High DNA melting activity of extremophile *Eutrema salsugineum* cold shock domain proteins EsCSDP1 and EsCSDP3

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**A B S T R A C T**

Plant cold shock domain proteins (CSDP) participate in maintenance of plant stress tolerance and in regulating their development. In the present paper we show that two out of three extremophile plant *Eutrema salsugineum* proteins EsCSDP1-3, namely EsCSDP1 and EsCSDP3, possess high DNA-melting activity. DNA-melting activity of proteins was evaluated using molecular beacon assay in two ways: by measuring Tm parameter (the temperature at which half of the DNA beacon molecules is fully melted) and the beacon fluorescence at 4 °C. As the ratio protein/beacon was increased, a decrease in Tm was observed. Besides DNA-melting activity of full proteins, activity was measured for three isolated cold shock domains EsCSD1-3, C-terminal domain of EsCSDP1 (EsZnF1), as well as a mixture of EsCSD1 and EsZnF1. The Tm reduction efficiency of proteins formed the following sequence: EsCSDP3 ≈ EsCSDP1 > (EsCSD1 + EsZnF1) > EsZnF1 > EsCSDP2. Only full proteins EsCSDP3 and EsCSDP1 demonstrated DNA-melting activity at 4 °C. The presented experimental data indicate that: interaction of EsCSDP1-3 with beacon single-stranded region is obligatory for efficient melting; ii: cold shock domain and C-terminal domain with zinc finger motifs should be present in one protein molecule to have high melting activity.

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1. Introduction

The term "cold shock domain proteins" combines several families of small proteins present in different prokaryotic and eukaryotic organisms. The common feature of such proteins is the presence of cold shock domain (CSD) in their structure [1]. CSD is closely related to bacterial cold shock proteins (CSP). All bacterial CSPs are highly homologous and consist of 69–74 amino acid residues. A typical CSP contains two characteristic motifs RNP1 (consensus K/NAGAF/YAGAFA/V) and RNP2 (VAFAVAHAF), which determine the ability of CSP to bind RNA and single-stranded DNA [Horn et al. 2007] [1]. Bacterial CSPs function as transcription antiterminators and RNA chaperones, affecting gene transcription and translation of different proteins [2–6].

*E. coli* genome contains nine genes coding cold shock proteins (CspA to CspI). Four of these genes (CspA, CspB, CspG, CspI) are induced in response to the reduction of ambient temperature to the values lower than optimal for *E. coli* (below 20 °C) [1]. Cells of *E. coli* strain BX04 with the genes of these four proteins deleted demonstrate significantly slower growth with decreasing temperature. However, their ability to grow can be restored by overexpressing any of nine Csp, except CspD. It is assumed that the recovery effect is due to the ability of CSP to induce melting of RNA hairpin structures forming at lower temperatures and preventing RNA translation [7].

More complex cold shock domain proteins (CSDPs), containing cold shock domain, have been observed in animals. Numerous studies present a variety of CSDPs functions in animals cells at the level of transcription and translation [8]. CSDP genes were also found in the genomes of plants from different systematic groups such as lower plants, monocots, dicots, including various woody plants [9]. Different plant CSDPs have a similar structure: the N-terminal part consists of cold shock domain, the C-terminal part contains "zinc finger" domains of CCHC-type (ZnF) separated by glycine-rich regions [10]. The individual proteins differ in the number of ZnF ranging from one to seven [9]. Large volume of accumulated experimental data indicate that CSDPs are involved in a number of molecular mechanisms of plant resistance to adverse environmental factors [11–13] and the regulation of plant development [14–17].

Plant CSDPs exhibit a greater affinity for RNA and single-stranded DNA than for double-stranded DNA [15], so until very recently, their function in plants was associated mainly with RNA-binding activity. Nevertheless, there is fragmentary evidence that some plant CSDPs, such as wheat protein WCSP1 [18] and Arabidopsis protein AtCSDP1 [12], are able to promote melting of double-stranded DNA structure in vitro. In the present study, we have

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analyzed DNA-melting activity of three proteins from extremophyte plant *Eutrema salugineum* EsCSDP1, EsCSDP2 and EsCSDP3 containing six, two and seven zinc finger motifs, respectively [19] and their isolated cold shock domains (Fig. S1A).

