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Fusion to an endoglucanase allows alkaline phosphatase to bind to cellulose

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Endoglucanase CenA of *Cellulomonas fimi* comprises an N-terminal cellulose-binding domain and a C-terminal catalytic domain joined together by a sequence of 23 proline and threonine residues (the Pro-Thr box). The domains function independently when separated by proteolysis. Tn*phoA* has been used to generate *cen*A'-'*pho*A fusions. CenA'-'PhoA fusion polypeptides which contain the entire cellulose-binding domain of CenA bind to cellulose, allowing their purification from periplasmic extracts in a single, facile step. This result has implications for purification or immobilisation of chimeric proteins on a cheap cellulose matrix.

Endoglucanase; Cellulose-binding domain; Alkaline phosphatase; Fusion protein; Purification

1. INTRODUCTION

Endoglucanase CenA of *Cellulomonas fimi* comprises two discrete, functionally independent domains joined by a hinge-like sequence of proline and threonine residues (the Pro-Thr box). The N-terminal domain of 111 amino acids binds the enzyme to cellulose; the C-terminal domain of 284 amino acids is the catalytic domain (fig.1B) [1,2]. Each domain retains its function when the enzyme is cleaved proteolytically at the carboxyl end of the Pro-Thr box [2]. The gene for CenA, *cen*A, has been cloned in *Escherichia coli* [3]. Its product can be purified from cell extracts of *E. coli* by affinity chromatography on cellulose [2]. *E. coli* exports a significant fraction of the CenA it produces to the periplasm [3].

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Abbreviations: Amp, ampicillin; CenA, endoglucanase A; CM-cellulose, carboxymethylcellulose; Kan, kanamycin; XP, 5-bromo-4-chloro-3-indolyl phosphate

The properties of the cellulose-binding domain of CenA make it very attractive for use in hybrid proteins which can be purified by affinity chromatography on cellulose. Cellulose is available in a variety of forms: powder; paper; cotton. The conditions for binding of CenA to and its elution from cellulose are very mild, and the enzyme can be obtained in a concentrated solution free of low molecular mass ligands [2,4]. We demonstrate here the use of the cellulose-binding domain of a cellulase to confer specific adhesive properties to a recombinant protein. This is the first report of the construction of a cellulose-binding chimeric protein and offers a new generic technique for the immobilisation or purification of proteins on an inexpensive inert support.

2. EXPERIMENTAL

2.1. *Bacterial strain, plasmids, bacteriophage and medium*

E. coli CC118 [5], plasmids pUC18-1.6*cen*A [6] and pB-1 [5], and bacteriophage λTn*phoA*-1 [7] were described previously. Cultures were grown in LB medium [8]. Solid medium contained 1.5% agar (Difco). Kanamycin was used at both 50 and

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300 μg/ml (6 ×), and ampicillin at 75 μg/ml. PhoA activity was detected by including XP at 40 μg/ml in the plating medium [5]. CenA activity was detected by including 1% CM-cellulose in the plating medium and staining the agar with Congo red [9] after incubation.

2.2. *Construction of* cenA'-'phoA *gene fusions*

Plasmid pUC18-1.6*cen*A in the PhoA$^-$ strain *E. coli* CC118 was the target for insertional mutagenesis with Tn*pho*A [5]. The transposition event was mediated by infection of *E. coli* CC118/pUC18-1.6*cen*A with a λ suicide phage, λTn*pho*A-1 [7], carrying the transposon. After infection, cells were plated on (6 ×)Kan-Amp-XP plates. Clones expressing PhoA activity were pooled and used to prepare plasmid DNA [10]. The plasmid preparation was used to transform *E. coli* CC118 [10], and transformants were selected on Kan-Amp-XP plates. PhoA$^+$ clones were screened for CenA activity after growth on CM-cellulose-containing plates. Plasmid DNA was prepared from PhoA$^+$ CenA$^-$ clones and the position of Tn*pho*A insertion determined by restriction analysis with *Eco*RI.

2.3. *Binding of CenA'-'PhoA fusion proteins to filter paper*

Cells from cultures of *E. coli* CC118 CenA'-'PhoA fusion strains were concentrated by centrifugation. Periplasmic proteins were released from the cells with chloroform [11] and applied to discs of Whatman 541 filter paper, which had been pre-incubated with 50 mM Tris-HCl (pH 7.5), containing 5% bovine serum albumin to prevent non-specific binding of proteins. One filter from each pair was washed exhaustively in 50 mM Tris-HCl (pH 7.5) and in 0.5 M NaCl. All filters were stained for PhoA activity [12] for 15 min with 1 mg/ml naphthol AS-MX phosphate, 2 mg/ml Fast Red TR salt in 50 mM Tris-HCl (pH 8.0). Three controls were used: purified PhoA from *E. coli*, a periplasmic fraction of *E. coli* CC118/pB-1, which expresses a Bla'-'PhoA fusion protein [5], and a periplasmic fraction of *E. coli* CC118.

