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Short communication

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The role of hydrophobic amino acids of K-segments in the cryoprotection of lactate dehydrogenase by dehydrins

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Abstract

Dehydrins, which are group 2 late embryogenesis abundant (LEA) proteins, accumulate in plants during the development of the embryo and exposure to abiotic stresses including low temperature. Dehydrins exhibit cryoprotection of freezing-sensitive enzymes, e.g. lactate dehydrogenase (LDH). Although it has been reported that K-segments conserved in dehydrins are related to their cryoprotection activity, it has not been determined which sequence features of the K-segments contribute to the cryoprotection. A cryoprotection assay using LDH indicated that 13 K-segments including 12 K-segments found in *Arabidopsis* dehydrins and a typical K-segment (TypK, EKKGIMEKIKEKLPG) derived from the K-segments of many plants showed similar cryoprotective activities. Mutation of the TypK sequence demonstrated that hydrophobic amino acids were clearly involved in preventing the cryoinactivation, cryoaggregation, and cryodenaturation of LDH. We propose that the cryoprotective activities of dehydrins may be made possible by the hydrophobic residues of the K-segments.

Keywords

Cryoprotection

Dehydrin

K-segment

Late embryogenesis abundant (LEA) proteins

1. Introduction

Dehydrins, which are also known as group 2 late embryogenesis abundant (LEA) proteins, are common stress-related proteins in plants (Close, 1996; Battaglia et al., 2008; Hundertmark and Hincha, 2008; Eriksson and Harryson, 2011; Graether and Boddington, 2014). Dehydrins accumulate during embryogenesis and exposure to stresses such as drought, cold, and high salinity. Diverse subcellular localizations of dehydrins, such as in the cytoplasm, nucleus, plasma membrane, tonoplast, plastid, mitochondrion, endoplasmic reticulum, etc., suggest that these proteins universally protect plant cells from stresses. Dehydrins possess one or more conserved K-segment(s) whose typical sequence is EKKGIMEKIKEKLPG (Svensson et al., 2002). The K-segment is predicted to form an amphipathic helix, and this feature is believed to be related to the dehydrin functions (Koag et al., 2009). Some dehydrins also have a Y-segment (a frequent sequence; DEYGNP) and S-segment (LHRSGSSSSSSSEDD or related sequences), but these sequences do not occur in all dehydrins. A shorthand using the K, Y, and S-segments is commonly applied to classify dehydrins, e.g. SKn, KnS, Kn, YnSKn, YnKn, etc. Dehydrins are considered to be intrinsically disordered proteins, because they show a highly flexible structure

due to the presence of many hydrophilic residues. This structural feature has been determined by various methods of analysis including circular dichroism (CD), Fourier-transform infrared spectroscopy (FTIR), and nuclear magnetic resonance (NMR) (Rahman et al., 2011; Szalainé Ágoston et al., 2011).

Investigations of transgenic plants have revealed that the expression of dehydrins enhanced the low-temperature (Hara et al., 2001; Puhakainen et al., 2004; Houde et al., 2004; Yin et al., 2006; Xing et al., 2011; Ochoa-Alfaro et al., 2012), osmotic-stress (Cheng et al., 2002; Figueras et al., 2004; Brini et al., 2007), and high-salinity (Shekhawat et al. 2011) tolerances in various species. Concomitantly, diverse *in vitro* studies have been conducted to elucidate the molecular functions of dehydrins. Dehydrins inhibited enzyme inactivation caused by freezing and dehydration, and interacted with phospholipids, water, calcium, heavy metals, and nucleic acids (Close, 1996; Battaglia et al., 2008; Hundertmark and Hincha, 2008; Hara et al., 2009; Eriksson and Harryson, 2011; Graether and Boddington, 2014). Among these functions, the cryoprotection of freezing-sensitive enzymes has been confirmed by a large number of studies using dehydrins from various plant species such as SoCOR85 from spinach (*Spinacia oleracea*) (Kazuoka and Oeda, 1994), TaWCS120 (Houde et al., 1995) and TaDHN-5 (Drira et al., 2013) from wheat (*Triticum aestivum*), PpPCA60 from peaches (*Prunus persica*) (Wisniewski et al., 1999), CuCOR19 (Hara et al., 2001) and CrCOR15 (Sanchez-Ballesta et al., 2004) from citrus species (*Citrus unshiu* and *Citrus clementina* Hort. Ex Tanaka x *Citrus reticulata* Blanco), HvP-80/Dhn5 from barley (*Hordeum vulgare*) (Bravo et al., 2003), GmDHN26 and GmDHN27 from soybeans (*Glycine max*) (Momma et al., 2003), VrYSK2 from riverbank grapes (*Vitis riparia*) (Hughes and Graether, 2011; Hughes et al., 2013), VvcDHN1a from table grapes (*Vitis vinifera*) (Rosales et al., 2014), ERD10 (Reyes et al., 2008), ERD14 (Tantos et al., 2009), and AtHIRD11 (Hara et al., 2014) from *Arabidopsis thaliana*, TsDHN-2 from *Thellungiella salsuginea* (Hughes et al., 2013), RsDHN from radishes (*Raphanus sativus*) (Hara et al., 2014), RcDhn5 from *Rhododendron catawbiense* (Reyes et al., 2008), and OpsDHN-1 from *Opuntia streptacantha* (Hughes et al., 2013). The cryoprotective activities of ERD10, RcDhn5, and TaDHN-5 for lactate dehydrogenase (LDH) were reduced by truncating the K-segments (Reyes et al., 2008; Drira et al., 2013). The K-segment itself showed cryoprotection of LDH (Hughes et al., 2013). These findings indicate that K-segments at least partially contribute to the cryoprotective activities of dehydrins.

Recently, the mechanisms of the cryoprotection of LDH by dehydrins have been discussed. The major hypothesis is a molecular shield model (Hughes and Graether, 2011; Chakrabortee et al. 2012) in which dehydrins with large hydrodynamic radii form molecular shields between the target proteins to prevent collisions between them. On the other hand, an interaction model which predicts that protection will occur by means of direct interaction between dehydrins and

the target proteins was also proposed (Cuevas-Velazquez et al. 2014). However, it is unknown which amino acids of the K-segments participate in the cryoprotective activities of K-segments. Here we report that the typical K-segment lost its cryoprotective activity when the hydrophobic amino acids were mutated. We discuss the mechanism of the cryoprotection of freezing-sensitive enzymes by dehydrins by comparing previously proposed models with our results.