The melting assay was done using fluorescent DNA probes in the form of “molecular beacon”. For EsCSDP1 protein, DNA-melting activity of isolated C-terminal part containing six ZnF and its mixture with CSD was studied in more detail.

### 2. Experimental

#### 2.1. Genetic constructs

Oligonucleotide sequence encoding HaloTag protein and the linker with TEV protease site was amplified from plasmids pFC20K HaloTag<sup>®</sup> T7 SP6 Flexi<sup>®</sup> (Promega, USA) using primers P1 and P2 (Table S1). Restriction sites Ncol and EcoRI were added at the 5′-end of P1. Restriction site Xhol was added at the 5′-end of P2 primer after the stop codon. Amplified HaloTag gene was cloned into the vector Pet28α- at the restriction sites Ncol and Xhol. Amplification of EsCSDP1-3, EsCSD1-3, EsZnF1, CspA nucleotide sequences was performed using primers P3-P14 (Table S1) from genomic DNA. The following restriction sites were added at the 5′-end of forward primers: Nco1 for genes EsCSDP1, EsCSDP3 and their deletion variants EsCSD1, EsZnF1 and EsCSD3; PaqI for EsCSDP2 and EsCSD2; PciI for CspA. Amplified DNA fragments were inserted into the vector pET28a+ with HaloTag at restriction sites Ncol and EcoRI. To obtain a genetic construct encoding the mutant protein EsCSDP3, primers P10 and P11 were used to amplify a fragment of synthetic DNA sequence encoding protein EsCSDP3 carrying point mutations in the CSD (W17A, F26A, F37A and ZnF (C96A, C128A, C158A, C192A, C223A, C251A, C278A which adversely affect the ability of CSDP to bind nucleic acids, according to published data [20,21]. The mutant gene EsCSDP3 was synthesized by “ATG Service Gene” (Russia). All of the genetic constructs used in the study were checked by DNA sequencing.

#### 2.2. Preparation of recombinant proteins

*E. coli* strain BL21 cells were transformed by the genetic constructs obtained. Extraction and purification of recombinant proteins was performed using HaloTag Protein Purification Kit (Promega) according to the manufacturer instruction. Proteins were isolated from 50 ml overnight *E. coli* culture. The culture was incubated at 37 °C until the optical density of 1.1 to 1.2 was reached, then IPTG was added into the medium at a final concentration of 1.7 mM and incubation was continued at 27 °C overnight. Bacterial cells were precipitated by centrifugation at 6000 g for 15 min. The precipitate was resuspended on ice in 5 ml of lysis buffer containing standard buffer (SB; 50 mM HEPES, 150 mM NaCl, 1 mM DTT, 10 mM MgSO<sub>4</sub>, pH 7.5 with 10 mM lysozyme (Sigma, USA)), 50 µl of RO1 RNase-Free DNase (Promega), 50 µl of Protease Inhibitor Cocktail (Promega). Suspension was sonicated on ice using 5-second bursts with 5-second cooling time in between, for a total of 2 min. The sonicated lysate was centrifuged for 30 min at 10,000 g, supernatant was collected and incubated with 1 ml of HaloLink Resin at room temperature for 1 h with continuous stirring. HaloLink Resin with chimeric protein bound was washed successively with SB buffer, SB+0.5 M NaCl, SB+2 mM ATP+10 mM MgSO<sub>4</sub> and finally 4 times with SB buffer. When EsCSDP1C-terminal fragment was extracted, arginine was added to the lysis buffer to a final concentration of 40 mM, and 0.05% IGEPAL detergent (Promega) was added into the first washing buffer. Proteins were recovered from the resin using TEV-protease according to the manufacturer instruction. Purified proteins in SB buffer, supplemented with 10% glycerol, were frozen in liquid nitrogen and stored at −70 °C. The protein concentration was measured by Bradford method. The purity of proteins was determined using PAAG-electrophoresis according to the method by Laemmli (Fig. S2).

