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ARTICLE *in* FEMS MICROBIOLOGY LETTERS · JULY 2002

Impact Factor: 2.12 · DOI: 10.1016/S0378-1097(02)00705-X · Source: PubMed

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The substrate-binding protein TeaA of the osmoregulated ectoine transporter TeaABC from *Halomonas elongata*: purification and characterization of recombinant TeaA

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Received 17 January 2002; received in revised form 9 April 2002; accepted 16 April 2002

First published online 20 May 2002

Abstract

TeaABC is a novel, osmoregulated secondary transport system for compatible solutes found in the halophilic proteobacterium *Halomonas elongata* DSM 2581^T. Sequence comparisons have confirmed that TeaABC belongs to the family of TRAP transporters and as such it consists of two putative transmembrane proteins (TeaB, TeaC) and a putative periplasmic substrate-binding protein (TeaA). TeaABC is the only osmoregulated transporter for ectoines found in *H. elongata*. By overexpressing TeaA-(6)His tag in *Escherichia coli* we demonstrated that TeaA is processed as predicted and exported to the periplasm. Furthermore, it was proven that TeaA is indeed a periplasmic ectoine-binding protein. The functionality of the purified protein as an ectoine-binding protein was tested employing a modified binding assay.

Keywords: Ectoine-binding assay; Protein purification; Substrate-binding protein; TRAP transporter; Halophilic bacterium; *Halomonas elongata*

1. Introduction

Halophilic bacteria are adapted to environments with high inorganic salt concentrations, which result in high ionic strength and low water potential. As water flows from high to low potential and the cell membrane is freely permeable to water, cytoplasm with higher water potential than the environment will lose its free water [1]. To avoid the loss of cytoplasmic water and to achieve a cytoplasm of similar osmotic strength to the surrounding medium, halophilic bacteria accumulate osmoprotective compounds like amino acids and/or amino acid derivatives [2]. These non-ionic, highly water-soluble compounds do not disturb the metabolism and are therefore named compatible solutes [3]. Many halophilic organisms are able to synthesize their own compatible solutes such as glycine-betaine [4] or ectoine [5] and in addition are equipped with special transport systems for the uptake of compatible solutes or their precursors [6]. Such osmoregulated transport systems have mostly been studied in non-halophilic bacteria like *Escherichia coli*, *Corynebacterium glutamicum*, *Bacillus subtilis*, and some halotolerant microorganisms [7–9]. These organisms either use high affinity binding protein-dependent ABC transporters or secondary permeases consisting of only one single transmembrane protein.

Halomonas elongata is a moderately halophilic proteobacterium of the γ-subdivision [10] that synthesizes ectoine as its major compatible solute [11]. *H. elongata* possesses the osmoregulated transport system TeaABC for the uptake of ectoines leaking through the cytoplasmic membrane [12]. Sequence comparisons have shown that TeaABC belongs to the family of TRAP transporters and that it comprises three putative proteins: a small and a large transmembrane protein (TeaB and TeaC, respectively) and a periplasmic substrate-binding protein (TeaA). Sequence analysis of TeaA revealed that this protein consists of 341 amino acids of which the 25 N-terminal residues form a potential signal sequence for the Sec-dependent secretion into the periplasm. In this study, we were able to overexpress and to purify TeaA to confirm its secretion into the periplasm and to prove its function as an ectoine-binding protein.

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0378-1097/02/$22.00
PII: S0378-1097(02)00705-X

2. Materials and methods

2.1. Bacterial strains and plasmids

H. elongata DSM 2581^T was obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). *E. coli* DH5α [13] and *E. coli* BL21(DE3) were used as hosts for plasmid pLTB1 (pET22b(+)MCS::*teaA*) [14]. *E. coli* BL21(DE3) and plasmid pET22b(+) were obtained from Novagen Inc. (Madison, WI, USA).