2. Materials and methods

2.1. Chemicals

8-Anilino-1-naphthalene sulfonic acid (ANS) was purchased from Sigma (Tokyo, Japan). L-LDH (recombinant, rabbit muscle) and nicotine adenine dinucleotide (NADH) were obtained from Oriental Yeast (Tokyo, Japan).

2.2. Peptide synthesis

Peptides were chemically synthesized by an automated solid phase peptide synthesizer (Tetras, Advanced ChemTech, KY, USA). The peptides were purified using C18 (AlltimaTM 4.6 x 250 mm) reversed-phase column chromatography (UFLC-20AB, Shimadzu, Kyoto, Japan) with a linear gradient of acetonitrile (from 5% to 95%) in 0.05% trifluoroacetic acid solution over 25 min. The purified peptides were identified by mass spectrometry (MS) (LCMS-2020, Shimadzu). The peptides were concentrated and lyophilized.

2.3. Cryoprotective assay

Cryoprotective activities were measured using our previous method (Hara et al. 2001) with modifications. Twenty microliters of LDH (12 μg mL^{-1}, 0.34 μM as a monomer) and 30 μL of peptides (0, 1, 10, 50, 100, 500, and 1000 μg mL^{-1}) were combined in 1.5-mL plastic tubes. The tubes were immersed in liquid N_2 for 1 min. The frozen tubes were immediately floated in the water bath which was kept at 25±2°C. After ensuring that the ice in the tubes had totally melted, the tubes were kept in the same water bath for an additional 3 min. Then the tubes were frozen again. The freeze-thaw cycles were repeated 3 times, and then the LDH activities were determined. A reaction reagent contained 9.5 mM Tris-HCl pH 7.5, 0.58 mM sodium pyruvate, and 60 μM NADH. A reaction was started by adding 4 μL of the LDH-peptide mix to the reaction reagent (196 μL). The absorbance at 340 nm was monitored with a microplate reader

(Varioskan Flash, Thermo Fisher Scientific, Tokyo, Japan) at 25 ^{o}C. The three freeze-thaw cycles reduced the LDH activity to 15 to 20% of the initial activity. If the LDH activity after the freeze-thaw cycles was equal to the activity of an unfrozen sample, the protection was 100%. The value of a 50% protection dose (PD_{50}) was determined to assess the cryoprotective activities.

2.4. Light scatter

The light scatter method was performed as in the previous report (Hughes and Graether, 2011). A 2.5-µL volume of LDH (15 mg mL^{-1}) and 2.5 µL of peptides (0, 0.1, 1, and 10 mg mL^{-1}) were added to 245 µL of 10 mM Tris-HCl pH 7.5 in 1.5-mL plastic tubes (0.25 mL in total). The final concentrations of the peptides were 0, 1, 10, and 100 µg mL^{-1}. After three freeze-thaw cycles, the absorption at 415 nm was recorded with an iMark Microplate Absorbance Reader (Bio-Rad, Tokyo, Japan).

2.5. Protein folding assay

The fluorescence assay using ANS was conducted using the method previously described (Reyes et al. 2008), but partially modified. A 2.5- µL volume of LDH (14 mg mL^{-1}), 2.5 µL of peptides (0, 0.1, 1, and 10 mg mL^{-1}), and 5 µL of ANS (0.2 mM) were added to 240 µL of 10 mM sodium phosphate buffer pH 7.0 in 1.5-ml plastic tubes (250 µL in total). The samples were subjected to three freeze-thaw cycles, and then the ANS fluorescence at Ex 350 nm/Em 470 nm was measured (Varioskan Flash).

2.6. Statistical analysis

Data for *P* values were analyzed by Student's *t* test and one-way analysis of variance (ANOVA) at a significance level of 0.05.

3. Results and discussion

3.1. Cryoprotection by diverse K-segments

Since the K-segment of riverbank grape dehydrin was shown to have cryoprotective activity for LDH (Hughes et al., 2013), we determined the cryoprotective activities of K-segments from various dehydrins to reveal whether the variation in the sequences of the K-segments affects

their cryoprotective activities. We collected K-segments from the *Arabidopsis* dehydrins. *Arabidopsis* has 10 dehydrin genes which possess 24 K-segments (Supplemental Fig. 1). The minimum number of K-segments is 1 (At1g54410 and At4g39130) and the maximum number is 6 (At3g50970). Phylogenic analysis suggested that the 24 K-segments were separated into 12 groups; 1 K-segment was chosen from each group (Supplemental Fig. 2). Thus, we prepared 12 *Arabidopsis* K-segments (from At1g20440_K3 to At1g20450_K1 in Fig. 1). In addition, we also synthesized EKKGIMEKIKEKLPG (TypK in Fig. 1), which is the most frequent sequence out of 247 K-segments determined by Svensson et al. (2002). The 13 K-segments had similar cryoprotective activities for LDH (Fig. 1B), suggesting that either some sequence characteristics related to cryoprotective activities may be conserved in these K-segments or the expression of cryoprotective activity does not depend on their sequences.

3.2. Cryoprotective activities of mutant K-segments

Since various K-segments showed similar cryoprotective activities, we used TypK for the following experiments. The main sequence characteristics found in the K-segments were as follows: (1) an abundance of K (5 Ks), (2) the presence of 4 hydrophobic residues, and (3) the presence of 3 Es. So these residues were changed, and the cryoprotective activities for LDH were determined (Fig. 2A). The replacement of 5 Ks with 5 Ts (TypK_K/T) and of 3 Es with 3 Ts (TypK_E/T) little affected the activities. However, the substitution of 4 hydrophobic residues by 4 Ts (TypK_Hphob/T) drastically lowered the activity. The same results were obtained when the 4 hydrophobic residues were replaced by 4 Ks (TypK_Hphob/K) and 4 Es (TypK_Hphob/E). This showed that the hydrophobic residues were involved in the cryoprotective activities.

