other than d-OVA may result in different relative chaperone efficiencies and thus may contribute to our understanding of the proper use of molecular chaperones for each target protein. Further studies along these lines are under way and will be reported in due course.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.11.081.

References

[1] F.U. Hartl, A. Bracher, M. Hayer-Hartl, Molecular chaperones in protein folding and proteostasis, Nature 475 (2011) 324–332.
[2] A.L. Fink, Chaperone-mediated protein folding, Physiol. Rev. 79 (1999) 425–449.
[3] C.M. Fraser, J.D. Gocayne, O. White, M.D. Adams, R.A. Clayton, R.D. Fleischmann, C.J. Bult, A.R. Kerlavage, G. Sutton, J.M. Kelley, R.D. Fritchman, J.F. Weidman, K.V. Small, M. Sandusky, J. Fuhrmann, D. Nguyen, T.R. Utterback, D.M. Saudek, C.A. Phillips, J.M. Merrick, J.F. Tomb, B.A. Dougherty, K.F. Bott, P.C. Hu, T.S. Lucier, S.N. Peterson, H.O. Smith, C.A. Hutchison 3rd., J.C. Venter, The minimal gene complement of *Mycoplasma genitalium*, Science 270 (1995) 397–403.
[4] J. Buchner, M. Schmidt, M. Fuchs, R. Jaenicke, R. Rudolph, F.X. Schmid, T. Kiefhaber, GroE facilitates refolding of citrate synthase by suppressing aggregation, Biochemistry 30 (1991) 1586–1591.
[5] U. Jakob, H. Lilie, I. Meyer, J. Buchner, Transient interaction of Hsp90 with early unfolding intermediates of citrate synthase. Implications for heat shock in vivo, J. Biol. Chem. 270 (1995) 7288–7294.
[6] G.J. Lee, A.M. Roseman, H.R. Saibil, E. Vierling, A small heat shock protein stably binds heat-denatured model substrates and can maintain a substrate in a folding-competent state, EMBO J. 16 (1997) 659–671.
[7] Y. Saito, Y. Ihara, M.R. Leach, M.F. Cohen-Doyle, D.B. Williams, Calreticulin functions in vitro as a molecular chaperone for both glycosylated and non-glycosylated proteins, EMBO J. 18 (1999) 6718–6729.
[8] B.C. Freeman, A. Michels, J. Song, H.H. Kampinga, R.I. Morimoto, Analysis of molecular chaperone activities using in vitro and in vivo approaches, Methods Mol. Biol. 99 (2000) 393–419.
[9] R.E. Mares, S.G. Melendez-Lopez, M.A. Ramos, Acid-denatured green fluorescent protein (GFP) as model substrate to study the chaperone activity of protein disulfide isomerase, Int. J. Mol. Sci. 12 (2011) 4625–4636.
[10] L. Llauger-Bufi, S.J. Felts, H. Huezo, N. Rosen, G. Chiosis, Synthesis of novel fluorescent probes for the molecular chaperone Hsp90, Bioorg. Med. Chem. Lett. 13 (2003) 3975–3978.
[11] V. Zhou, S. Han, A. Brinker, H. Klock, J. Caldwell, X.J. Gu, A time-resolved fluorescence resonance energy transfer-based HTS assay and a surface plasmon resonance-based binding assay for heat shock protein 90 inhibitors, Anal. Biochem. 331 (2004) 349–357.
[12] X. Ding, H. Li, H. Xie, Y. Huang, Y. Hou, Y. Yin, G. Li, A novel method to assay molecular chaperone activity of HSP70: evaluation of drug resistance in cancer treatment, Biosens. Bioelectron. 47 (2013) 75–79.
[13] P. Maattanen, K. Gehring, J.J. Bergeron, D.Y. Thomas, Protein quality control in the ER: the recognition of misfolded proteins, Semin. Cell Dev. Biol. 21 (2010) 500–511.
[14] S.-H. Nam, M.K. Walsh, Characterization of interactions between *Escherichia coli* molecular chaperones and immobilized caseins, Prep. Biochem. Biotechnol. 33 (2003) 321–339.
[15] C. Kumagai, Y. Kitagawa, Potential molecular chaperones involved in laminin chain assembly, Cytotechnology 25 (1997) 173–182.
[16] S.K. Nigan, A.L. Goldberg, S. Ho, M.F. Rohde, K.T. Bush, M.Y. Sherman, A set of endoplasmic-reticulum proteins possessing properties of molecular chaperones includes Ca^{2+}-binding proteins and members of the thioredoxin superfamily, J. Biol. Chem. 269 (1994) 1744–1749.