2.2. Media, growth conditions and cell fractionation

E. coli strains were grown aerobically at 37°C in Luria–Bertani (LB) medium [15] or in M9 minimal medium [16]. For the growth of plasmid carrying strains, ampicillin was added to a final concentration of 100 μg ml^{-1}. *H. elongata* DSM 2581^T was grown aerobically at 30°C on MM63 minimal medium [17] or on nutrient broth (Merck, Darmstadt, Germany) complex medium with 3% NaCl.

In order to induce TeaA protein expression in *E. coli* pLTB1, IPTG (isopropyl 1-thio-β-D-galactopyranoside) was added to final concentrations of 25 μM, 50 μM, 100 μM, and 1 mM. After 2 h cells were harvested by centrifugation and fractionated into periplasmic components, soluble and insoluble cytoplasmic components. The application of the osmotic shock procedure [18] for the purification of TeaA-(6)His tag from the periplasm was problematic as the outer membrane became destabilized after the addition of shock buffer containing 20% sucrose, whereas destabilization should proceed only after subsequent addition of low salt buffer. Part of the periplasmic protein was therefore already released into the shock buffer and could be detected in the supernatant after centrifugation. Therefore the supernatants of both buffers were collected after centrifugation and named periplasmic fractions 1 and 2. To obtain cytoplasmic components, cells were lysed by lysozyme treatment (100 μg ml^{-1}) and subsequent sonication. Afterwards soluble and insoluble proteins were separated by centrifugation. The precipitated, insoluble proteins were dissolved in 1% SDS. Proteins in medium and periplasmic fractions were concentrated by TCA precipitation.

2.3. Methods used with nucleic acids

Chromosomal DNA from *H. elongata* was prepared by a modified method based on the technique of Marmur [19]. Routine manipulation of DNA, plasmid isolation, construction of recombinant plasmids, polymerase chain reaction, electrophoresis of DNA, and transformation were carried out according to standard procedures [16]. DNA sequencing was carried out by Sequiserve (Vaterstetten, Germany) based on the Sanger procedure.

2.4. Protein purification

E. coli BL21pLTB1 was grown in 100 ml LB medium at 37°C and expression was initiated by addition of IPTG to exponentially growing cells. After 2 h cells were harvested by centrifugation and medium was used for purification of TeaA-(6)His tag. A portion of 100 ml of medium was concentrated overnight with PEG20000 (polyethylene glycol) and supplemented with binding buffer (5 mM imidazole, 500 mM NaCl). The solution was applied to a Ni^{2+}-IDA column (iminodiacetic acid), which had been pre-equilibrated with three bed volumes of binding buffer. Unbound protein was removed by washing with buffer containing 30 mM imidazole. TeaA-(6)His tag was eluted from the column with 1 M imidazole buffer. After dialysis for 3 h against phosphate buffered saline with 2% NaCl, the protein was loaded onto an ion exchange column (MonoQ HR 5/5, Pharmacia, Uppsala, Sweden), which had been pre-equilibrated with 10 mM Tris buffer, pH 7.8. The resin is a strong anion exchanger with quaternary amines as a functional group. After application of sample unbound protein was removed by washing with 10 mM Tris buffer, 340 mM NaCl, pH 7.8 for 10 min (10 bed volumes). Afterwards TeaA-(6)His tag was eluted with 10 mM Tris buffer containing 1 M NaCl. The purification process was monitored by SDS–PAGE analysis. Protein was determined by the BCA protein assay (Pierce Chemical Co., Rockford, IL, USA).

2.5. Ectoine-binding assay

Ectoine binding of TeaA-(6)His tag was examined by a modified retention test. Purified TeaA-(6)His tag was incubated with ectoine for 5 min at 30°C and centrifuged through a Microcon filter device with a molecular mass cutoff of 10 000 Da. The flow-through was analyzed by high performance liquid chromatography (HPLC) according to the method of Frings et al. [20] using a UV detector (220 nm) to determine the fraction of unbound ectoine. Ectoine was purified from cell extract of *H. elongata* as described by Galinski et al. [21].