2.4. *Purification of* cenA'-'phoA *fusion proteins by binding to cellulose*

Three protein samples were used: purified PhoA from *E. coli*, a periplasmic fraction of *E. coli* CC118/pB-1, and a periplasmic fraction of an *E. coli* CC118/pUC18-1.6*cen*A::Tn*pho*A strain encoding a CenA'-'PhoA fusion protein containing the cellulose-binding domain and part of the Pro-Thr box. The samples were adjusted to the same PhoA activity/ml. Assay of PhoA activity has been described [13]. Avicel PH-101, a microcrystalline cellulose, was autoclaved, washed with distilled water and resuspended in 50 mM Tris-HCl (pH 7.5), prior to use. After incubation of the protein samples with Avicel at 0°C for 1 h, the Avicel was collected by centrifugation. The supernatant was removed and an aliquot mixed with gel loading buffer. The Avicel was washed with 0.5 M NaCl and with 10 mM Tris-HCl (pH 8.0). The material remaining bound to the Avicel was extracted into loading buffer. The samples were boiled for 1 min and analysed by SDS-PAGE [14], using two gels (7.5% acrylamide). One gel was stained with Coomassie blue. The second, which contained 10 μg bovine serum albumin/ml to facilitate enzyme renaturation, was incubated in 2.5% Triton X-100 for 1 h to remove SDS, then equilibrated in 50 mM Tris-HCl (pH 8.0), containing 2 μM $ZnCl_2$ for 1 h; finally, it was stained for PhoA activity for 15–30 min, as described in section 2.3.

3. RESULTS

3.1. *Fusion proteins*

The enzyme chosen to make hybrids with the CenA cellulose-binding domain was alkaline phosphatase (PhoA) of *E. coli*. This allowed the

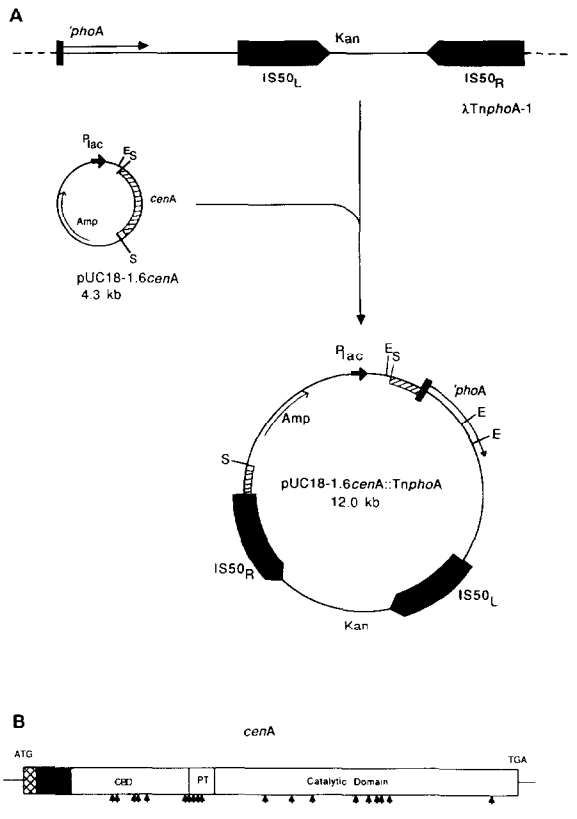

Fig.1. Construction of *cen*A'-'*pho*A gene fusions. (A) Scheme of Tn*pho*A transposition into *cen*A. The cleavage sites for *Eco*RI are shown. The dashed lines represent phage DNA. Kan, kanamycin resistance; Amp, ampicillin resistance; P_{lac}, *lac* promoter; S, *Sst*I; E, *Eco*RI; kb, kilobase. (B) Sites of Tn*pho*A insertions in *cen*A. The *cen*A gene is shown with regions coding for the signal peptide (shaded black), cellulose-binding domain (CBD), Pro-Thr box (PT) and catalytic domain of CenA. The cross-hatched box represents the coding region for 10 amino acids from LacZ fused to the N-terminus of CenA. Processing of the CenA leader peptide by *E. coli* has been previously demonstrated [6]. Translational start and stop codons are shown. The arrows show positions of Tn*pho*A insertion into *cen*A, determined by *Eco*RI restriction analysis.

use of Tn*phoA* [5] to generate a set of CenA'-'PhoA fusion polypeptides. If Tn*phoA* inserts in the correct orientation and translational reading frame into a gene encoding an exported protein, such as CenA, a hybrid protein with alkaline phosphatase activity can result. A set of fusions was obtained in which Tn*phoA* was inserted progressively further downstream of the translational start site in the CenA coding sequence (fig.1). The approximate points of insertion of Tn*phoA* within *cen*A were determined by restriction endonuclease mapping.