#### 2.3. DNA melting assay

DNA-melting activity of proteins was measured using “molecular beacon” assay. Four different DNA-beacons were used, each of them containing two 40- bp DNA chains which had nine paired complementary bases (Fig. 1). Fluorescently-labeled oligodeoxynucleotides were synthesized by “Syntol” (Russia). The chains of DNA-beacon were labeled by 6-carboxyrhodamine-6G fluorophore (R6G; Syntol, Russia) at the 5′-end of one chain and by fluorescence quencher BHQ-2 (Glen Research, USA, Cat. no. 20-5932) at the 5′-end of a complementary chain. Before the assay, R6G- and BHQ-2-labeled oligonucleotides were mixed in SB buffer at the ratio 1:2, incubated 4 min at 95 °C and transferred to ice. Melting curves of DNA-beacons were obtained at the thermal cycler CFX-96 (Bio-Rad, USA) in 50 µl of SB buffer, supplemented with 10% glycerol, in three replicates (excitation 526 nm, emission 555 nm,

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**Fig. 1.** Molecular beacons BC1, BCnn, BCstem, BCfullstem. BC1 beacon consisted of 9 bp double-stranded segment and two single-stranded segments of 30 and 31 bp length. BCnn was different from BC1 by the sequence of singlestranded segments being degenerate. Unlike BC1, BCstem contained double-stranded part of BC1 only. BCfullstem was a complementary 40-bp sequence containing a nick between nucleotides 31 and 32 in the chain linked to the fluorescence quencher.
heating mode 2 °C/5 s). All values were expressed as mean ± SE. The final concentration of DNA-beacon for melting curve measurement was 100 nM. The effect of protein concentration on DNA-beacon melting was analyzed at the fixed temperature of 4 °C with LS55 fluorometer (Perkin Elmer, USA). The fluorometer was equipped with a temperature-controlled cuvette holder connected to a water thermostat. First, a protein sample in SB buffer with 10% glycerol was injected into 100-μl cuvette with optical path length of 10 mm, then DNA-beacon was added at a final concentration of 50 nM, solution was mixed and fluorescence was measured at 526/555 nm. The measurements were repeated in three independent experiments. All values were expressed as mean ± SE.

3. Results

3.1. EsCSDP1–3 decrease $T_m$ of DNA-beacon

DNA-melting activity of proteins was studied in the “molecular beacon” assay system (Figs. 1 and S3). The structure of BC1 beacon was adopted from the paper [18] where it was used to compare curves with and without protein. RFU was EsCSDP2, whereas the activities of EsCSDP1 and EsCSDP3 were about the same. Interestingly, the range of efficient concentration causing BC1 melting did not differ significantly among the proteins EsCSDP1–3. The main difference was observed in the values of $T_m$ maximum shift being more than 10 °C for EsCSDP1, EsCSDP3 and 5 °C for EsCSDP2. As a control, the mutant protein EsCSDP3M was used (see Experimental), which had the main nucleic acid binding sites destroyed by mutations. In all of the experiments performed, EsCSDP3M protein was found completely inactive (Fig. S4).

The melting curve for EsCSDP1 incubated at 95 °C for 20 min before the melting experiment is presented in Supplemental (Fig. S5a). The fact that the melting curves of BC1 beacon obtained in the presence of the native protein and the protein exposed to elevated temperature look similar indicates that the protein EsCSDP1 is thermally stable. Similarly, we have tested and verified the thermal stability of the other two proteins EsCSDP2 and EsCSDP3 (Fig. S5b,c). The finding of EsCSDP1–3 thermal stability was not unexpected, as it was formerly shown for a wheat protein with cold shock domain WCSP1 [22].