2.6. SDS–polyacrylamide gel, immunological detection of TeaA and Edman degradation

SDS–PAGE was performed according to the method of Laemmli. Protein solution (100 μl) was supplemented with the same volume of reducing sample buffer and heated at 70°C for 3 min prior to application to a 13% SDS–polyacrylamide gel. Proteins were stained with Coomassie brilliant blue R250 or by using a modified silver staining procedure [22]. The molecular standard used was the synthetic Perfect Protein Western Marker by Novagen (Madison, WI, USA). Western transfer was performed after the method of Towbin et al. [23] using nitrocellulose (0.2 μm) for the electrophoretical transfer. TeaA-(6)His tag was de-

tected by a monoclonal mouse antibody against His tags and an alkaline phosphatase-conjugated goat anti-mouse antibody (Novagen). Concentration of the monoclonal antibody was 100 ng ml^{-1}. N-Terminal sequencing of TeaA was performed by TOPLAB (Martinsried, Germany) by automated Edman degradation.

2.7. Computer analysis

The hydropathy profile of TeaA was constructed by the method of Kyte and Doolittle [24]. Sequence analysis of TeaA was performed on an Apple Macintosh computer using DNA-Strider™ 1.2 [25].

3. Results and discussion

3.1. Synthesis and subcellular location of TeaA in E. coli BL21pLTB1

The *teaA* ORF was amplified from *H. elongata* DSM 2581^T total DNA by PCR using forward primer 5′-ACC ACA CAT ATG AAG GCA TAC AAG CTG-3′ and reverse primer 5′-GGC TCC CTC GAG GCC CTC GCT CTC GCT-3′. The forward primer was designed to create an *Nde*I site (underlined) at the inition codon, and the reverse primer was designed to create an *Xho*I site (underlined) in front of the stop codon of *teaA*. The *teaA* PCR product was cut with *Nde*I and *Xho*I and inserted into the MCS (multiple cloning site) of plasmid pET22b(+), which was linearized using the corresponding enzymes. This employed vector was designed to amplify the expression of *teaA* by placing it under the control of a strong, IPTG-inducible promoter of T7 bacteriophage (Novagen). Expression from the resulting plasmid pLTB1 led to a TeaA fusion protein containing six additional histidine residues at the C-terminus ((6)His tag), allowing for the purification of TeaA by nickel chelate affinity chromatography.

After induction, a protein with an apparent molecular mass of 38 kDa was present in *E. coli* BL21pLTB1. This protein cross-reacted with a monoclonal antibody against the His tag (Fig. 1), suggesting that the synthesized protein was TeaA. A somewhat smaller protein was detected, when the IPTG concentration used for the induction of *teaA* expression was lowered, indicating that TeaA might exist in an unprocessed and a processed periplasmic form. DNA sequence analysis of *teaA* had predicted that the N-terminus of TeaA contains a leader sequence for the transport by the Sec pathway. The unprocessed form of TeaA has a calculated molecular mass of 38 257 kDa, whereas the putative processed form has a calculated molecular mass of 35 665 kDa [12].

In order to prove that TeaA is exported to the periplasm, we examined different cell fractions (Fig. 2). A total cell protein plus cytoplasmic fraction contained only the