[17] M.E. Evans, B. Huhse, V.I. Titorenko, W.H. Kunau, F.-U. Hartl, W. Harder, M. Veenhuis, Affinity purification of molecular chaperones of the yeast *Hansenula polymorpha* using immobilized denatured alcohol oxidase, FEBS Lett. 321 (1993) 32–36.
[18] S. Baksh, M. Michalak, Expression of calreticulin in *Escherichia coli* and identification of its Ca^{2+} binding domains, J. Biol. Chem. 266 (1991) 21458–21465.
[19] J.A. Huntington, P.E. Stein, Structure and properties of ovalbumin, J. Chromatogr. B Biomed. Sci. Appl. 756 (2001) 189–198.
[20] M. Weihers, P.A. Barneveld, M.A. Cohen Stuart, R.W. Visschers, Heat-induced denaturation and aggregation of ovalubumin at neutral pH described by irreversible first-order kinetics, Protein Sci. 12 (2003) 2693–2703.
[21] S. Photchanachai, A. Mehta, N. Kitabatake, Heating of an ovalbumin solution at neutral pH and high temperature, Biosci. Biotechnol. Biochem. 66 (2002) 1635–1640.
[22] U.B. Ericsson, B.M. Hallberg, G.T. Detitta, N. Dekker, P. Nordlund, Thermofluor-based high-throughput stability optimization of proteins for structural studies, Anal. Biochem. 357 (2006) 289–298.
[23] F.H. Niesen, H. Berglund, M. Vedadi, The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability, Nat. Protoc. 2 (2007) 2212–2221.
[24] T.H. Steinberg, L.J. Jones, R.P. Haugland, V.L. Singer, SYPRO orange and SYPRO red protein gel stains: one-step fluorescent staining of denaturing gels for detection of nanogram levels of protein, Anal. Biochem. 239 (1996) 223–237.
[25] J.A. Huntington, P.A. Patston, P.G. Gettins, S-ovalbumin, an ovalbumin conformer with properties analogous to those of loop-inserted serpins, Protein Sci. 4 (1995) 613–621.
[26] M. Weijers, P.A. Barneveld, M.A. Cohen Stuart, R.W. Visschers, Heat-induced denaturation and aggregation of ovalbumin at neutral pH described by irreversible first-order kinetics, Protein Sci. 12 (2003) 2693–2703.
[27] C.A. Haskard, E.C.Y. Li-Chan, Hydrophobicity of bovine serum albumin and ovalbumin determined using uncharged (PRODAN) and anionic (ANS^-) fluorescent probes, J. Agric. Food Chem. 46 (1998) 2671–2677.
[28] L.A. Fothergill, J.E. Fothergill, Thiol and disulphide contents of hen ovalbumin. C-terminal sequence and location of disulphide bond, Biochem. J. 116 (1970) 555–561.
[29] M.E. Paquet, M.R. Leach, D.B. Williams, In vitro and in vivo assays to assess the functions of calnexin and calreticulin in ER protein folding and quality control, Methods 35 (2005) 338–347.
[30] Y. Takeda, K. Totani, I. Matsuo, Y. Ito, Chemical approaches toward understanding glycan-mediated protein quality control, Curr. Opin. Chem. Biol. 13 (2009) 582–591.
[31] K. Duus, N. Sandhu, C.S. Jorgensen, P.R. Hansen, A. Steino, M. Thaysen-Andersen, P. Hojrup, G. Houen, Interaction of the chaperone calreticulin with proteins and peptides of different structural classes, Protein Pept. Lett. 16 (2009) 1414–1423.
[32] C.S. Jørgensen, N.H.H. Heegaard, A. Holm, P. Højrup, G. Houen, Polypeptide binding properties of the chaperone calreticulin, Eur. J. Biochem. 267 (2000) 2945–2954.
[33] H.J. Jeong, Y.G. Kim, Y.H. Yang, B.G. Kim, High-throughput quantitative analysis of total n-glycans by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Anal. Chem. 84 (2012) 3453–3460.
[34] D.J. Harvey, D.R. Wing, B. Kuster, I.B.H. Wilson, Composition of N-linked carbohydrates from ovalbumin and Co-purified glycoproteins, J. Am. Soc. Mass. Spectrom. 11 (2000) 564–571.
[35] K.E. Matlack, B. Misselwitz, K. Plath, T.A. Rapoport, BiP acts as a molecular ratchet during posttranslational transport of prepro-alpha factor across the ER membrane, Cell 97 (1999) 553–564.
[36] G.S. Hotamisligil, Endoplasmic reticulum stress and the inflammatory basis of metabolic disease, Cell 140 (2010) 900–917.