Lectin-based affinity tag for one-step protein purification

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The production of pure protein is indispensable for many applications in life sciences, however, protein purification protocols are difficult to establish, and the experimental procedures are usually tedious and time-consuming. Therefore, a number of tags were developed to which proteins of interest can be fused and subsequently purified by affinity chromatography. We report here on a novel lectin-based affinity tag using the D-mannose-specific lectin LecB from Pseudomonas aeruginosa*. A fusion protein was constructed consisting of yellow fluorescent protein and LecB separated by an enterokinase cleavage site. This protein was overexpressed in* Escherichia coli *Tuner (DE3), and the cell extract was loaded onto a column containing a mannose agarose matrix. Electrophoretically pure fusion protein at a yield of 24 mg/L culture was eluted with a D-mannose containing buffer. The determination of equilibrium adsorption isotherms revealed an association constant of the lectin to the mannose agarose matrix of* $K_a = 3.26 \times 10^5/M$*. Enterokinase treatment of the purified fusion protein resulted in the complete removal of the LecB-tag. In conclusion, our results indicate that the lectin LecB of* P. aeruginosa *can be used as a tag for the high-yield one-step purification of recombinant proteins.*

INTRODUCTION

The expression and purification of recombinant proteins still is an essential tool for various applications in life science, including enzyme characterization, crystallization, and antibody production. The purification of native proteins is often a problematic and time-consuming process, which has greatly been facilitated by the development of affinity tags. In the last few years, a number of different tags became commercially available (1). Generally, affinity tags can be divided into two categories: small peptide tags like poly-His (2) or Strep-tag (3) are less immunogenic than large proteins. They are considered not to affect the activity of the fused protein and, at least for many applications, their removal is not required. On the other hand, fusions to larger protein tags like glutathione S-transferase (4) or maltose binding protein (MBP) (5) can increase the solubility of the respective recombinant protein, but for many downstream applications the tag has to be removed, usually by hydrolysis using site-specific proteases. In view of the inherent drawbacks of the existing tag-systems, the development of new affinity tags still appears attractive.

Lectins represent a specific class of carbohydrate binding proteins different from enzymes or antibodies (6). They are found in a wide range of organisms including viruses, bacteria, plants, and animals and belong to one of several different families, many of which have been characterized structurally (7–10). LecB (alternatively PA-IIL) synthesized by the Gram-negative bacterium *Pseudomonas aeruginosa* is a tetrameric lectin with each monomer having a molecular mass of 11.73 kDa (11,12). This lectin exhibits a remarkably high binding affinity for L-fucose with an association constant in the micromolar range and also binds D-mannose, albeit with lower affinity (13). The structural basis of carbohydrate recognition and binding by LecB has recently been characterized in detail (14–16).

Presently, only few affinity purification systems are available, that are based on proteins with an intrinsic carbohydrate affinity; these include the cellulose binding domains (17), the MBP (5), and the chitin binding-domain (18). Here, we have used the lectin LecB as a fusion tag allowing for the one-step purification of a target protein by affinity chromatography.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5α was used for cloning experiments, and *E. coli* Tuner (DE3) was used as a heterologous expression host for plasmid-encoded YFP-LecB fusion protein.

Cloning of *yfp*::*lecB*

After digestion of the *lecB* carrying plasmid pEC2 with *Nde*I/*Bam*HI, the resulting 345-bp fragment encoding LecB was ligated into the same sites of pET19, giving pET19-*lecB*. Following hybridization of the two oligonucleotides PUREUP (5′-CATGGGCCATCATCATCATCATCATATGCCCGGGGAGCTCCTCGAGGACGACGACGACAAGGC-3′) and PUREDWN

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Table 1. Bacterial Strains and Plasmids