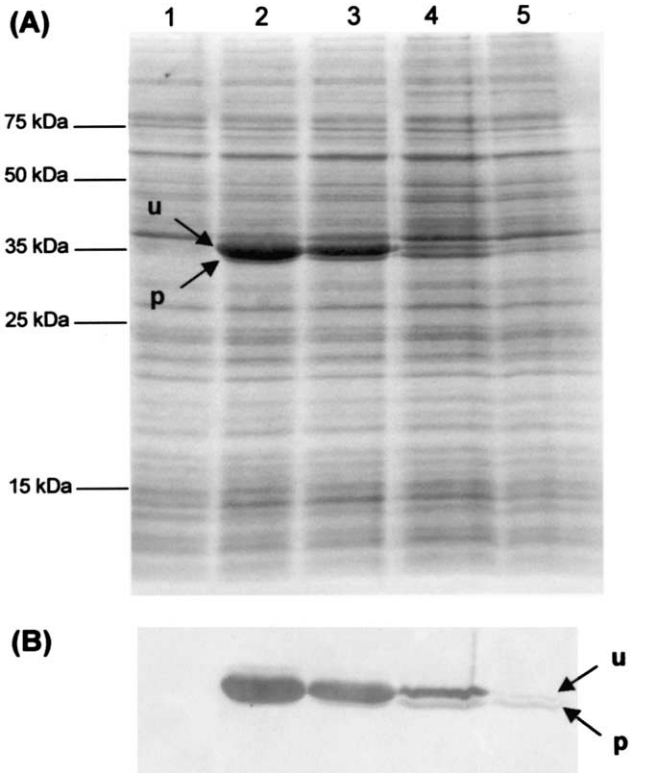

Fig. 1. Synthesis of TeaA-(6)His tag in *E. coli* BL21pLTB1 after induction with IPTG in various concentrations. A: SDS–PAGE analysis, Coomassie blue stained. B: Western blot analysis, using monoclonal antibody against His tags. Total cell protein was harvested 2 h after induction with 1 mM (lane 2), 100 μM (lane 3), 50 μM (lane 4) and 25 μM (lane 5) IPTG and applied to a 13% SDS–polyacrylamide gel (20–25 μg per lane). Lane 1 contains total cell protein before induction. A gel identical to that in A was blotted onto nitrocellulose. After induction with IPTG, TeaA became visible in its unprocessed (u) and processed (p) form (indicated by arrows). Lowering of the IPTG concentration resulted in the increase of processed protein in comparison to unprocessed protein.

unprocessed form of TeaA, but both forms of TeaA were found in the periplasm fraction (fraction 1). As described above, two periplasmic fractions were obtained when using the osmotic shock procedure. This can be explained by the fact that strong overexpression of TeaA-(6)His tag destabilized the outer membrane leading to a disruption as soon as the shock buffer (20% sucrose) was added to the cells. Hence periplasmic proteins, including processed TeaA, were released prematurely into the buffer, which we refer to as periplasmic fraction 1. The unprocessed protein detected in periplasmic fraction 1 might be explained by a destabilization of the cytoplasmic membrane due to the treatment with sucrose shock buffer and EDTA. In periplasmic fraction 2, obtained after adding a low salt buffer (osmotic downshock), only unprocessed TeaA could be detected. According to Vazquez-Laslop et al. [26] osmotic downshock of EDTA treated cells is thought to damage the bacterial envelope, thereby forming a molecular sieve through which cytoplasmic proteins (< 100 kDa) are released from the cell. This would explain why only unpro-

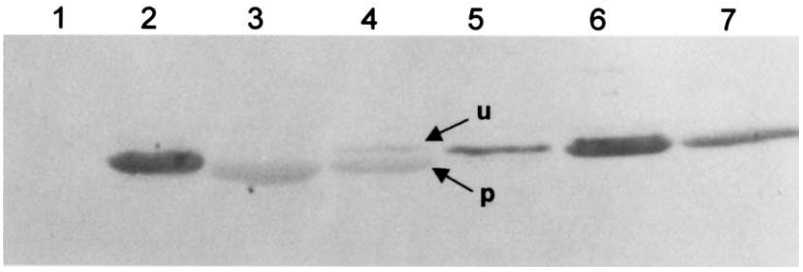

Fig. 2. Western blot analysis of *E. coli* BL21pLTB1 cell fractions 2 h after induction with 50 μM IPTG. TeaA-(6)His tag was detected by a monoclonal antibody against His tags. Each lane contains either 20–25 μg of total cell protein (lane 1, before induction; lane 2, 2 h after induction) or 20 μl medium (lane 3) or 20 μl of cell fractions (lane 4, periplasmic fraction 1; lane 5, periplasmic fraction 2; lane 6, cytoplasmic fraction; lane 7, insoluble cytoplasmic fraction). Processed TeaA could be detected in the medium and periplasmic fraction 1. All other fractions contained only the unprocessed form of TeaA. Arrows indicate the unprocessed (u) and processed form (p) of TeaA.

