A novel approach for purification of recombinant proteins using the dextran-binding domain

Kuniyoshi Kaseda[a], Takao Kodama[b], Kazuhiro Fukui[c], Keiko Hirose[c],*

[a]Gene Discovery Research Center, National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1 Higashi, Tsukuba, Ibaraki 305-8562, Japan
[b]Laboratory of Molecular Enzymology, Department of Biochemical Science and Engineering, Kyushu Institute of Technology, Ihzuka, Fukuoka 820-8502, Japan
[c]Department of Microbiology, Okayama University Dental School, Okayama 700-8525, Japan

Abstract Using the dextran-binding domain (DBD) of a type of glucosyltransferase (GTF) from *Streptococcus sobrinus*, we have developed a novel method for purifying recombinant proteins. DBD-tagged green and red fluorescent proteins as well as the parent GTF and DBD moiety were adsorbed well to commercially available cross-linked dextran (such as Sephadex beads and Sephacryl beads), and eluted efficiently with water-soluble dextran. The purity of the eluted proteins after this one-step affinity purification was ∼90% or better. The results suggest that DBD can be used as a powerful carrier for purification of various recombinant proteins. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Dextran; Dextran-binding domain; Glucosyltransferase; Purification; Recombinant protein; Tag

1. Introduction

Thanks to the development of genetic engineering techniques, it is now possible to prepare a protein of interest as a recombinant protein relatively easily, rapidly, and reproducibly, in host cells such as *Escherichia coli*, yeast, baculovirus, or mammalian cells. One of the most powerful techniques is the use of carrier sequences. The carrier sequences are fused to the proteins of interest as a 'tag', to be used for their purification, immobilization, immunological detection, localization study in vivo, and screening of the proteins that interact with the target proteins [1–6].

There are several advantages to each of the existing tag systems, such as a maltose-binding protein (MBP), glutathione-*S*-transferase (GST), or consecutive histidine residues. However, some target proteins are difficult to obtain using these tags, due to inhibited expression of the fusion protein

or formation of insoluble aggregate. In some cases, the trouble can be avoided by utilization of a different tag. Therefore, in order to prepare various proteins efficiently, it is advantageous to have a large variety of tag systems.

The dextran-binding domain (DBD) is a functional moiety of glucosyltransferase (GTF) from oral streptococci (see Fig. 1a). DBD is known to interact with dextran reversibly [7–10]. The DBD-binding unit of a single dextran chain consists of approximately 15 glucosyl residues, and the binding constant to the unit (2×10^5 M^{-1}) is relatively low. However, insoluble, cross-linked dextrans such as Sephadex beads or Sephacryl beads have multiple binding sites for DBD. As a result, DBD binds to these beads almost irreversibly in the absence of water-soluble dextran. In fact, in our recent work [10], specific and irreversible interaction between fluorescently labeled GTF and Sephadex G-100 was directly imaged under a fluorescence microscope. Adsorbed GTF molecules were easily eluted from the beads with water-soluble dextran in excess. Moreover, GTF from oral bacteria, *Streptococcus sobrinus* 6715, was purified by binding to insoluble dextrans followed by elution using soluble dextran or denaturing solvents such as guanidine hydrochloride [11,12]. These observations suggest the possibility of collecting recombinant proteins fused to DBD specifically from the cell lysates, and eluting them by addition of water-soluble dextran.

In the present work, we demonstrate that the DBD-tag is in fact a powerful tool for rapid purification of target proteins. We characterized some features of the DBD-tag system for its efficient use. One of the advantages of the DBD purification method is its low cost compared with other tag systems, because expensive resins are not required. Also, dextran is an inactive molecule so that it has little effect on the functions of the target proteins.

2. Materials and methods

2.1. Chemicals
Dextrans of different sizes were purchased from the following sources: dextran (18.1 kDa, industrial grade), from Sigma; dextran T-10, T-500, and T-2000 (10, 500, and 2000 kDa, respectively), for enzymatic studies, from Pharmacia; dextran (5–150 kDa, for gel-filtration analysis), from Fluka; and dextran (4000–5000 kDa, for molecular weight standard), from Polyscience. Sephadex G-200, Sephadex G-100 (Superfine), Sephadex G-25 (Fine), and Sephacryl S-500 HR were from Pharmacia. All cross-linked dextrans were well soaked in buffer before use. Other reagents were of analytical grade.