Strain or Plasmid	Genotype/Phenotype	Reference or Source
Strains		
Escherichia coli		
DH5α	*supE44* Δ(*lacZYA-argF*)*U196* (ϕ80Δ*lacZM15*) *hsdR17*(r_K^-, m_K^+) *recA1 endA1 gyrA96 thi-1 relA1 deoR*	26
Tuner (DE3)	*F⁻ ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (λ*cIts857 ind1 Sam7 nin5 lacUV5-T7 gene1*)	Novagen®; EMD Biosciences, Madison, WI, USA
Plasmids		
pET19b	T7-expression vector for *E. coli*, Ap^r	Novagen
pET19-*lecB*	pET19 containing *lecB* gene	This study
pEC2	pET22b containing the 345 bp *Nde*I/*Bam*HI PCR product encoding LecB	15
pFF19-EYFP	plant expression vector carrying the *yfp* gene	27
pURE	pET19-*lecB* containing an artificial multiple cloning site and the sequences encoding a hexahistidine tag and the enterokinase cleavage site	This study
pURE-*yfp*	pURE containing 720 bp *Nco*I/*Xho*I PCR product encoding yellow fluorescent protein (YFP)	This study

(5′-TAGCCTTGTCGTCGTCGTCC TCGAGGAGCTCCCCGGGCATAT GATGATGATGATGATGGCC-3′) a 60-bp fragment was obtained carrying recognition sites for the restriction enzymes *Nco*I, *Sma*I, *Sac*I, and *Xho*I and additionally encoded an enterokinase cleavage site and a hexahistidine tag. This fragment was cloned into the *Nco*I/*Nde*I sites of pET19-*lecB* to give plasmid pURE. A 720-bp DNA fragment carrying the *yfp* gene excluding its stop codon was amplified by PCR using plasmid pFF19-EYFP as the template with oligonucleotide primers YFPXhoI (5′-CGC**CTCG AG**CTTGTACAGCTCGTC-3′) and YFPNcoI (5′-CATG**CCATGG**TGA GCAAGGGCGAG-3′), introducing an *Xho*I and *Nco*I site, represented in bold, respectively. This fragment was digested with *Xho*I/*Nco*I and cloned into the LecB-tag carrying plasmid pURE, resulting in plasmid pURE-*yfp*, which was then transferred into *E. coli* Tuner (DE3).

Overexpression of *yfp*::*lecB*

Expression cultures were grown at 30°C in 1 L Luria-Bertani medium containing 0.4% glucose and 100 µg/mL ampicillin in 5-L Erlenmeyer flasks to an absorbance of 0.6 and then induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG). The production of the YFP-LecB fusion protein was monitored by fluorescence measurements at excitation and emission wavelengths of 485 and 514 nm, respectively. After 16 h of growth, cells were harvested by centrifugation at 3000× *g* for 10 min and suspended in 100 mL 100 mM Tris-HCl, pH 8.0.

Purification of YFP-LecB by Affinity Chromatography

The LecB-tagged yellow fluorescent protein (YFP; Clontech, Mountain View, CA, USA) was purified as described previously for the native LecB protein (15). Bacterial cells were disrupted by freezing for at least 1 h at -20°C and subsequent sonication. The lysate was centrifuged at 10,000× *g* for 30 min, and the following steps were carried out at 37°C; after equilibration with 100 mL 100 mM Tris-HCl, pH 8.0, 50 mL of the cleared cell extract were loaded onto a mannose agarose column (volume, 20 mL; Sigma-Aldrich, St. Louis, MO, USA). The column was washed with 100 mL 100 mM Tris-HCl, pH 8.0, containing 150 mM NaCl to remove nonspecifically bound proteins. The YFP-LecB fusion protein was eluted with 30 mL 100 mM Tris-HCl, pH 8.0, containing 20 mM D-mannose, and the eluate was concentrated by ultrafiltration using Vivaspin 20 microconcentrators (M_r cut-off, 10 kDa; Sartorius AG, Goettingen, Germany) and then washed with 100 mM Tris-HCl, pH 8.0. Protein concentration was determined by absorption measurements at 280 nm (ε_{280} = 0.98), and the purified protein was stored at -20°C.

Removal of the LecB-Tag by Enterokinase Cleavage

The YFP-LecB fusion protein (500 µg) was incubated in 20 mM Tris-HCl, pH 8.0, containing 50 mM NaCl, 2 mM $CaCl_2$, and 4 ng enterokinase (New England Biolabs, Ipswich, MA, USA) for 48 h at 16°C. After incubation periods of 0.5, 1, 4, 6, 8, 20, 22, 24, 28, 30, and 48 h, samples each containing 10 µg YFP-LecB fusion protein were withdrawn and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Gels were stained with Coomassie® Brilliant Blue R-250 (Serva, Heidelberg, Germany) (19).