cessed TeaA protein appeared in the second fraction and no free periplasmic TeaA protein, which was already released into the sucrose buffer of periplasmic fraction 1. As total cell protein contains proteins from both the cytoplasm and periplasm, processed TeaA was also found in total cell protein by Western blot analysis. However, to detect the processed form, the colorimetric reaction had to be stopped after a few seconds (not shown). Otherwise the strong signal of unprocessed TeaA would overlap the second signal of processed TeaA.

Processed TeaA-(6)His tag was excreted into the medium most probably by unspecific export, which is known to occur when proteins are overexpressed (see below). The sequencing of the first eight N-terminal amino acids of protein, which was purified from the medium, proved that TeaA was indeed processed at the putative signal sequence for Sec-dependent secretion as predicted by computer analysis [12] and that TeaA therefore represents a periplasmic protein (Fig. 3).

The fact that both forms run closely together in the electrophoretical separation can be explained by the strong hydrophobic character of the N-terminal signal sequence (Fig. 3). Due to a higher affinity of SDS to hydrophobic amino acids, the unprocessed form should run faster than expected in the SDS–PAGE. Fig. 1 shows that the amount of protein transported over the cytoplasmic membrane is raised when the IPTG concentration is lowered. It has been suggested that the translocation machinery for the Sec-dependent transport is overloaded with recombinant protein when there is more protein synthesized than can be transported [27]. This also explains why strong overproduction weakens the outer membrane, resulting in the leakage of processed TeaA into the medium. According to the model of Barron et al. [28], unprocessed TeaA accumulates at the translocation machinery impeding the translocation of envelope proteins and resulting in an unstable outer membrane. In order to maximize the amount of processed protein, an IPTG concentration of 50 μM was used for all following experiments.

3.2. Purification of TeaA

The protocol used for the purification of TeaA-(6)His tag was comprised of two steps: purification by nickel chelate affinity chromatography and ion exchange chromatography. In a typical experiment 3 mg of TeaA-(6)His tag were purified by nickel chelate affinity chromatography from 100 ml medium. Gene expression was induced with 50 μM IPTG and cells were harvested 2 h after induction. Since TeaA carrying a C-terminal (6)His tag binds only weakly to the resin, the imidazole concentration in the washing buffer was lowered from 60 mM to 30 mM to enable binding of TeaA during the washing step. The obtained protein was between 95 and 99% pure as judged from a silver-stained SDS gel (Fig. 4). Dialysis, performed in order to remove imidazole, resulted in the degradation of protein TeaA and an emerging of oligopeptides in SDS–PAGE analysis. To remove the residual contaminating peptides, ion exchange chromatography was applied. Because of its high excess of negatively charged amino acids, the stability of TeaA is dependent on the NaCl concentration. Dialysis against Tris buffer without NaCl led to a denaturation of TeaA preventing a binding to the strong anion exchanger used for the purification (not shown). Eisenberg and Wachtel [29] have shown that purification of halophilic proteins by ion ex-