Partial replacements of the 4 hydrophobic residues by Ts were tested (Fig. 2B). The single replacements resulted in little (TypK_5I/T and TypK_6M/T) or slight (TypK_9I/T and TypK_13L/T) reduction of the cryoprotective activities. However, the double replacements (TypK_5I+6M/T and TypK_9I+13L/T) and triple replacements (TypK_5I+9I+13L/T and TypK_6M+9I+13L/T) severely reduced the protective properties. Therefore, at least 3 out of 4 hydrophobic residues were necessary to maintain the cryoprotective activities of TypK.

The following experiments were performed to collect more evidence that TypK is more efficient than TypK_Hphob/T for the cryoprotection of LDH. It has been reported that LDH formed aggregates and altered its tertiary structures during the freeze-thaw cycles (Reyes et al., 2008; Hughes et al., 2011). Light scattering was used to detect the aggregation of LDH. Although the absorbance at 415 nm of the LDH solution approximately tripled after the freeze-thaw cycles, the addition of TypK to the LDH solution at the concentration of 58 μM lowered the absorbance to the level observed before freezing (Fig. 3A). This absorbance

reduction did not occur when TypK_Hphob/T was tested. The change in tertiary structures concomitant with the exposure of hydrophobic residues can be detected by the fluorescence probe ANS, which exhibits strong fluorescence when associated with the hydrophobic residues. The freeze-thaw cycles drastically enhanced the fluorescence of ANS, but TypK quenched the fluorescence emission at 5.8 and 58 μM (Fig. 3B). TypK_Hphob/T did not show such effects, just as in the case of light scatter.

3.3. Putative structures of TypK and TypK_Hphob/T

The structural distinction between TypK and TypK_Hphob/T was analyzed using PEP-FOLD3 software (http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/), which can predict the structures of linear peptides with 5-50 amino acids in aqueous solution (Lamiable et al. 2016). The cartoon models suggested that TypK and TypK_Hphob/T had similar loose helix structures, whereas TypK_Hphob/T was slightly looser than TypK (Fig. 4). The structural looseness was also predicted by IUPred software (http://iupred.enzim.hu/) (Dosztányi et al., 2005), which analyzes the intrinsically disordered states of peptides (Supplementary Fig. 3). Although both peptides appeared disordered, TypK_Hphob/T was somewhat more disordered than TypK because the disorder tendency of TypK_Hphob/T was higher than that of TypK. The ball-and-stick models showed that the hydrophobic residues of TypK, i.e. the 5th I, 6th M, 9th I, and 13th L, were oriented to one side of the loose helix in this order (Fig. 4A). This prediction is supported by the HeliQuest (http://heliquest.ipmc.cnrs.fr/) analysis (Gautier et al. 2008) (Supplementary Fig. 4). Taken together, these findings suggest that the cryoprotective activity of TypK might depend on the presence and orientation of the hydrophobic residues rather than the potential loose structure.

3.4. Roles of hydrophobic amino acids in cryoprotection

In this paper we demonstrated that hydrophobic amino acids contribute to the cryoprotective activities of the K-segment, which is a conserved sequence of dehydrin. The typical K-segment is 15 amino acids long and has 4 hydrophobic residues at the 5th, 6th, 9th, and 13th positions of the sequence. Although changing the 5th or 6th hydrophobic residue to T did not affect the cryoprotective activity, changing the 9th or 13th hydrophobic residue to T significantly reduced the cryoprotective activity, indicating that the 5th or 6th residue may not need to be hydrophobic to show cryoprotective activity. This explains why some *Arabidopsis* K-segments e.g. At3g50980_K1 (KKKGITEKIKEKLPG), At3g50970_K6 (ENKSTMDKIKEKLPG), At2g21490_K1 (RKKSIKEKIKEKFGS), etc., which lacked hydrophobicity at the 5th or 6th

position, still showed sufficient cryoprotective activities despite having only 3 hydrophobic residues in the K-segments (Fig. 1).

Various molecular mechanisms of the protein protection provided by dehydrins have been proposed. The most accepted mechanism is a molecular shield model (Chakrabortee et al. 2012). Dehydrins act as molecular shields which occupy the space between the target proteins to reduce collisions between them. In this model, dehydrins do not interact with the target proteins; the large hydrodynamic radius of dehydrins has a space-filling effect. Another model, the entropy transfer model, is a derivative of the molecular shield model (Kovacs et al. 2008). According to the entropy transfer model, dehydrins may form a protective barrier on the surface of the target proteins by loosely associating with the target proteins to prevent aggregation. Also, an interaction model in which dehydrins can directly bind to the target proteins to inhibit their denaturation was recently proposed (Cuevas-Velazquez et al. 2014). Since in our results the cryoprotective activities of TypK clearly depended on the hydrophobic residues in the sequence, specific interactions might occur between the hydrophobic amino acids and LDH in the process of denaturation. The NMR study demonstrated that K2, which is a peptide containing 2 K-segments, did not show direct binding to LDH (Hughes and Graether, 2011). Therefore, interaction between TypK and LDH could be transient rather than permanent. Intense molecular motion during the thaw process could cause unfavorable exposure of the hydrophobic residues of LDH. It is postulated that TypK might be a passive chaperone which prevents the exposure of hydrophobic residues by transient interaction in order to return the hydrophobic residues to their original space in LDH using the entropic energy during the thaw process. If so, TypK might show cryoprotective activities according to the entropy transfer model as well as the interaction model. This may be called the transient hydrophobic interaction model. However, the molecular shield model should also be considered, because a large excess of K-segment molecules (PD_{50} was 20 μM) was needed to cryoprotect 0.137 μM of the LDH monomer (Fig. 1).

Polyethylene glycol (PEG) has potent cryoprotective activities for LDH. PEG is a polymer which repeats [CH_3-CH_3-O] units. This indicates that PEG is a hydrophilic polymer, but it partially possesses hydrophobic sites. Not only the molecular shield model but also the transient hydrophobic interaction model may be applied to explain the mechanisms of the cryoprotection of LDH by PEG. Taken together, these findings lead us to propose that the role of hydrophobic amino acids should be considered when studying the cryoprotective mechanisms shown by dehydrins for LDH.