E-mail: kaseda@nair.go.jp
E-mail: kodama@bse.kyutech.ac.jp
E-mail: kfukui@dent.okayama-u.ac.jp
*Corresponding author. Fax: (81)-298-61 3049.
E-mail: khirose@nair.go.jp

2.2. Plasmid construction

To obtain DBD consisting of 3.5 repetitive units (referred as A repeats, [13]), the plasmid that encodes GTF I of *S. sobrinus* 6715 (pAB2) was digested by *Hin*dIII as described by Abo et al. [8]. The resultant fragment was ligated into the *Hin*dIII site of the pUC 18 vector, which is in phase with the lacZ reading frame. Here we refer to this plasmid as 'pDBD'. DNA fragments encoding a green fluorescent protein (GFP) and a red fluorescent protein (RFP) were subcloned into the *Bam*HI site of pDBD using 5′ *Bgl*II and 3′ *Bam*HI restriction sites engineered by a polymerase chain reaction to obtain pGFP–DBD and pRFP–DBD. The gene products of pGFP–DBD and pRFP–DBD are referred as GFP–DBD and RFP–DBD, respectively.

2.3. Protein expression and purification

The proteins were expressed in *E. coli* BL21(DE3) cells freshly transformed with the plasmids, pAB2, pDBD, pGFP–DBD, and pRFP–DBD. Overnight cultures of bacteria were diluted 1:200 in TPM medium [14] supplemented with ampicillin (100 μg/ml), and grown with shaking at 37°C until the absorbance at 600 nm reached 0.4. The cells were shaken for another 15 min at 20°C before induction with isopropyl β-D-thiogalactopyranoside (0.5 mM). After incubation for 4 h at 20°C, the cells were harvested by centrifugation (2 min at 2000×g), and washed with buffer A (10 mM Tris-acetate (pH 8.0), 1 mM magnesium acetate, and 250 mM potassium acetate). The bacterial pellets were re-suspended in buffer A supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 10 μg/ml Na-*p*-tosyl-L-arginine methyl ester, and 1.0 μg/ml aprotinin), disrupted by sonication, and then centrifuged (30 000×g, 30 min, 4°C). 1/20 volume of a 50% (v/v) slurry of Sephadex G-100 beads was added to the supernatant, and mixed gently for 2 h at 4°C. The beads were then collected by brief centrifugation (30 s at 500×g), and washed four times with 30 volumes of buffer A. Finally, the proteins were eluted by applying an equal volume of 50 mg/ml dextran (18.1 kDa) to the beads, and analyzed by SDS–PAGE/Coomassie brilliant blue staining.

It has been shown that GTF can be purified from oral streptococci using dextran beads [11,12]. We confirmed the results in our recombinant purification system as above, and also checked to see if DBD is also purified efficiently using the same method. GTF and DBD were only 1–3% of the total proteins in the soluble fractions of the bacterial cell extract (Fig. 1b, lanes 1 and 3). After the purification step using Sephadex G-100 beads, both GTF and DBD appeared as single bands on SDS–PAGE/Coomassie brilliant blue staining (Fig. 1b, lanes 2 and 4), suggesting high feasibility of this system for purifying various DBD-tagged proteins.

2.4. The binding capacities of cross-linked dextrans for DBD-fusion proteins

The binding capacities of cross-linked dextrans beads were examined for GTF and DBD, and for GFP–DBD in cell extract. GTF or DBD was purified in advance by the method described previously [9]. Proteins (0.5 mg/ml) were incubated with 0.75–5% (v/v) cross-linked dextran beads (Sephadex G-100, G-200, G-25, or Sephacryl S-500 HR) in buffer A, with gentle mixing for 3 h at 25°C. After sedimenting the beads (30 s at 500×g), the concentration of the protein in the supernatant was determined by the Bradford method [15] to calculate the amount of GTF and DBD adsorbed to the dextran beads.

In order to examine the binding capacity in an actual purification procedure, cell extract containing GFP–DBD was used. After high speed centrifugation of the extract, 1/25 volume of Sephadex G-100 beads were added to the supernatant, incubated for 2 h at 4°C, and then sedimented by centrifugation (30 s at 500×g). The concentration of GFP–DBD in the supernatant was directly determined by optical measurement (OD$_{488}$).