MKAYKLLTTASIGALMLGMSTAAYSDNWRYAHEEYEGDVQDVFAQAFKGYVEDNSDHTVQVYRFGEL
GESDDIMEQTQNGILQFVNQSPGFTGSLIPSAQIFFIPYLMPTDMDTVLEFFDESKAINEMFPKLYAEHG
LELLKMYPEGEMVVTADEPITSPEDFDNKKIRTMTNPLLAETYKAFGATPTPLPWGEVYGGLQTGIIDGQ
ENPIFWIESGGLYEVSPNLTFTSHGWFTTAMMANQDFYEGLSEEDQQLVQDAADAAYDHTIEHIKGLSE
ESLEKIKAASDEVTVTRLNDEQIQAFKERAPQVEEKFIEMTGEQGQELLDQFKADLKAVQSESEG

Fig. 3. Amino acid sequence of unprocessed TeaA as predicted by the genetic sequence. The dark shaded amino acids represent the N-terminal signal sequence, which is cut off following secretion into the periplasm; the light shaded amino acids have been sequenced by Edman degradation in order to confirm the identity of the purified protein.

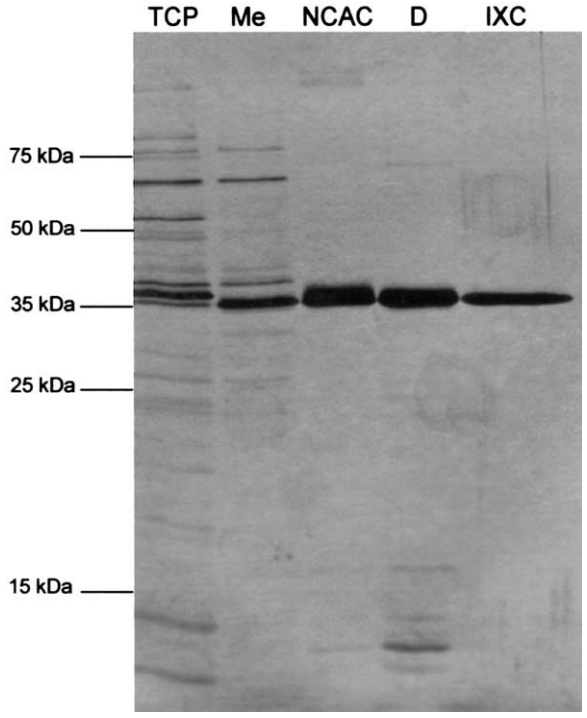

Fig. 4. SDS–PAGE analysis of the purification process of TeaA-(6)His tag. A normalized amount of protein was applied to a polyacrylamide gel (13%) containing SDS and detected by silver staining. Total cell protein (TCP) and medium (Me) were diluted 1/20 and 1/5, respectively. After dialysis (D) of protein obtained from nickel chelate affinity chromatography (NCAC) degradation products appear. These were removed by ion exchange chromatography (IXC).

change chromatography is difficult to accomplish because of the observed salt dependence of these proteins for stability. Thus, we adapted the purification protocol enabling the purification of TeaA-(6)His tag by ion exchange chromatography. A prerequisite is a minimum salt concentration allowing for protein stability, but lower than necessary for elution of protein from the column. In the case of TeaA-(6)His tag 340 mM NaCl buffer was used for protein stabilization, with the elution of protein conducted at a concentration of 500 mM NaCl (3%).

Additional studies showed that a linear NaCl gradient was not suitable for the concentrated elution of protein, since TeaA eluted steadily over a wide range of NaCl concentrations. Furthermore, most of the impurities eluted closely to TeaA, as they represented degradation products from this protein and therefore had a similar surface charge. The protocol that proved to be most effective for TeaA purification was carried out by washing with 340 mM NaCl for 5 min followed by a sudden increase in buffer salinity from 340 mM to 1 M NaCl. Washing with 340 mM NaCl buffer removed all protein impurities and the steep rise of the NaCl concentration enabled us to elute TeaA-(6)His tag in concentrated form. The obtained protein was electrophoretically pure as judged from a silver-stained SDS gel (Fig. 4). Resulting pure protein yield was approximately 1.3 mg of the originally harvested 3 mg of protein.