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Figure legends

Fig. 1. Cryoprotective activities of K-segments. (A) Amino acid sequences of the *Arabidopsis* K-segments and TypK. (B) Cryoprotective activities of the K-segments for LDH are represented by PD_{50}. PD_{50}-values and bars represent means ± SD (four individual experiments). No significant differences ($p < 0.05$) were observed as determined by one-way analysis of variance (ANOVA) in a comparison between TypK and other K-segments.

Fig. 2. Cryoprotective activities of TypK and its related peptides. (A) Ks, Es, and hydrophobic residues of TypK were changed. (B) Hydrophobic residues of TypK were partially changed. Marks "-", "+", and "Φ" represent negatively charged Es, positively charged Ks, and hydrophobic amino acids, respectively. Hydrophobic amino acids are shaded. Cryoprotective activities are represented by PD_{50}. PD_{50}-values and bars represent means ± SD (four individual experiments). Asterisks show significant differences ($p < 0.05$) as determined by Student's *t*-test in a comparison between TypK and other K-segments. Broken bars indicate that the PD_{50}s were more than 350 μM. In those cases, Student's t-test was not performed because the data were not defined.

Fig. 3. Effects of TypK and TypK_Hphob/T on the cryoprotection of LDH. (A) Light scatter. (B) ANS fluorescence. Open and grey columns indicate before and after triple freeze-thaw cycles, respectively. Values and bars represent means ± SD (four individual experiments). Asterisks show significant differences ($p < 0.05$) as determined by Student's *t*-test in a comparison between no treatment (0 μM) and peptide treatments.

Fig. 4. Prediction of the structures of TypK (A) and TypK_Hphob/T (B). PEP-FOLD3 (http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/) was used. The cartoon models are shown on the left side. N and C indicate the N-terminus and C-terminus, respectively. The ball-and-stick models are depicted on the right side. I_5, M_6, I_9, and L_{13} indicate the 5th I, 6th M, 9th I, and 13th L, respectively.

A

At1g20440_K3	EKKGILEKIKEKLPG	At3g50970_K2	EKKGMTEKVMEQLPG
At1g20440_K1	DKKGLVEKIKEKLPG	At3g50970_K4	EKKSVTEKVMEKLPG
At1g20450_K2	EKKGFMDKIKEKLPG	At2g21490_K1	RKKSIKEKIKEKFGS
At3g50980_K1	KKKGITEKIKEKLPG	At1g54410_K1	HKEGIVDKIKDKIHG
At4g38410_K1	KEKGFMEKIKDKLPG	At1g20450_K1	NQGVMDRIKEKFPLG
At4g39130_K1	EKKGFFKKIKEKLSG	TypK	EKKGIMEKIKEKLPG
At3g50970_K6	ENKSTMDKIKEKLPG		

B

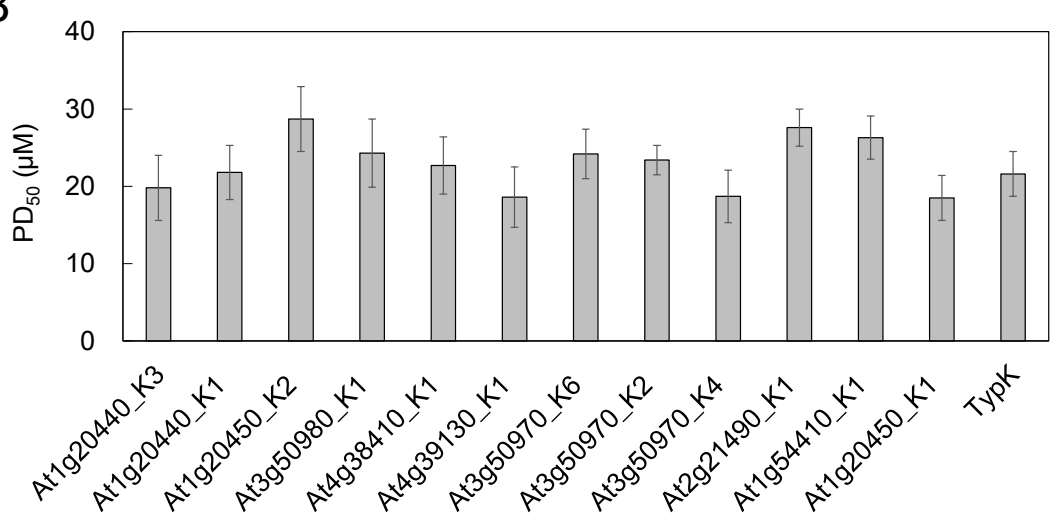


Fig. 1 Hara et al.