2.5. Efficiency of elution of DBD-fusion proteins from cross-linked dextran beads

Crude cell extract was incubated with Sephadex G-100 beads as above to adsorb expressed GFP–DBD to the beads. After the beads were washed with buffer A, a part of the beads solution was analyzed by SDS–PAGE. The amount of GFP–DBD adsorbed to the beads was estimated by densitometry of the Coomassie brilliant blue-stained gel. The protein was eluted by addition of an equal volume of water-soluble dextran to the beads solution. After centrifugation (30 s at 500×g), the amount of the recovered protein was determined by measuring the concentration of GFP–DBD in the supernatant.

To examine how the amount of the eluted protein depends on the concentration of dextran, 0–50 mg/ml (at a final concentration) dextran (18.1 kDa) was used. To investigate the size dependency, dextrans with the relative molecular mass of 1–5000 kDa (at a final concentration of 30 mg/ml) were examined.

3. Results and discussion

3.1. Purification of DBD-tagged fusion proteins

As described in Section 2.3, recombinant GTF and DBD expressed in *E. coli* BL21 (DE3) cells were obtained with the purity better than 95% by the one-step affinity purification. In order to check whether DBD can be generally used as a tag for purifying recombinant proteins, we used crude cell extract containing two constructs, GFP–DBD and RFP–DBD, as test specimens. Both DBD-tagged proteins were purified by the batch processing as above. As shown in Fig. 1b (lanes 5–8), these proteins were also purified to the level of 89% (for GFP–DBD) and 92% (for RFP–DBD). Similar results were obtained using Sephadex G-200 and Sephacryl S-500 HR (data not shown). These results strongly suggest that the DBD–dextran beads system is a powerful tool for purification of various recombinant proteins.

3.2. Estimation of the binding capacities of cross-linked dextrans for DBD-fusion proteins

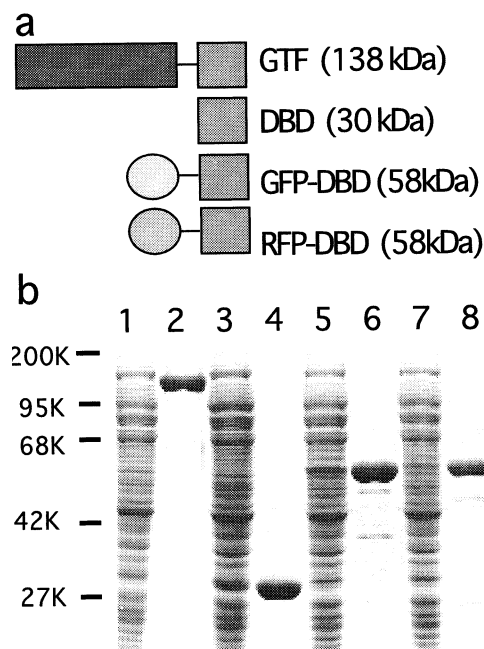In order to perform the purification procedure efficiently,

Fig. 1. One-step purification of recombinant proteins using DBD as the tag. Schematic representation of the recombinant proteins (a) and SDS–PAGE analysis of the protein preparations (b). The calculated molecular weight of each protein is indicated in parentheses. The parent GTF (lanes 1 and 2), its functional moiety DBD (lanes 3 and 4), the DBD-tagged proteins GFP–DBD (lanes 5 and 6) and RFP–DBD (lanes 7 and 8) were adsorbed with Sephadex G-100 beads and eluted with 25 mg/ml dextran (18.1 kDa). The SDS–PAGE patterns of the proteins before (lanes 1,3,5 and 7) and after (lanes 2,4,6 and 8) this one-step purification step are shown. After the purification, the SDS gel shows mainly single strong bands approximately corresponding to the expected molecular weights. The purity of those proteins were 95%, 96%, 89%, and 92% for GTF, DBD, GFP–DBD, and RFP–DBD, respectively.
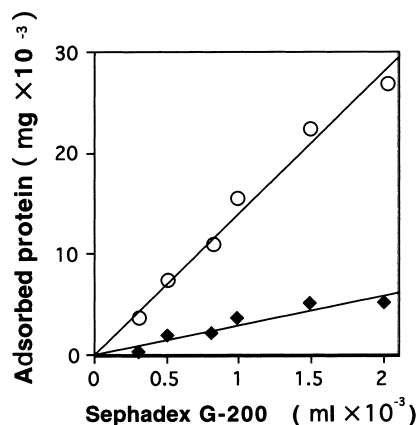
Fig. 2. The binding capacity of Sephadex G-200 for DBD containing proteins. A Sephadex G-200 solution was mixed with an excess amount of purified GTF or DBD (0.5 mg/ml) in buffer A, and centrifuged as described in Section 2.4. The amount of GTF and DBD adsorbed to the dextran beads was estimated by measuring the concentration of the protein in the supernatant. The adsorbed GTF (open circles) and DBD (closed diamonds) increased linearly with the volume of the added beads. 1 ml of beads has the capacity to adsorb 14 mg GTF or 3 mg DBD.