3.3. Structural and functional analysis of TeaA

TeaA consists of 341 amino acids of which the N-terminal 25 represent a signal sequence for the secretion into the periplasm (Fig. 2). This signal sequence is cut off when TeaA is transported over the cytoplasmic membrane resulting in a processed periplasmic protein with a length of 316 amino acids. The calculated molecular mass of 35 665 kDa was confirmed by SDS–PAGE analysis. Hydrophathy profiles revealed that TeaA is an overall hydrophilic protein, which is consistent with its location in the bacterial periplasm. Only two hydrophobic stretches are detectable comprising amino acids 1–25 and 90–110. The first stretch represents the signal sequence with 60% hydrophobic amino acids in contrast to only 47.3% in the processed TeaA protein. Furthermore, TeaA shows an excess of acidic over basic amino acids (19.3% and 7.9%, respectively) which contributes to its instability in the absence of salt. TeaA is not forming multimers by disulfide links, since it does not contain any cysteines. As indicated by native PAGE analysis, TeaA is supposed to be a monomeric protein (not shown).

In order to show that TeaA is indeed an ectoine-binding protein, we have modified a retention assay for the verification of ectoine binding by TeaA. This assay was also used to test the functionality of purified TeaA-(6)His tag. Ectoine at a concentration of 100 μM was incubated with purified TeaA-(6)His tag at a concentration of 150 μM for 5 min at 30°C and centrifuged through a filter device with a molecular mass cutoff of 10 000 Da. Ectoine in the flow-through was detected by HPLC using a UV detector. For control experiments with bovine serum albumin (BSA) or no protein at all, 86.2% and 82.6%, respectively, of the applied ectoine remained in solution (Fig. 5). When

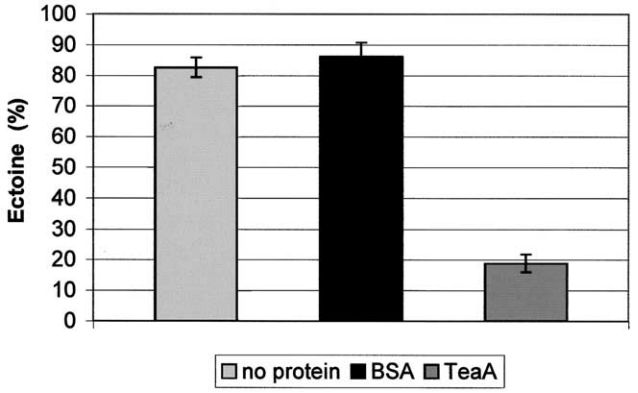

Fig. 5. Percentage of ectoine in the flow-through of the ectoine-binding assay. Each experiment was carried out at least three times to calculate the mean deviation. TeaA and BSA were employed at a concentration of 150 μM, ectoine at a concentration of 100 μM (100%). When TeaA was added to the assay, at least 60% were bound to the substrate-binding protein.

TeaA was added to the assay, only 18.9% ectoine could be detected in the flow-through indicating that up to 80% were bound to the substrate-binding protein. These results show that TeaA is indeed a substrate-binding protein. However, it cannot be excluded that the His tag interferes with the affinity of TeaA towards its substrate as there is no information available at present about the influence of the His tag on the ectoine-binding properties of TeaA. Also, we can only speculate why 20% of the ectoine did not bind to TeaA in the binding assay although the protein was applied in excess. This might be explained by the presence of denatured protein in the assay, which lost ectoine-binding capability after elution from the ion exchange column. In addition, at present no statements can be made about substrate specificity and affinity of TeaA. The protein purification procedure described should help to obtain TeaA for X-ray crystallography and to further study substrate-binding mechanisms.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (Tr 133/27-1). We are grateful to Christiane Dahl for access to equipment. The careful technical assistance of Marlene Stein is gratefully acknowledged. We thank Sharon Taylor and Erwin A. Galinski for critical reading of the manuscript.

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