3.2. *Binding of fusion proteins to cellulose*

Binding of the PhoA activity from permeabilised cells to filter paper afforded a convenient screening procedure for cellulose binding of the CenA'-'PhoA fusions. The fusion polypeptides bound to filter paper if they contained the entire cellulose-binding domain of CenA (fig.2).

3.3. *Purification of fusion proteins*

Fusion polypeptides could be purified extensively from periplasmic extracts by adsorption to microcrystalline cellulose. A CenA'-'PhoA fusion containing the entire cellulose-binding domain bound to cellulose while purified PhoA and a fusion of β-lactamase (Bla) with PhoA remained in the supernatant (fig.3). Migration of the PhoA activity band for the Bla'-'PhoA fusion was similar to that of purified PhoA, suggesting that some proteolytic degradation of the fusion protein may have occurred. However, as this genetic construct was also derived from Tn*phoA* insertion [5] and PhoA activity was retained, this polypeptide still provided a valid negative control. For the CenA'-'PhoA fusion, at least 68% of the applied PhoA activity bound to cellulose and was not removed by buffer washes. This activity could be quantitatively removed by washing with distilled water (not shown).

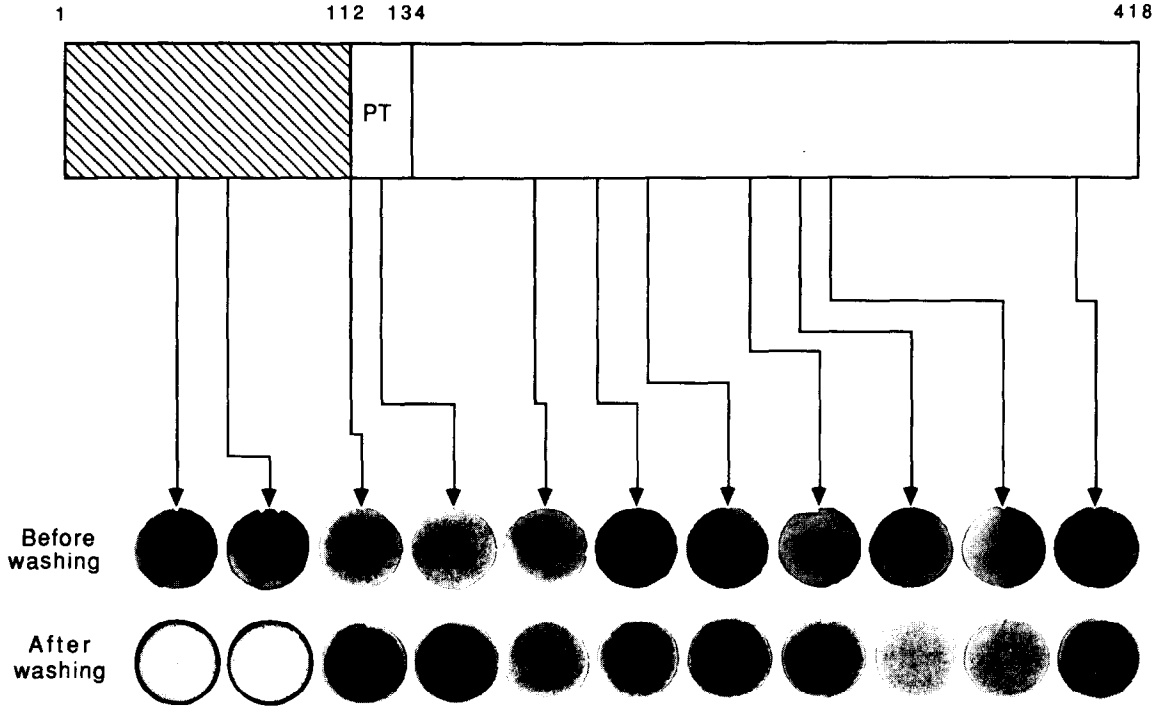

Fig.2. Binding of CenA'-'PhoA fusion proteins to filter paper. Mature CenA is shown, with numbered amino acid residues delineating the domains (see fig.1B). The arrows indicate the junctions of CenA with PhoA for the various fusions examined. For each fusion protein, filter paper disks stained for PhoA activity before and after buffer washing are shown. Controls for this assay (not shown) were: (i) periplasmic fraction of the PhoA$^-$ host, *E. coli* CC118 – negative before and after washing; (ii) purified *E. coli* PhoA – positive before washing, negative after washing; (iii) periplasmic fraction of cells containing a Bla'-'PhoA fusion protein – positive before washing, negative after washing.