Determination of Adsorption Isotherms

Adsorption isotherms were determined with purified LecB protein in triplicate batch experiments with varying starting protein concentrations in 1.5-mL reaction tubes. A vacuum plaque device was used to generate

equally sized adsorbent plaques with a volume of 8.8 µL each (20). The plaques were equilibrated within the plaque device with 100 mM Tris-HCl buffer, pH 8.0. The particle plaques were transferred into the tubes, and 800 µL protein solution were added. After 20 h of incubation at 20° or 37°C on an overhead shaker, the tubes were centrifuged at 2000× *g* for 1 min, and the protein concentration in the supernatant was determined photometrically (ε_{280} = 0.57). The adsorption isotherm was calculated from the mass balances given in Equation 1

$$q_{eq} = \frac{V_{system} \times (c_{init} - c_{eq})}{V_{adsorber}} \quad \text{[Eq. 1]}$$

where q_{eq} is the capacity in adsorption equilibrium, V_{system} is the total reaction volume, $V_{adsorber}$ is the volume of stationary phase, c_{init} is the initial protein concentration in liquid phase, and c_{eq} is the final protein concentration in liquid phase.

The isotherm was fitted to the Langmuir isotherm model (21) using the computer program Origin 7G (OriginLab, Northampton, MA, USA) for determination of the maximum capacity q_{max} and the affinity constant k_a (Equation 2).

$$q_{eq} = q_{max} \times \frac{k_a \times c_{eq}}{1 + k_a \times c_{eq}} \quad \text{[Eq. 2]}$$

RESULTS

Adsorption Isotherms

Binding isotherms of pure LecB to mannose agarose were determined at 20° and 37°C, with the latter being close to the optimum temperature for hemagglutination activity of LecB (Figure 1). At 20°C, the binding constant was determined to be 11.09 mL resin/mg LecB (±3.18) with a saturation loading capacity of 29.48 (±1.30) mg LecB/mL of resin. For the LecB tetramer with a mass of 46.92 kg/mol, the resulting affinity constant is $K_a = 5.20 \times 10^5$/M. At 37°C, a loading capacity of 31.98 (±1.05) mg LecB/mL of resin was determined, and LecB binds to the mannose agarose matrix with an affinity constant of 6.94 (±1.20) mL resin/mg LecB, which corresponds to $K_a = 3.26 \times 10^5$/M.

Construction and Overexpression of an *yfp*::*lecB* Fusion

An artificial multiple cloning site was introduced into plasmid pURE to fuse a protein of interest to an N-terminal histidine hexapeptide and/or to a C-terminal LecB-tag. In addition, this plasmid contains a sequence encoding an enterokinase cleavage site allowing the removal of the LecB-tag subsequent to protein purification. Each monomer of the C-terminally LecB-tagged YFP consists of 362 amino acids with a molecular mass of 39.74 kDa (Figure 2) and is encoded by a 1089-bp gene.

The plasmid pURE-*yfp* was introduced into *E. coli* Tuner (DE3). Intracellular fluorescence was determined to monitor the synthesis of the YFP-LecB fusion protein before and after induction of gene expression with IPTG at 1, 2, 3, and 16 h. At 16 h after induction of the *yfp::lecB* expression, the strain showed a 32-fold higher fluorescence as compared with *E. coli* Tuner (DE3) containing plasmid pURE, which served as negative control (Figure 3).

Affinity Purification of LecB-Tagged YFP

The YFP-LecB protein in the cleared cell lysate was purified by affinity chromatography on mannose agarose utilizing the intrinsic specificity of LecB for D-mannose. In accordance with the hemagglutination optimum of this lectin, the purification process was carried out at a temperature of 37°C (22). Elution of the YFP-LecB fusion protein was initiated at a concentration of 20 mM D-mannose in the elution buffer and occurred in a single sharp peak as shown in a representative purification chromatogram (Figure 4A). The fluorescence was measured to confirm the presence of the fusion protein in the peak fractions, which were subsequently analyzed by SDS-PAGE (Figure 4B). The fluorescence signal obtained with the cleared cell extract was set as 100% (Figure 4B); 11% of the total YFP-LecB protein was detected in the flow through, and 6% was present in the wash fractions. Consequently, 83% of the fusion protein had bound to the mannose agarose. Extinction measurements at 280 nm revealed an average yield of 24 mg pure YFP-LecB protein/L culture.