Besides complete proteins, we tested the DNA-melting activity of isolated cold shock domains EsCSD1–3. C-terminal domain EsCSDP1 containing six ZnF (EsZnF1), and an equimolar mixture of EsCSD1 and EsZnF1 (EsMix1). As a comparison, the major cold shock protein of E. coli CspA was used. In the range of protein concentrations studied, none of the cold shock domains EsCSD1–3 showed any visible DNA-melting activity. On the contrary, EsZnF1, EsMix1 and CspA were able to melt BC1 beacon (see Fig. 3B). The concentration of protein reducing $T_m$ by one half the maximal value was at the ratio of 1:2:4 for EsCSDP1:EsMix1:EsZnF1. Thus the highest DNA-melting activity was demonstrated by EsCSDP1 complete protein, the lowest one by EsZnF1. Although cold shock domain EsCSD1 had no DNA-melting activity by itself, adding it to EsZnF1 significantly increased the activity of the latter. Unlike the other proteins, CspA showed a marked DNA-melting activity at concentrations over 1 μM.

3.2. Single-stranded segment of DNA-beacon is required for EsCSDP1 and EsCSDP3 to demonstrate DNA-melting activity

To investigate the role of single-stranded segments of BC1 beacon in the DNA-melting activity of EsCSDP1–3 proteins, three beacons BCstem, BCfullstem and BCnn were created based on the BC1 sequence (see Fig. 1). The single-stranded segments of BCnn beacon consisted of random nucleotide sequence (degenerate sequence). BCstem was an isolated 9-bp double-stranded part of BC1 beacon. BCfullstem was a complementary 40-bp sequence containing a nick between nucleotides 31 and 32 in the chain linked to the fluorescence quencher. Due to the nick, the $T_m$ value was equal for both BCstem and BCfullstem beacons.

Melting experiments have demonstrated that full proteins EsCSDP1–3 were unable to reduce $T_m$ of BCstem and BCfullstem beacons at concentrations up to 8 μM (Fig. 4). In the experiments with BCnn beacon, EsCSDP1 and EsCSDP3 proteins had significant DNA-melting activity (Fig. 3C), whereas EsCSDP2 had none. Fragment EsZnF1 and mixture EsMix1 were active (Fig. 3D), and the fragments EsCSD1 and EsZnF1 showed slight synergism, same as in BC1 beacon experiments. However, it should be mentioned that the synergistic effect was observed at the protein concentrations below 1 μM and disappeared at the increasing concentrations.

3.3. DNA-melting activity of EsCSDP1–3 at 4 °C

When the melting effects were analyzed at 4 °C (Fig. 5), BC1 fluorescence in the presence of full proteins EsCSDP1 and EsCSDP3 was growing sharply as the protein : beacon ratio was increased higher than 10:1, i.e. to the range at which $T_m$ value is

![Fig. 2. Melting curves of BC1 beacon in the presence of EsCSDP1 protein in variable concentrations: line 1 – 0 μM, line 2 – 0.25 μM, line 3 – 1 μM, line 4 – 4 μM. Lower right corner insert: $T_m$ shift in the presence of 1 μM EsCSDP1. $T_m$ shift is estimated as a difference between derivative curve $d$RFU$/$dT peak positions for melting curves with and without protein. RFU – relative fluorescence units.](image-url)
almost unchanged (Fig. 3A). The diagram in Fig. 6 presents the data on beacon BC1 melting at constant temperature 4°C at the protein : beacon ratio of 80:1 (4 μM protein to 50 nM beacon). It is clearly seen that proteins EsCSDP1 and EsCSDP3 showed the highest melting activity. The least active protein was EsCSDP2. C-terminal fragment of EsCSDP1 had significantly lower melting activity than the full protein. The melting activity of EsCSDP1 and EsCSDP3 full proteins was lower for BCnn than for BC1 beacon. CspA had relatively small DNA-melting activity for BC1 and no activity for BCnn beacon.

During DNA-melting activity measuring at 4°C, we noticed that after mixing EsCSDP1 with BC1 beacon in the measuring cuvette, fluorescence level was not set immediately but reaching a steady-state level in more than 5 min (Fig. 7). With increasing temperature, this delay was reduced down to 1 min at 15°C.

