4356–4357 Nucleic Acids Research, 1994, Vol. 22, No. 20

Differential effects of detergents on enzyme and DNA-binding activities of a glutathione S-transferase – homeodomain fusion protein

Laurent Seroude* and David L.Cribbs
Centre de Biologie du Développement, 118 route de Narbonne, Bâtiment 4R3, 31062 Toulouse Cedex, France

Received April 21, 1994; Revised and Accepted August 31, 1994

Recombinant proteins are widely utilised to investigate mechanisms of protein–DNA or protein–protein interactions. Expression of a recombinant protein in *E.coli* as a fusion with glutathione S-transferase (GST) is one of the most popular and easiest methods as it allows a single-step purification of the fusion protein by affinity chromatography on immobilised glutathione (5). However one can encounter the serious technical problems that some DNA-binding fusion proteins cannot be solubilized, are inefficiently fixed by the affinity matrix, or have very low or no DNA-binding activity. To overcome these difficulties when we tried to express and purify a GST fusion with a polypeptide containing the homeodomain of the homeotic protein proboscipedia (pb) (1), we tested the influence of different detergents on purification measured by GST specific activity, as compared with pb DNA-binding activity.

Overnight cultures (~30 h at 30°C instead of 37°C) of *E.coli* strain XL1Blue harboring pGEX-B (6) or pGEX-HDpb plasmids (Table 1) were used to inoculate 400ml 2TY medium to a density of 0.1 OD_{600nm}. When cultures reached an OD_{600nm} of 0.4–0.5, IPTG was added to a final concentration of 0.1mM. After 1 h of induction, cells were pelleted and resuspended in PBS (140mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, 10mM DTT pH 7) to a final concentration of 20 OD_{600nm}units/ml. After 60 min incubation at 4°C with 1mg/ml lysosyme (Sigma) cells were pelleted and resuspended in PBS or PBS supplemented with 0.03% SDS (Biorad), 1% Tween-20 (Biorad), or 0.2% NP40 (Sigma). After sonication and 60 min incubation on ice, lysed cells (crude extract) were subjected to centrifugation at 11600g at 22°C. To purify the GST-containing proteins, each supernatant was then mixed with glutathione Sepharose 4B (Pharmacia, 0.025µl bed volume/µl of supernatant) and incubated 30 min with agitation at 22°C. Beads were collected by brief centrifugation (1mn, 5000g, 22°C) and washed three times with ten bed volumes of PBS buffer. GST and GST-HDpb were eluted

Table 1. Effects of detergents on purification

Protein :	GST				GST-HDpb			
Detergent :		SDS	Tween	NP40		SDS	Tween	NP40
Protein concentration (mg/ml)								
Crude extract :	2.8	2.1	3.8	3.2	2.6	2.8	4.2	3.0
Soluble fraction :	2.6	1.1	3.7	2.9	2.4	1.4	3.8	2.9
Elution :	0.8	0.3	1.0	1.2	0.5	0.3	0.6	0.5
GST specific activity (u/mg)								
Crude extract :	15.5	7.5	17.2	19.5	3.3	2.6	4.2	4.3
Soluble fraction :	18.9	13.8	17.6	20.8	3.5	3.3	4.6	3.8
Elution :	203.0	253.0	187.9	192.7	15.2	8.0	39.6	39.2
Purification factor								
Solubilization :	**1.2**	**1.8**	**1.0**	**1.1**	**1.1**	**1.3**	**1.1**	**0.9**
Elution :	**10.7**	**18.4**	**10.7**	**9.3**	**4.3**	**2.4**	**8.5**	**10.3**
DNA binding activity (cpm/µg)								
Elution :					**12862.5**	**8543.7**	**22831.3**	**66784.8**

Plasmid pGEX-B(no insert) produces GST alone with an expected size of 26kDa. pGEX-HDpb, obtained by cloning in PGEX-B a 525pb BamHI–XmaI pb cDNA fragment(exon2-exon6) including the entire homeodomain, produces a fusion protein with an expected size of 47.2kDa. The solubilization purification factor is the ratio of soluble fraction GST specific activity versus crude extract GST specific activity. The elution purification factor is the ratio of elution GST specific activity versus soluble fraction GST specific activity. DNA-binding activity of GST-HDpb proteins was determined as the radioactivity (Cerenkov cpm) retained by membrane-bound protein (corrected by subtraction of radioactivity retained with GST alone).

* To whom correspondence should be addressed

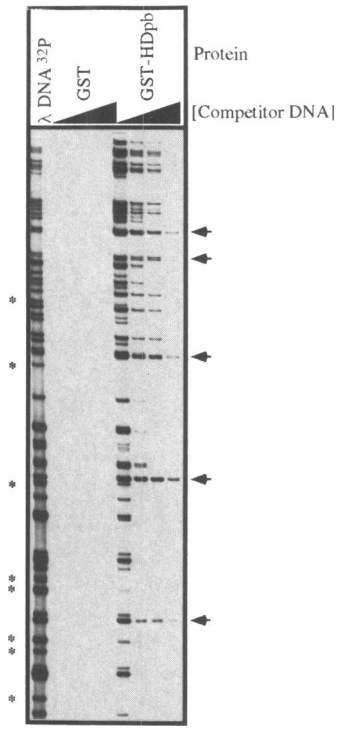

Figure 1. DNA binding activity test. Purified GST was used as negative control of DNA binding activity as it does not bind to DNA. 200µl binding reaction contained 0.5µg of nitrocellulose membrane (Schleicher & Schuell BA85) immobilised GST or GST-HDpb purified protein (without detergent), 30ng ^{32}P-lambda DNA Sau3AI fragments (2.10^7cpm/µg), plus competitor DNA (mass ratio of competitor/^{32}P-lambda DNA = 0, 10, 100 and 1000). Following washes and elution as described (4), retained fragments were separated on a 6% sequencing gel. Fragments specifically retained at the highest competitor DNA concentrations are indicated by arrows (right), while fragments poorly if at all retained without competitor are marked with asterisks (left).