we quantitatively estimated the amount of DBD-tagged proteins bound to the dextran beads. First, the maximum binding capacities were studied using purified proteins, GTF and DBD, which were obtained by established methods [9]. When incubated with Sephadex G-200 beads at different concentrations, the amount of GTF (Fig. 2, open circles) or DBD (Fig. 2, closed diamonds) adsorbed to the beads was proportional to the volume of the added beads. The binding capacities of Sephadex G-200 calculated from the graph were 3 mg protein/ml beads for DBD, and 14 mg of protein/ml beads for GTF. Considering the molecular weight of DBD (30 kDa) and GTF (138 kDa), these two proteins were adsorbed to Sephadex G-200 at nearly the same molar levels ($\sim$100 μmol protein/ml beads). The binding capacity determined here is as good as or better than obtained with the histidine-tag or GST-tag methods [16,17].
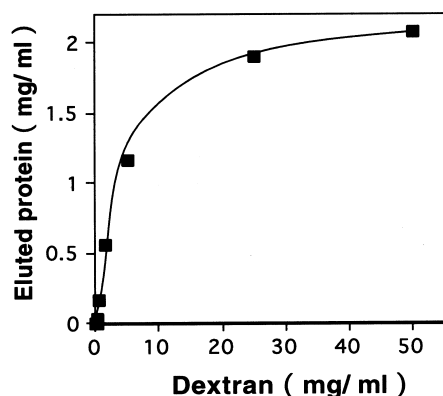
Sephadex G-100 and Sephacryl S-500 HR beads also

Fig. 3. Effect of the concentration of dextran for the elution step. The soluble fraction containing GFP–DBD was mixed with Sephadex G-100, and the protein was eluted by the addition of water-soluble dextran (18.1 kDa) to the final concentrations of 0–50 mg/ml, and the concentration of the eluate was measured by the optical method. The amount of eluted GFP–DBD was nearly maximal with 30 mg/ml dextran.
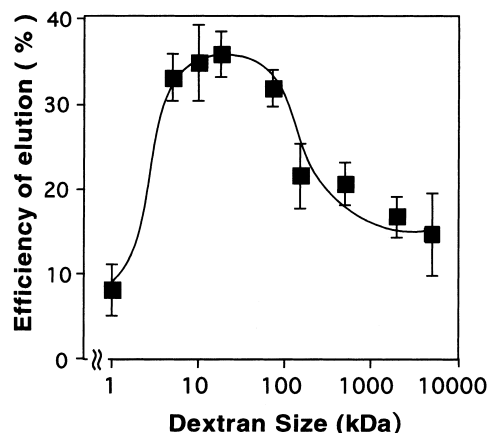

Fig. 4. Dependency of the elution efficiency on the size of dextran. After incubation with Sephadex G-100, GFP–DBD was eluted with water-soluble dextrans of various sizes. The percentages of the recovered protein are shown. Dextrans with a size of 5–70 kDa showed high efficiency.

showed high binding capacities, especially for DBD: the binding capacities of Sephadex G-100 were 11 and 17 mg protein/ml beads for DBD and GTF, respectively, and those of Sephacryl S-500 HR were 14 and 5 mg protein/ml beads for DBD and GTF, respectively. In contrast, Sephadex G-25 beads that have a smaller pore size (fractionation range for globular proteins in gel-filtration: 1×10^3–5×10^3) did not retain either GTF or DBD at all. This is probably because DBD-tagged recombinant proteins can bind in the pore of the dextran beads only if the pore is sufficiently large. Since Sephadex G-100, G-200, and Sephacryl S-500 HR all showed high binding capacities for purified GTF and DBD, we used Sephadex G-100 for the following experiments.

The results in Fig. 1 showed that DBD-tagged GFP and RFP were collected efficiently from the cell extract by dextran beads, even though the original extract contained only a few percent of the DBD-tagged proteins. Using Sephadex G-100, we determined the fraction of GFP–DBD adsorbed to the beads. More than 30% of the soluble GFP–DBD was collected by incubation with 1/25 volume of Sephadex G-100 beads in buffer A at 4°C for 2 h (5 mg of GFP–DBD/ml of beads). The fraction was increased to 70% when the incubation time was extended to 15 h.