4. Discussion

In the present study, high DNA-melting activity of cold shock domain proteins from E. salsugineum plant was found. DNA-melting activity of plant cold shock domain proteins was demonstrated previously; however, those measurements were performed at fixed temperature and high concentration of protein (up to 20 μM). In such conditions, the melting activity of plant cold shock domain proteins detected was significantly lower than the activity of E. coli cold shock protein CspA. Melting activity of E. salsugineum proteins EsCSDP1-3 investigated at different concentrations and variable temperature significantly exceeded that of CspA, which was especially notable at the protein concentrations of less than 1 μM.

The decrease of beacon melting temperature Tm observed in the presence of proteins EsCSDP1-3 indicates, firstly, that the proteins studied interact with DNA-beacons and, secondly, that this interaction is weakening bonds in beacon double-stranded segment. It is
important to note that EsCSDP3m protein containing mutations in the motifs responsible for interaction with nucleic acid chains did not demonstrate any melting activity. This fact confirms that the DNA-beacon melting observed is not an artifact but definitely results from the interaction of CSDP with nucleic acid.

One characteristic feature of DNA-beacon melting curves in the presence of CSDPs is increased level of fluorescence of the fully denatured free beacon (Fig. 2). This fact indicates that protein-DNA binding remains at least with one chain of the beacon when the temperature is increased. It is likely that in this case there are structural constraints on the interaction of dye (R6G) and quencher (BHQ2) groups present, which are absent in fully denatured beacon.

The range of effective concentrations inducing \( T_m \) decrease for all of the three proteins and for EsCSDP1 C-terminal domain is practically identical and equal to 0.1–2 \( \mu \)M. Effective concentrations for EsCSDP1 protein and its separate C-terminal domain EsZnF1 are equal, whereas EsCSD1 domain is lacking any melting activity. These facts indicate that the observed decrease in \( T_m \) is largely due to the interaction of EsZnF1 with the beacon chains. As the cold shock domains EsCSD2 and EsCSD3 have also shown no melting activity, the above conclusion about the role of C-terminal domain in DNA melting can be applicable to the other two proteins EsCSDP2 and EsCSDP3.

When analyzing the data of Fig. 3B and D we see a notable synergistic action between EsCSD1 and EsZnF1 studied in the equimolar mixture. Comparing the decrease in \( T_m \) values depending on the concentrations of EsZnF1 and EsMix1 mixture, we can see that adding EsCSD1 to EsZnF1 leads to a shift of \( T_m \) curve to smaller concentrations. The maximum values of \( T_m \) shift were close which is especially clear for BC1. Taking this into account, one could think that EsCSD1 binds both beacons and this binding increases EsZnF1 affinity to BC1 and BCnn. However, the melting activity of full protein EsCSDP1 is significantly higher than activity of mixture. A much greater difference between the melting activities of EsCSDP1, EsZnF1 and EsMix1 is observed when measuring BC1 melting at 4 °C (Fig. 6). The results of BC1 melting induced by EsCSDP1 protein and its individual domains show that high DNA-melting activity requires the presence of both cold shock domain and C-end part with “zinc finger” motifs in one molecule. Given the ability to bind nucleic acids characteristic for each of the CSDs and for ZnF motifs, it can be assumed that cooperative interaction of both EsCSDP1 domains, namely EsCSD1 and EsZnF1, with beacon determines high melting activity of the protein. This assumption is consistent with the observed time dependence of fluorescence (Fig. 7) especially noticeable at the lower temperatures (5–10 °C). It is possible that this temperature effect is related to the conformational mobility of the EsCSDP1 protein domain structure which contains at least two nucleic acid binding sites.

The data presented demonstrate that melting activity of EsCSDP1 and EsCSDP3 proteins is much higher than EsCSDP2 activity. Previously, relatively low DNA-melting activity was
observed for wheat protein WCSPl carrying 3 ZnF motifs, compared to CspA [18]. No melting activity was detected for AtCSDP2 protein from Arabidopsis thaliana with two ZnF, whereas the activity found in AtCSDP1 which contains seven ZnF was as high as in CspA [12]. Comparing the amino acid sequences of CSDPs with different DNA-melting activity, we noticed that the protein melting activity differs both in amount of “zinc finger” motifs and the length and composition of amino acid chain linking CSD with the first zinc finger (Fig. S1B).