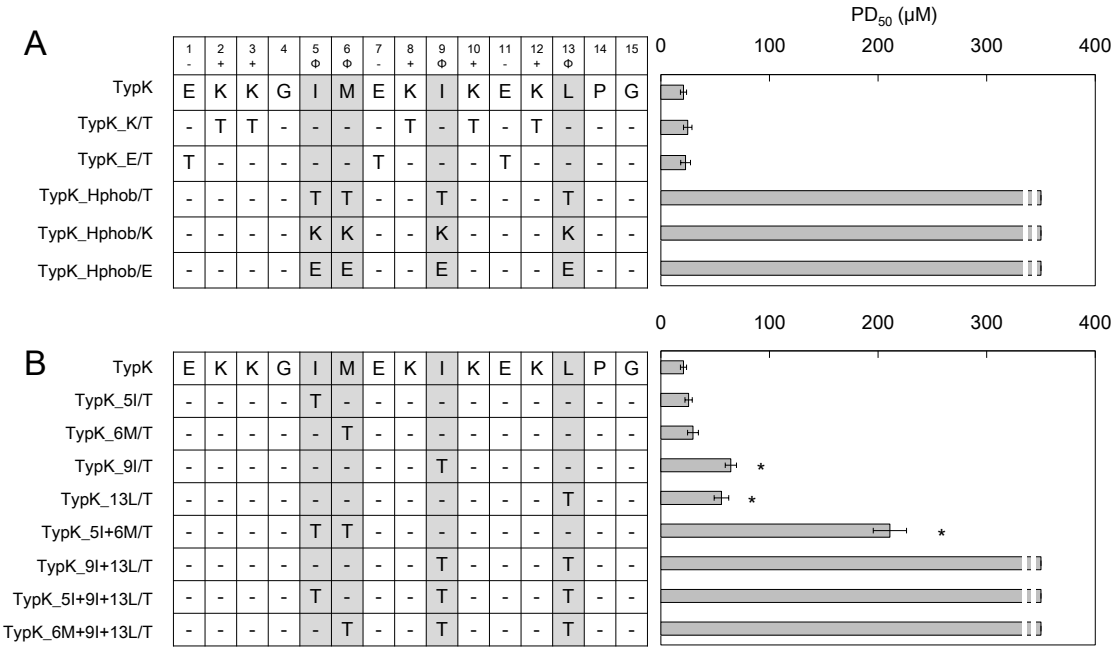


Fig. 2 Hara et al.

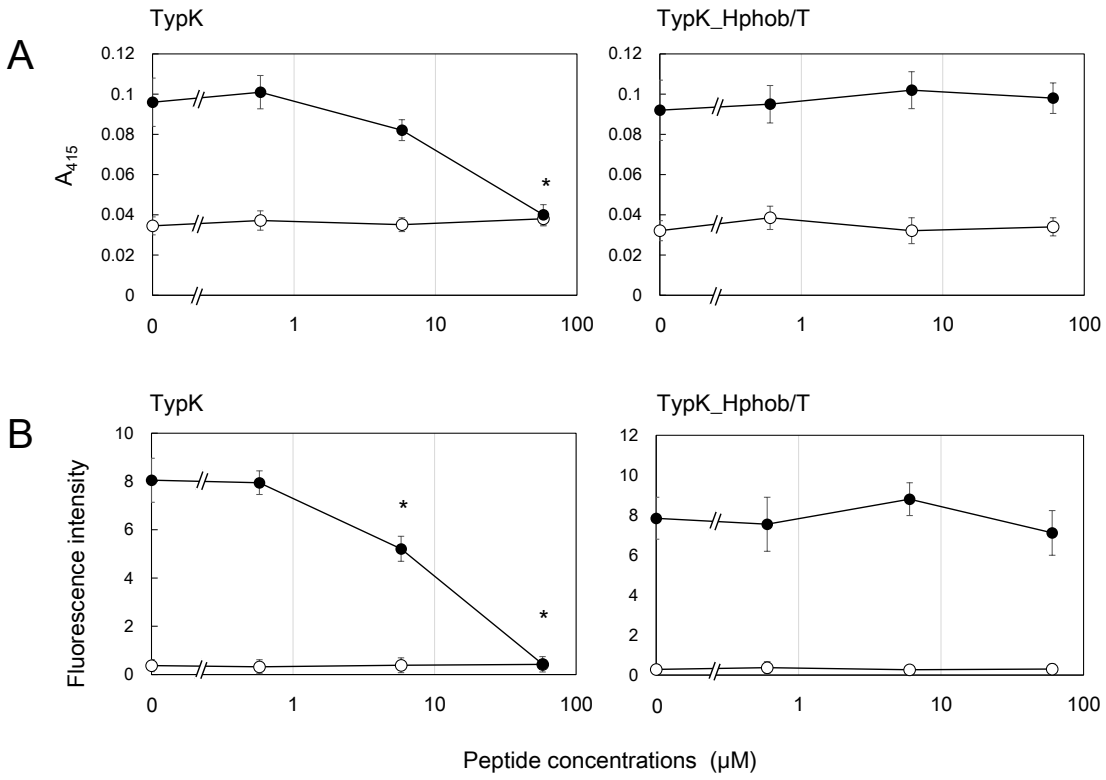


Fig. 3 Hara et al.

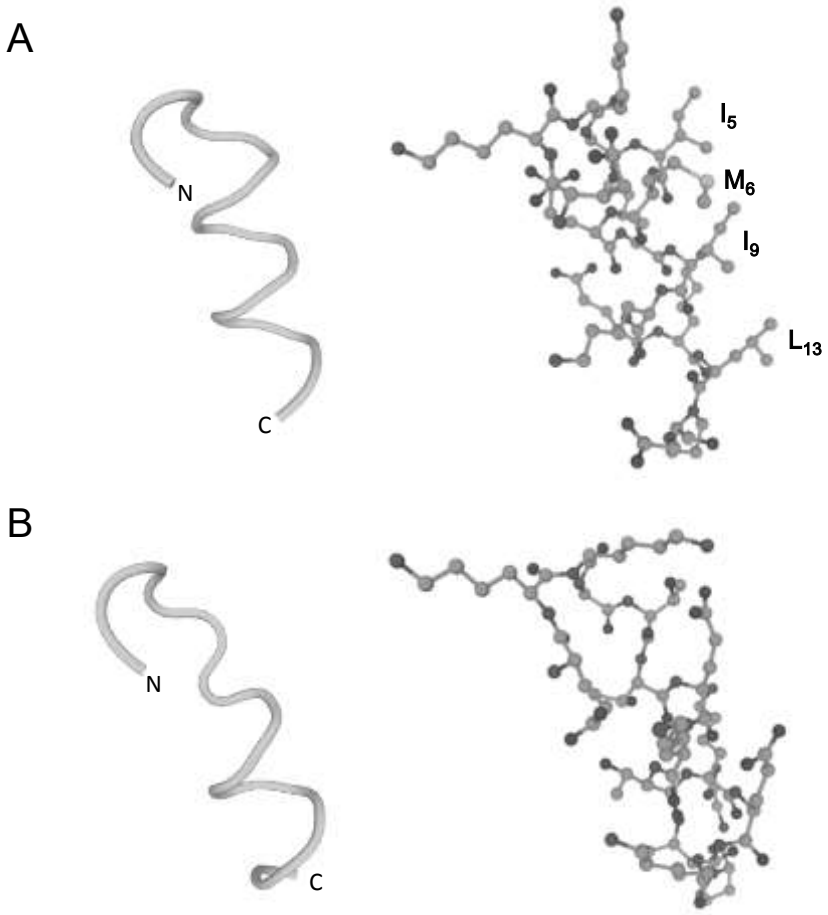


Fig. 4 Hara et al.