Enterokinase Cleavage of the LecB-Tag

For a number of reasons, it may be essential to isolate a target protein in its native conformation. Therefore, an enterokinase cleavage site was incorporated into plasmid pURE adjacent to the C terminus of the target protein. The kinetics of LecB-tag cleavage from the YFP-LecB protein were analyzed at 16°C and pH 8.0 and a concentration ratio of fusion protein to enzyme of 125,000:1. After an incubation period of 30 h, the LecB-tag was completely removed, as shown in Figure 5.

DISCUSSION

In recent years, the development of different affinity separation systems has greatly facilitated the expression and downstream processing of recombinant proteins. In contrast to conventional multistep protein purification protocols, affinity chromatography needs just a single highly selective binding step to purify and concentrate the target protein. However, each system has its intrinsic drawbacks, making it difficult to decide on the optimal affinity tag system for a particular protein of interest (1); therefore, the availability of novel tag systems will increase the experimental options.

A number of affinity purification systems have been described that utilize the intrinsic affinity of a fusion protein to different carbohydrates, among them are the cellulose binding domains (17), the MBP (5), and the chitin binding domain (18). Recently, Kavoosi et al. (23) have shown that proteins expressed in *E. coli* as a fusion with the family 9 carbohydrate binding module of xylanase 10A from *Thermotoga maritima* (CBM9) can efficiently be purified by affinity chromatography on a cellulose-based Perloza MT-100 column.

Here, we have evaluated the *P. aeruginosa* lectin LecB as a fusion tag for affinity protein purification. The LecB protein exhibits a remarkable

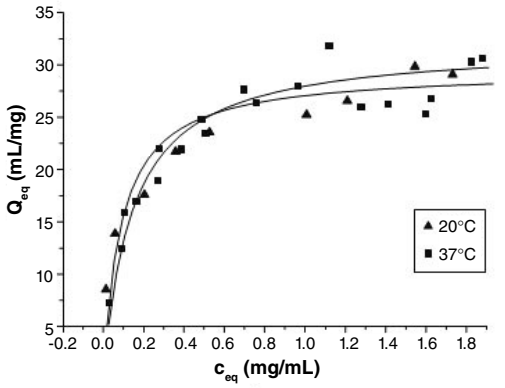

Figure 1. Equilibrium adsorption isotherms for binding of LecB to mannose agarose at 20° and 37°C. Q_{eq} is the bound protein concentration, and c_{eq} is the equilibrium of free protein in the liquid phase. Isotherm determination was done in triplicate.

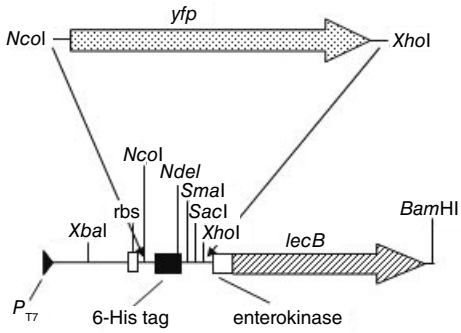

Figure 2. Map of plasmid pURE. The 6051-bp plasmid pURE carries the coding regions for a hexahistidine tag (6-His tag), an enterokinase cleavage site, and the LecB protein. The multiple cloning site contains unique recognition sites of four common restriction endonucleases. A 720-bp fragment containing the *yfp* gene was cloned into the *Nco*I/*Xho*I sites of the plasmid. The resulting YFP-LecB protein consists of 362 amino acids with a molecular mass of 39.74 kDa. P_{T7}, T7 promoter; rbs, ribosomal binding site; EK, enterokinase cleavage site.