None of the EsCSDPs studied was able to melt BCstem and BCfullstem DNA beacons consisting of paired bases only and not having in its composition single-stranded segments (Fig. 4). It means that binding of EsCSDP1 and EsCSDP3 to a single-stranded DNA region is required to melt double-stranded DNA structure. C-terminal domain of EsCSDP1 contains six CCHC-type ZnF motifs. The ability of this domain to melt BC1 beacon but not BCstem and BCfullstem suggests that one or more ZnF should interact with single-stranded DNA chain too. Replacement of BC1 single-stranded segments by chains with degenerate sequence did not result in disruption of melting activity of either EsCSDP1 and EsCSDP3 or EsCSDP1 C-terminal domain. However, the difference in \( \Delta T_{m} \) and the melting activity at 4 \( ^{\circ}C \) demonstrated by E. salsugineum CSDPs acting at BCnn compared to BC1 may be a sign of some nucleotide sequence preferences.

Thus EsCSDP1, EsCSDP2 and EsCSDP3 proteins are able to melt double-stranded DNA chains effectively by interacting with adjacent single-stranded regions. Yet the highest DNA-melting activity is demonstrated by EsCSDP1 and EsCSDP3. This kind of structures containing alternating single- and double-stranded核酸 acid segments is rather frequent in living cells forming due to the destabilization of DNA double helix during processes such as transcription and repair. EsCSDP1, EsCSDP2 and EsCSDP3 are relatively small proteins with molecular weight of 28, 20 and 31 kDa respectively, and they should be able to cross the nuclear membrane easily. Nuclear localization was demonstrated for homologous proteins AtCSDP1 [23], AtCSDP2 [15] and AtCSDPs [13] from A. thaliana plants. Probable nuclear localization of EsCSDP1, EsCSDP2 and EsCSDP3 and their DNA-melting activity indicate possible involvement of these proteins and their homologs from other plants in the mechanisms associated with DNA structure destabilization, such as transcription and repair.

Interestingly, some of the interactions between E. salsugineum CSDPs and DNA we found resemble those for animal CSDP YB-1. Same as plant CSDPs, human protein YB-1 contains the cold shock domain in its N-terminal part and an extended C-terminal part. However, unlike plant CSDPs, YB-1 C-terminal segment consists of alternating clusters of positively and negatively charged amino acid residues. YB-1 binding with different DNA sequences was demonstrated [24,25] preferentially with single-stranded chains [26], and the DNA-melting activity has also been shown [27]. The role of YB-1 in DNA-associated processes such as transcription and repair was discussed [28,29]. The similarity of structural organization and functional features of DNA interaction suggests that plant cold shock domain proteins, same as YB-1 may be involved in various aspects of DNA biology.

5. Conclusion

The DNA-melting activity of three cold shock domain proteins EsCSDP1-3 from extremophile plant E. salsugineum was studied in the system of DNA-beacons. The protein structures differ mainly in the number of zinc finger motifs in C-terminal part (6 repeats in EsCSDP1, 2 repeats in EsCSDP2, and 7 repeats in EsCSDP3). DNA-melting activity was investigated in a wide range of concentrations and temperatures. Comparing three proteins, the highest DNA-melting activity is demonstrated by EsCSDP1 and EsCSDP3. It was shown that single-stranded segments should be present in DNA molecules in order to elicit melting activity of the proteins. Both cold shock domain and the C-terminal part of the protein molecule are involved in DNA melting. Based on structural and functional similarities between proteins EsCSDP1, EsCSDP2, EsCSDP3 and animal protein YB-1, we can suppose that as YB-1, E. salsugineum CSDPs are involved in DNA-dependent processes such as transcription and repair.

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.02.004.

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