Arabidopsis thaliana

◆At1g20440 **COR47**
MAEEYKNNVPEHETPTVATEESPATTTEVTDRGLFDFLGKKEEEVKPQETTTLESEFDHKAQISEPEL
AAEHEEVKENKITLLEELQEKTEEDEENKPSVIEKLHRSNSSSSSSSDEEGEEKKEKKKKIVEGEEDKKG
LVEKIKEKLPGHHDKTAEDDVPVSTTIPVPVSESVVEHDHPEEEKKGLVEKIKEKLPGHHDEKAEDSPA
VTSTPLVVTEHPVEPTTELPVEHPEEKKGILEKIKEKLPGYHAKTTEEEVKKEKESDD

At1g20440_K1: DKKGLVEKIKEKLPG
At1g20440_K2: EKKGLVEKIKEKLPG
At1g20440_K3: EKKGILEKIKEKLPG

◆At1g20450 **ERD10**
MAEEYKNTVPEQETPKVATEESSAPEIKERGMFDFLKKKEEVKPQETTTLASEFEHKTQISEPESFVAK
HEEEEHKPTLLEQLHQKHEEEEENKPSLLDKLHRSNSSSSSSDEEGEDGEKKKKEKKKKIVEGDHVK
TVEEENQGVMDRIKEKFPLGEKPGGDDVPVVTTMPAPHSVEDHKPEEEEKKGFMDKIKEKLPGHSKK
PEDSQVVNTTPLVETATPIADIPEEKKGFMDKIKEKLPGYHAKTTGEEEKKEKVSD

At1g20450_K1: NQGVMDRIKEKFPLG
At1g20450_K2: EKKGFMDKIKEKLPG
At1g20450_K3: EKKGFMDKIKEKLPG

◆At1g54410 **AtHIRD11**
MAGLINKIGDALHIGGGNKEGEHKKEEEHKKHVDEHKSGEHKEGIVDKIKDKIHGGEGKSHDGEGKSHD
GEKKKKKDKKEKKHHDDGHHSSSSDSDSD

At1g54410_K1: HKEGIVDKIKDKIHG

◆At1g76180 **ERD14**
MAEEIKNVPEQEVPKVATEESSAEVTDRGLFDFLGKKKDETKPEETPIASEFEQKVHISEPEPEVKHESL
LEKLHRSDSSSSSSSEEEGSDGEKRKKKKEKKKPTTEVEVKEEEKKGFMEKLKEKLPGHKKPEDGSA
VAAAPVVVPPPVEEAHPVEKKGILEKIKEKLPGYHPKTTVEEEKKDKE

At1g76180_K1: EKKGFMEKLKEKLPG
At1g76180_K2: EKKGILEKIKEKLPG

◆At2g21490
MADLRDEKGNPIHLTDTQGNPIVDLTDEHGNPMYLTGVVSSTPQHKESTTSDIAEHPTSTVGETHPAA
APAGAGAATAATATGVSAGTGATTTGQQHHGSLEEHLRRSGSSSSSSSEDDGQGGRRKKSIKEKIKE
KFGSGKHKDEQTPATATTTGPATTDQPHEKKGILEKIKDKLPGHHNHNHP

At2g21490_K1: RKKSIKEKIKEKFGS
At2g21490_K2: EKKGILEKIKDKLPG

Supplemental Fig. 1. Amino acid sequences of *Arabidopsis* dehydrins and their K-segments. Bold letters such as "COR47" represent common names of the corresponding dehydrins. Red letters indicate K-segments. A name was given to each K-segment, e.g. At1g20440_K1, etc.
Title: The role of hydrophobic amino acids of K-segments in the cryoprotection of lactate dehydrogenase by dehydrins
Authors: Hara M, Endo T, Kamiya K, Kameyama A

◆At3g50970 Xero2/LTI30
MNSHQNQTGVQKKGITEKIMEKLPGHHGPTNTGVVHHEKKGMTEKVMEQLPGHHGATGTGGVHHE
KKGMTEKVMEQLPGHHGSHQTGTNTTYGTTNTGGVHHEKKSVTEKVMEKLPGHHGSHQTGTNTAY
GTNTNVVHHEKKGIAEKIKEQLPGHHGTHKTGTTTSYGNTGVVHHENKSTMDKIKEKLPGGHH

At3g50970_K1: QKKGITEKIMEKLPG
At3g50970_K2: EKKGMTEKVMEQLPG
At3g50970_K3: EKKGMTEKVMEQLPG
At3g50970_K4: EKKSVTEKVMEKLPG
At3g50970_K5: EKKGIAEKIKEQLPG
At3g50970_K6: ENKSTMDKIKEKLPG

◆At3g50980 XERO1
MESYQNQSGAQQTHQQLDQFGNPFPATTGAYGTAGGAPAVAEGGGLSGMLHRSGSSSSSSSEDDG
LGGRRRKKKGITEKIKEKLPGHHDSNKTSSLGSTTTAYDTGTVHHEKKGMMEKIKEKLPGGHH

At3g50980_K1: KKKGITEKIKEKLPG
At3g50980_K2: EKKGMMEKIKEKLPG

◆At4g38410
MADHPRSTEQQEADAAASKGCGMFDFLKKKPEDVHSSENARVTKEPKEEEKPSLAERFHLSDSSSSD
EEAGENGEKKEKKKKKKKKNEVAEDQCETEEKIPAGIGHEDGKEKGFMEKIKDKLPGGHNGKPEAEPH
NDKAKEKGFMEKIKEKLPGHTNDEKKKET