Proteins are often purified in the presence of bio-active reagents, to preserve the functions of the target proteins. Since some of these reagents might interfere with the interaction between DBD and dextran beads, various reagents were added to the control solution (10 mM Tris-acetate (pH 8.0) alone), and the amount of GFP–DBD adsorbed to the beads was compared with the control. Tested chemical reagents and the relative amount of the adsorbed GFP–DBD were: 100 mM NaCl (115%), 100 mM Imidazole (123%), 2 mM EGTA (135%), 2 mM CaCl$_2$ (106%), 2 mM EDTA (139%), 2 mM MgCl$_2$ (124%), 0.05% 2-mercaptoethanol (118%), 1 mM DTT (124%), 0.5% polyoxyethylene (10) octylphenyl ester (Triton X-100) (137%), 0.1% polyoxyethylenesorbitan monolaurate (Tween 20, 82%), 0.05% NaN$_3$ (135%), and 1 mM ATP (123%). Therefore, the resultant binding capacities were within 80–140% of the control, indicating that these reagents do not largely affect the capacity. We have also examined the effect of the ionic strength: when 0 to 0.5 M NaCl was added

to the control solution, the fraction of the adsorbed protein increased dose-dependently from 23% to 33% (data not shown).

3.3. Estimation of the elution efficiency of DBD-fusion protein from cross-linked dextran

DBD-tagged proteins bound to dextran beads were eluted using water-soluble dextran. We first estimated the optimum concentration of water-soluble dextran that is used for elution. After specific binding to Sephadex beads and extensive washing with buffer A, GFP–DBD was eluted with an equal volume of water-soluble dextran (18.1 kDa) at various concentrations. As shown in Fig. 3, the amount of the protein in the supernatant increased dose-dependently, and reached a plateau when the concentration of dextran was approximately 30 mg/ml.

Using 50 mg/ml dextran, 43% of the protein adsorbed to the beads was recovered. When the elution procedure was repeated, 31% was subsequently recovered with the second elution, and 17% with the third. Thus, almost all (91% in total) of the adsorbed protein was recovered by the three-step elution with 50 mg/ml dextran.

We then examined how the elution efficiency depends on the size of the water-soluble dextran. Fig. 4 shows that GFP–DBD was efficiently eluted with 5 to 70 kDa dextrans, but poorly eluted with smaller (1 kDa) or larger (>150 kDa) dextrans. As described in Section 3.2, it is likely that DBD-tagged proteins bind in the pore of the beads. Thus, to elute the bound protein efficiently, water-soluble dextran would have to enter the pore of the beads, and then bind tightly to DBD. Since the Sephadex G-100 beads used here is known to be suitable for separation of 1–100 kDa water-soluble dextran, it is expected that dextran with larger sizes can not enter the pore. Smaller dextran, on the other hand, would enter the pore but have only a weak affinity to DBD. In fact, our calorimetric experiments showed that the affinity of DBD to dextran remained unchanged when the molecular weight of the dextran was higher than 10 kDa, but decreased when it was lower than 5 kDa (Kaseda, K. and Kodama, T., unpublished data). These results indicate that the optimum size of water-soluble dextran for the elution step is 5–70 kDa, when Sephadex G-100 is used to collect DBD-tagged proteins.

3.4. Evaluation of the DBD purification method

The DBD-fusion method described here permits one-step purification of recombinant proteins from crude cellular lysates. This method has several advantages. Firstly, the cost of the purification procedure is lower than with most of other methods, since expensive resin modified with specific ligand is not needed. Secondly, because dextran is an inactive molecule, it does not necessarily have to be removed after the elution steps. This means that the time required for purification could be shortened. Another advantage is that DBD refolds easily after being denatured with chemicals including guanidine hydrochloride, urea, SDS, and by heating. Thus, even if proteins with DBD aggregate with each other to form an insoluble inclusion body, they can be recovered after solubilization with a denaturing agent. Additionally, DBD has no cysteinyl residue, so that we can specifically modify cysteine residues in the target protein with some thiol-reactive probes for functional and/or structural studies. Finally, it has been shown that DBD is still functional when truncated at the C-terminus to 25 kDa [8]. The minimum functional DBD is not known yet, but it is possible that a smaller domain of DBD can be used as a tag for purifying recombinant proteins.

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