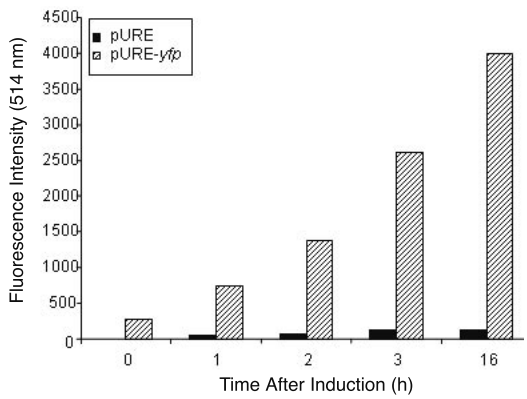

Figure 3. Intracellular fluorescence of *Escherichia coli* expressing YFP-LecB. Expression of the *yfp*::*lecB* gene fusion from plasmid pURE-*yfp* in *E. coli* Tuner (DE3) was induced by isopropyl-β-D-thiogalactoside (IPTG) at A_{580} = 0.6. The intracellular fluorescence intensity was determined before induction, as well as 1, 2, 3, and 16 h later, and compared with a strain containing the control vector pURE.

high affinity to L-fucose (K_a = 1.5 × 10^6/M) and also binds D-mannose, albeit with lower affinity (K_a = 3.1 × 10^2/M) (13). In addition, this lectin also binds several other sugars with varying binding constants. The crystal structure of the tetrameric LecB was solved recently (14–16). The structure revealed that the C terminus of LecB coordinates two essential Ca^{2+} ions and is directly involved in sugar binding, whereas the N terminus is surface-exposed and hence should be accessible for fusion to target proteins. The intrinsic affinity of LecB to D-mannose can be utilized to purify this protein. An *E. coli* expression culture gave a yield of 70 mg/L highly pure lectin protein obtained in a single chromatographic step on a mannose agarose column.

The adsorption isotherm for the lectin binding to mannose agarose at 37°C revealed an affinity constant of K_a = 3.23 × 10^5/M. This affinity is about 1000-fold higher than to monomeric D-mannose, which is most likely a consequence of the multivalency of this tetrameric lectin. The binding constant is slightly lower than the affinities of MBP to maltose (K_a = 3.5 × 10^6/M) (24) or of CBM9 to Perloza MT100 (K_a = 7.3 × 10^6/M) (23). The elution of the lectin from the column can be induced by relatively low concentrations of D-mannose (5–20 mM). The saturation loading capacity of the mannose agarose matrix was determined to be 31.98 mg LecB/mL resin. This value is in the same range as the binding capacity for another D-mannose-specific lectin, Concanavalin A (ConA) from *Canavalia ensiformis*, which is given by the manufacturer (Sigma-Aldrich) as 40–50 mg ConA/mL resin.

A C-terminal fusion of YFP with LecB was constructed to investigate whether LecB can be used as an affinity tag for purification. The fusion protein is encoded by plasmid pURE, which shares the following features: (*i*) any gene of interest can be fused to the *lecB* gene making use of unique restriction sites of four restriction enzymes, namely *Nco*I, *Sma*I, *Sac*I, and *Xho*I; (*ii*) as an alternative or in addition to LecB, the target protein can also be fused to an N-terminal histidine hexapeptide; (*iii*) an enterokinase recognition site is located adjacent to the C terminus of the target protein and allows the cleavage of the LecB-tag subsequent to purification. The recombinant fusion protein was purified from an *E. coli* cell extract on a commercially available mannose agarose column. The hemagglutination temperature profile of LecB suggested to carry out the purification was 37°C (22). Under these conditions, the fusion protein was eluted from the mannose agarose column in a single distinct peak after the addition of 20 mM D-mannose to the washing buffer. An average yield of 24 mg pure YFP-LecB protein was obtained per liter of bacterial culture. Additionally, we have observed that the purification efficiency decreased at lower temperatures (data not shown) with only 18% of the fusion protein bound to the column at 22°C as compared with 83% at 37°C. Interestingly, the adsorption isotherms did not significantly differ at both temperatures.

For many downstream applications, it is essential to remove an affinity tag once the fusion protein is isolated, since it may affect important properties of the target protein (1). The LecB-tag was removed from the fusion protein subsequent to purification by treatment with enterokinase. The kinetics of the cleavage reaction for the YFP-LecB fusion protein revealed that a processing period of 30 h was needed to completely remove the LecB-tag from the target protein under experimental conditions recommended by the manufacturer of the enterokinase. Afterwards, the target protein can be recovered by a second passage through the mannose agarose column. Alternatively, on-column cleavage of the tag may be performed with entero-