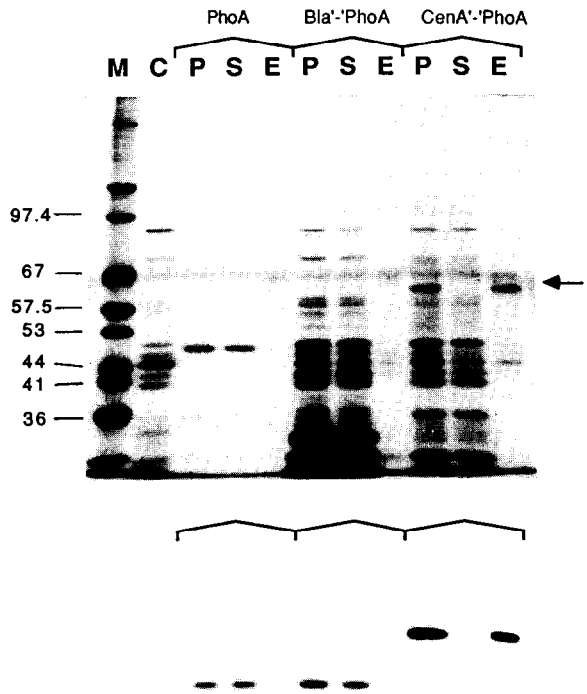

Fig.3. Coomassie blue-stained (top) and PhoA activity (bottom) SDS-polyacrylamide gels (7.5% acrylamide) showing purification of a CenA'-'PhoA fusion protein by adsorption to Avicel. M, molecular mass standards (sizes in kDa); C, periplasmic fraction of the PhoA$^-$ host, *E. coli* CC118; PhoA, purified *E. coli* PhoA; Bla'-'PhoA, periplasmic fraction of cells expressing a Bla'-'PhoA fusion protein; CenA'-'PhoA, periplasmic fraction of cells expressing a CenA'-'PhoA fusion protein containing the cellulose-binding domain and part of the Pro-Thr box (indicated by arrow); P, material applied to Avicel; S, supernatant after removal of Avicel; E, material recovered from Avicel.

4. DISCUSSION

Gene fusions encoding hybrid proteins with affinity for a particular matrix are used widely to facilitate the purification of polypeptides produced from cloned genes [15–19]. The majority of such hybrid polypeptides interact with a specific ligand which is coupled covalently to an inert support. These affinity matrices are usually complex and expensive. We demonstrate here the potential of cellulose, a cheap and readily available material, for affinity purification of hybrid polypeptides.

The filter paper assay (fig.2) indicates that fusion with the cellulose-binding domain of CenA could be used to immobilise an enzyme on cellulose, providing the fusion polypeptide retained activity. Certainly, fusion of a polypeptide of interest to the CenA domain will allow its facile purification from periplasmic extracts. A variety of methods is available for cleavage of fusion polypeptides at defined sites to release the desired fragment (see references in [15]). CenA fusion polypeptides may well be cleavable by specific proteases whilst bound to cellulose, thereby releasing the desired fragment into solution [1].

The Pro-Thr box clearly is not essential to the functioning of the cellulose-binding domain of CenA because some fusion polypeptides which lack part or all of the box still bind to cellulose (figs 2,3). The smallest fragment of the cellulose-binding domain which still allows binding to cellulose cannot be defined because all of the fusion polypeptides examined retain its N-terminus.

An exoglucanase, Cex, of *C. fimi* is structurally similar to CenA, but its cellulose-binding domain, which is also about 100 amino acids long, is at the C-terminus of the enzyme [2,20]. Fusion polypeptides which have the Cex cellulose-binding domain at their C-termini also bind to cellulose (Ong, E., personal communication). Thus, a given polypeptide could be fused at either its N- or C-terminus to a cellulose-binding sequence. This would be advantageous for polypeptides in which fusions at one but not at the other terminus affected stability, solubility or function. A further advantage of the cellulose-binding domains of the *C. fimi* cellulases is their small size relative to other so-called 'affinity tags' used for recombinant protein purification [15–19].

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