two times with one bed volume of elution buffer (20mM glutathione, 100mM Tris pH 8, 120mM NaCl). The first elution was performed at 22°C with 10 min agitation and the second 10 min at 37°C. We determined total protein concentration (Bradford assay – Biorad), GST activity (GST detection module – Pharmacia) and GST specific activity on crude extracts, soluble fractions and matrix elution fractions (Table 1). Detergents tested, other than SDS, have little effect on purification of GST alone and so probably do not drastically affect solubilization, binding to the affinity matrix or GST activity while SDS improves the purification factor by ~2 fold. In contrast the effects of these reagents differed markedly when we purified GST-HDpb. The presence of Tween-20 or NP40 improves the purification factor (2 to 3 fold) compared to no detergents without diminishing the yield. In contrast, SDS reduced this purification factor by about half. There is no significant effect on solubilization since the specific activities of soluble fractions are similar. Thus it seems these detergents act either on fusion protein binding to the affinity matrix (probably by avoiding non specific interactions) or by preserving structure and so fusion protein activity (or for both reasons). These results show that these reagents' effects on GST alone cannot be extrapolated to GST fusion proteins, since for example SDS has contradictory effects on GST and GST-HDpb purification (Table 1).

We wished to ask how precisely GST activity correlates with DNA-binding activity. The DNA-binding activity of GST-HDpb fusion was therefore tested by the binding-site-selection (BSS) method (4). Eukaryotic homeodomain proteins generally recognise related sequences containing the motif TAAT (3). Hence a sufficiently complex DNA sequence, such as lambda DNA used here, should contain sequences specifically recognised (2) by a given homeoprotein such as pb. As seen in Figure 1, many but not all ^{32}P-labelled lambda restriction fragments are retained in the absence of added competitor. That only some fragments are retained in these conditions while others are not (marked to the left of input by asterisks) suggests a sequence preference to DNA retention. This is confirmed since with competitor in 1000 fold excess, only a handful of fragments are retained (indicated by arrows). This specificity of binding is almost certainly biologically relevant since the fragments specifically bound contain the reiterated TAAT motif typical of many homeodomain binding sequences (3), though it is worth noting that the fragments bound are different from those bound by the homeoprotein engrailed on the same DNA substrate (2). The raw quantity of radioactivity retained without competitor was found to correlate with band intensities at the different competitor concentrations employed (densitometric data not shown). This allowed us to compare different protein preparations simply by quantification of ^{32}P DNA retention on the membrane (Table 1). The detergents employed, and presumed to be eliminated by affinity column purification, do not affect protein binding capacity for nitrocellulose membrane (confirmed by western blot quantification of pb antigen bound to the membrane). GST and DNA binding activities correlate poorly in these preparations. Tween or NP40-treated samples possess GST activities that are roughly equivalent but DNA-binding activity is clearly improved by NP40. Moreover, while Tween-treated GST activity is greater than untreated, DNA-binding activity is lower.

We conclude that while GST fusion proteins are useful to purify DNA-binding proteins because of the technical simplicity of this approach, it is still preferable to use a DNA-binding activity test instead of GST activity to estimate and optimise recovery of DNA-binding activity. While choosing extraction conditions will likely require a case-by-case assessment, the detergents employed here, especially NP40, may offer a useful advantage towards extracting active DNA-binding fusion proteins.

ACKNOWLEDGEMENTS

L.S. was supported by a training fellowship from the Ministère de l'Enseignement Supérieur et de la Recherche (M.E.S.R). This work was supported by the C.N.R.S. and by grants from the M.E.S.R. and the Association pour la Recherche contre le Cancer.

REFERENCES

1. Cribbs,D.L., Pultz,M.A., Johnson,D. Mazzula,M. and Kaufman,T.C. (1992) *EMBO J.* **11**(4), 1437–1449.
2. Desplan,C., Theis,J. and O'Farrell,P.H. (1985) *Nature* **318**, 630–635.
3. Ekker,S.C., Kessler,D.P.v. and Beachy,P.A. (1992) *EMBO J.* **11**(11), 4059–4072.
4. Norby,P.L., Pallisgaard,N., Pedersen,F.S. and Jorgensen,P. (1992) *Nucl. Acids Res.* **20**(23), 6317–6321.
5. Smith,D.B. and Johnson,K.S. (1988) *Gene* **67**, 31–40.
6. Valle,D., Kun,J., Linss,J., Garcia,E.d.S. and Goldenberg,S. (1993) *Insect Biochem. Molec. Biol.* **23**(4), 457–465.