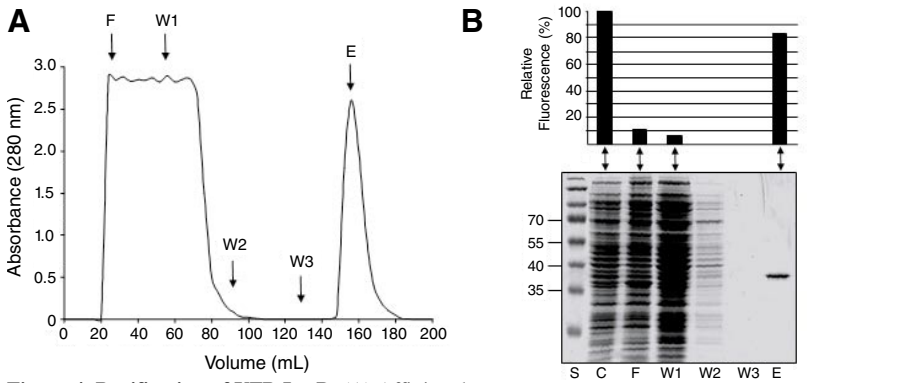

Figure 4. Purification of YFP-LecB. (A) Affinity chromatogram, and (B) fluorescence intensity and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of fractions obtained from panel A. The YFP-LecB fusion protein was purified from an *Escherichia coli* Tuner (DE3) cleared cell lysate on mannose agarose at 37°C. Fifty milliliters lysate were loaded on a 20-mL column packed with mannose agarose and washed with 100 mM Tris-HCl buffer containing 150 mM NaCl. Bound fusion protein was eluted with 20 mM D-mannose in 100 mM Tris-HCl buffer. The protein content was determined by measuring the absorbance at 280 nm; selected fractions were used to determine the fluorescence intensity and analyzed by SDS-PAGE. S, molecular mass standard in kDa; C, cleared cell lysate; F, column flow through; W1–3, selected wash fractions; E, pure YFP-LecB fusion protein eluted with 20 mM D-mannose in Tris-HCl buffer.

kinase, followed by elution of the target protein using a simple washing step.

Additional studies in our laboratory indicated that the lectin LecB tolerates unusual high concentrations of salt (<1.2 M NaCl) and detergent (<1%, v/v, Tween® 20 and Triton® X-100) as well as extreme pH values (pH 4.5–11.5). Furthermore, we have observed that the LecB-tag can be denaturated and refolded prior to affinity chromatography allowing the purification of inclusion body forming proteins (data not shown). Another interesting feature of the LecB protein is its remarkable temperature stability tolerating temperatures up to 80°C (25). This property may be applicable to tag and purify target proteins originating from thermophilic organisms.

In conclusion, we have clearly demonstrated here that the lectin LecB from *P. aeruginosa* can be used as a potent affinity tag that fulfils each of the following requirements (1): (*i*) the LecB-tag allows a one-step adsorption and purification of fused target proteins; (*ii*) the LecB-tag does not negatively affect the function of the target protein. This conclusion is based on the following results: the fluorescence spectra obtained with purified YFP-LecB protein and with native YFP were identical (data not shown). In addition, LecB was also fused to the lipase LipB of *Bacillus subtilis* and the cutinase of *Fusarium solani pisi*. In either case, the activity of the target enzyme was not affected by the LecB-tag (data not shown). (*iii*) The LecB protein can specifically be removed by cleavage with enterokinase to produce the native protein; (*iv*) highly specific antibodies against LecB are available (12) allowing the simple and specific detection of the recombinant protein to monitor its production; and (*v*) we have constructed LecB fusions of six different proteins, five of which have successfully been isolated essentially as described for the YFP-LecB protein.

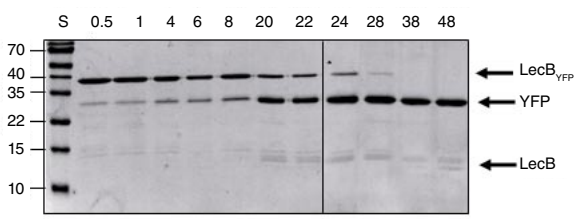

Figure 5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of LecB-tag removal by enterokinase cleavage. The cleavage reaction was carried out at 16°C with a fusion protein to enzyme ratio of 125,000:1. YFP, yellow fluorescent protein.

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D.T. and F.R. have contributed equally to this work. We are grateful to Monika Gehsing for her excellent technical assistance.

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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