At4g38410_K1: KEKGFMEKIKDKLPG
At4g38410_K2: KEKGFMEKIKEKLPG

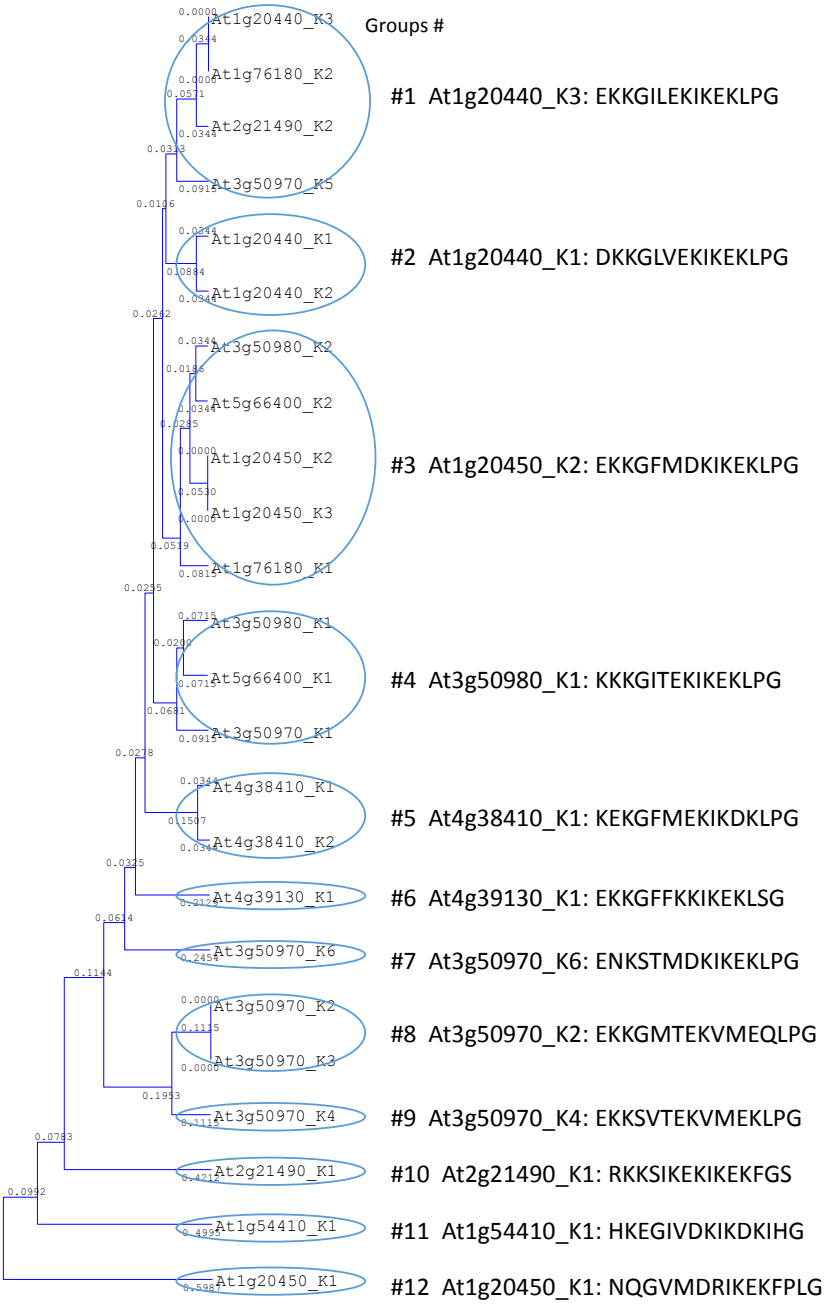
◆At4g39130
MADLKDERGNPIYLTDAHGEPAQLMDEFGNAMHLTGVATTVPHLKESSYTGPHPITAPVTTTNTPHH
AQPISVSHDPLQDHDLRWFGTSSTEENGEGVGRKTNITDETKSKLGVDKPSAATVTGSGSGSVHEKK
GFFKKIKEKLSGHHNDL

At4g39130_K1: EKKGFFKKIKEKLSG

◆At5g66400 RAB18
MASYQNRPGGQATDEYGNPIQQQYDEYGNPMGGGGYGTGGGGGATGGQGYGTGGQGYGSGGQGY
GTGGQGYGTGTGTEGFGTGGGARHHGQEQLHKESGGGLGGMLHRSGSGSSSSSEDDGQGGRRKKGI
TQKIKEKLPGHHDQSGQAQAMGGMGSGYDAGGYGGEHHEKKGMMDKIKEKLPGGGR

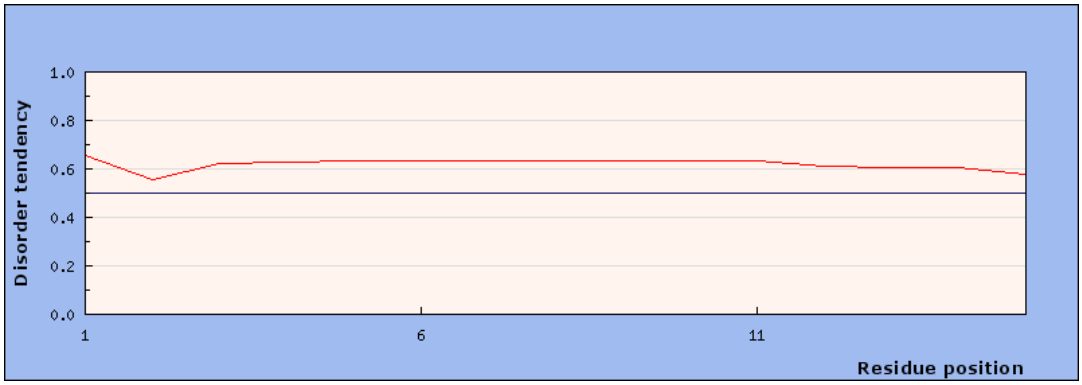
At5g66400_K1: RKKGITQKIKEKLPG
At5g66400_K2: EKKGMMDKIKEKLPG

Supplemental Fig. 1. (continued)

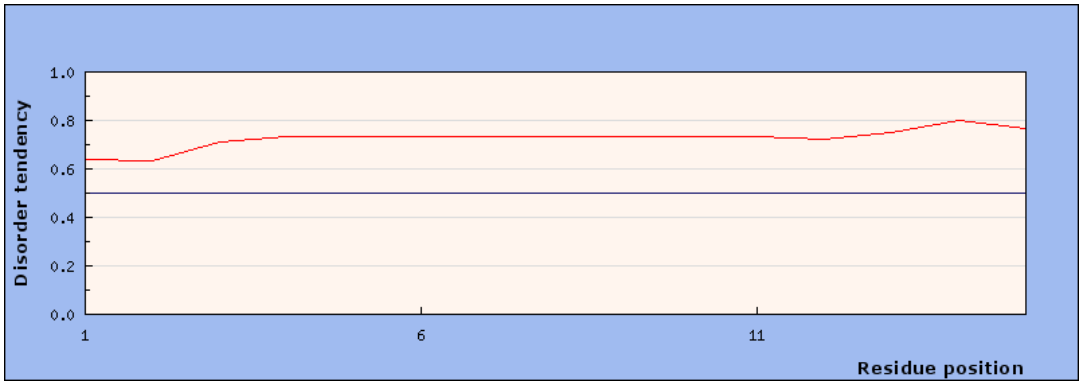


Supplemental Fig. 2. *Arabidopsis* 24 K-segments were grouped using an alignment tool in GENETYX software (Software Development Co, Tokyo, Japan). Circles indicate subgroups which were formed when differences were smaller than 0.1. Group numbers are shown (from #1 to #12). Twelve K-segments were chosen from the 12 groups.
Title: The role of hydrophobic amino acids of K-segments in the cryoprotection of lactate dehydrogenase by dehydrins
Authors: Hara M, Endo T, Kamiya K, Kameyama A

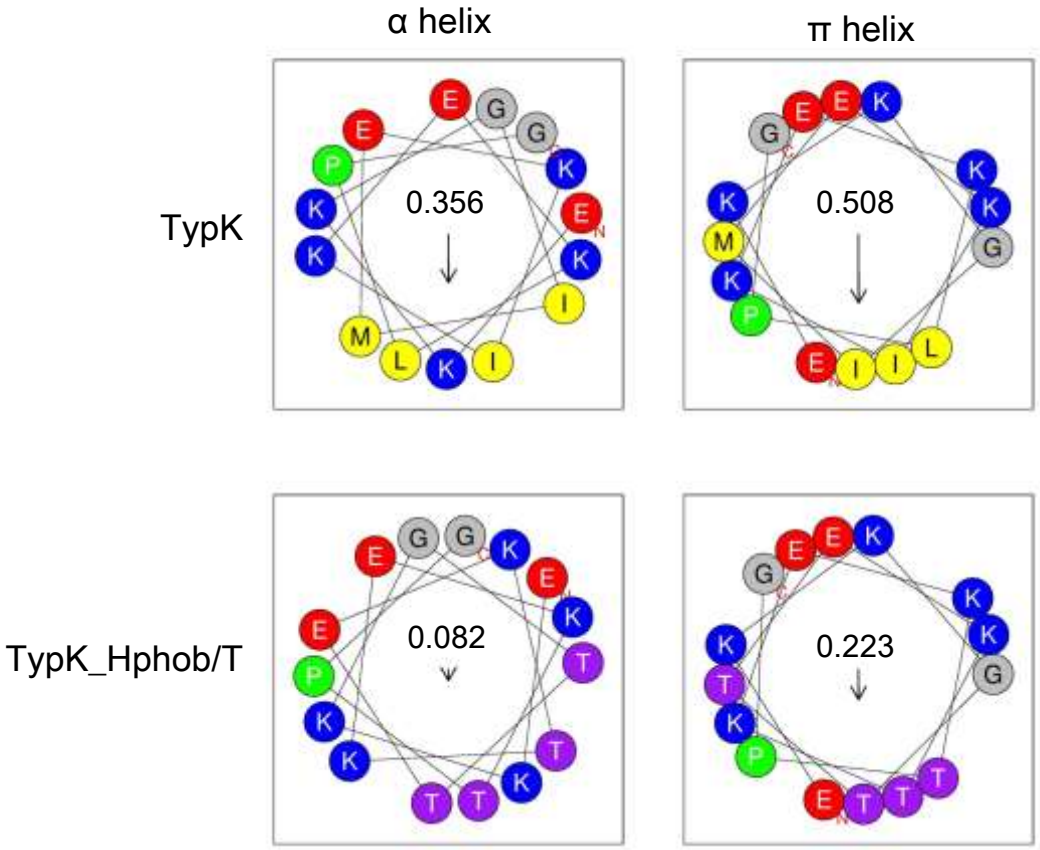
TypK



TypK_Hphob/T



Supplemental Fig. 3. Assessment of intrinsically disordered states in TypK and TypK_Hphob/T using IUPred software (http://iupred.enzim.hu/) (Dosztányi et al. 2005, IUPred: web server for the prediction of intrinsically unstructured regions of proteins based on estimated energy content. Bioinformatics 21:3433-3434). The sequences of TypK and TypK_Hphob/T are EKKGIMEKIKEKLPG and EKKGTTEKTKEKTPG, respectively. Both peptides appear disordered, although it is likely that TypK_Hphob/T is more disordered than TypK.
Title: The role of hydrophobic amino acids of K-segments in the cryoprotection of lactate dehydrogenase by dehydrins
Authors: Hara M, Endo T, Kamiya K, Kameyama A



Supplemental Fig. 4. Helical wheel analysis using HeliQuest software (http://heliquest.ipmc.cnrs.fr/) (Gautier et al. 2008, HELIQUEST: a web server to screen sequences with specific alpha-helical properties. Bioinformatics 24:2101-2102). Yellow residues represent hydrophobic amino acids. Arrows indicate hydrophobic moments (μH). The sequences of TypK and TypK_Hphob/T are EKKGIMEKIKEKLPG and EKKGTTEKTKEKTPG, respectively. TypK shows amphiphilicity in both α helix and π helix models.
Title: The role of hydrophobic amino acids of K-segments in the cryoprotection of lactate dehydrogenase by dehydrins
Authors: Hara M, Endo T, Kamiya K, Kameyama A